Maternal Effects in Nematodes:

Evidence, Relevance & Importance

by

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CONTENTS

List of Abbreviations

ABZ – Albendazole

CVT – Cyclically Varying Temperature

DMSO – Dimethyl Sulfoxide

EPN – Entomopathogenic Nematode

IJ – Infective Juvenile

L1-4 – Caenorhabditis elegans Larval Stages

LB – Luria-Bertani Broth

LRS – Lifetime Reproductive Success

MW – Mealworm (Tenebrio molitor Larvae)

nAChR – Nicotinic Acetylcholine Receptor

NB – Nutrient Broth

NGM – Nematode Growth Medium

NTO – Non-target Organism

PYR – Pyrantel

r – Intrinsic Rate of Population Increase

RKN – Root-Knot Nematode

WHO – World Health Organisation

WM – Wax moth (Galleria mellonella larvae)

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ABSTRACT

Maternal effects are ubiquitous in free-living organisms, with parent-environment interactions affecting offspring life-history traits and fitness. These effects have been demonstrated in a wide variety of organisms, including mammals, insects and plants. Despite this, there is little evidence of maternal effects existing in parasites. If maternal effects are so prevalent in free-living organisms then it is unlikely that they do not exist in parasites. Maternal effects are important because they influence progeny fitness, measured by fecundity, longevity and developmental time. If they exist in parasite then they likely result in increased virulence, greater persistence (especially in terms of soil dwelling nematodes) and may be a driving influence in selection for resistance. Here, maternal effects are demonstrated in the free-living nematode Caenorhabditis elegans and the maternal effect of temperature demonstrated under constant and variable conditions. Similarly, the fitness effects of environmental temperature are explored in entomopathogenic nematodes and the effects of both maternal temperature and host species are demonstrated to cause changes in offspring development. Finally, C. elegans is used to demonstrate the importance of maternal effects in parasite life history and their potential impact on parasite control. The implications of altered life-history strategies that come about as a result of mothers tailoring their reproductive strategies in response to environmental cues for agricultural and medical parasite control are discussed. Critically, the effects of low parental exposure to anthelmintic compounds and nematicidal plant extracts on the fitness of offspring are demonstrated. This work provides evidence for the existence of maternal effects in free-living and parasitic species and highlights the importance of the recognition of such effects in multiple fields.

Chapter One

Introduction

1.1 Life history theory and maternal effects

Historically, Development, as an area of research, has been considered separately from Evolutionary Biology as a whole. The `Central Dogma_ of Molecular Biology, that is the idea that DNA leads to RNA through the mechanism of transcription, which then leads to protein expression through the mechanism of translation, by definition does not allow gene activity to be influenced by the environment due to this one-way flow of genetic information (Gottlieb, 2000). By explaining heredity and development in this simplistic view (i.e. DNA mutation leads to altered transcription and ultimately a change in protein expression) we exclude phenomena that lead to protein expression changes that aren˘t reliant on mutation ¯ i.e. a change in phenotype that is not a direct consequence of genotypic change and is instead a consequence of epigenetic change. Such changes can have dramatic effects on development, yet researchers have been reluctant to imply that the parental environment and behavioural and phenotypical characteristics can be inherited by offspring without a direct mutational cause, likely due to the similarity to Lamarckian principles (i.e. the theory of acquired characteristics), now long refuted as being inaccurate (Bjorklund, 2006). However, increasing evidence is now being presented that supports the idea that in addition to genetic inheritance, an organisms behaviour and environment can indeed influence development and such changes can be heritable (Jablonka et al., 2009).

All organisms respond to changes in their environment, with all aspects of an individual˘s life history potentially affected by both biotic and abiotic elements in the juvenile and parental environment. Offspring phenotype, directly influenced by the parental genotype, can also be affected by change in both the progeny and parental environments. Evolutionarily speaking, the success of an organism can be determined by its ability to survive for long enough to reproduce and thus pass on its genes to the following generation. The optimal life-history strategy will be one in which offspring are given the best possible chance of survival. Life-

history theory suggests that organisms have the ability to adapt both morphologically and physiologically in response to changes in the environment, with offspring phenotype being influenced not only by genes inherited maternally or paternally but also directly and indirectly by the environment that both offspring and parents experience, respectively.

Life history theory is concerned with explaining how organisms optimise their reproduction and survival and maximise their reproductive potential for the environment they experience (Fabian & Flatt, 2012), thus maximising fitness, and seeks to explain the variation in traits both between species and within species and individuals (Dhondt, 2001). Fitness is measured through the observation of various life history traits such as size at birth, size and age at reproduction, lifetime reproductive success (fecundity), offspring size and lifespan all of which are variable and can change according to biotic and abiotic environmental change. Life history traits can also reflect both genetic and physiological change within an organism. A central concept in life history theory is that there is always a cost to reproduction and there is always a trade-off between survivability (i.e. lifespan) and reproductive success due to there usually being a finite amount of resources that can be allocated to offspring. In females, this can usually be counted as the amount of resources invested into producing offspring plus parental care and in males in the amount of energy expended whilst searching for a mate (Roff, 1992) plus parental care, if appropriate for a given species. For example, in male wolf spiders (Hygrolycosa rubrofasciata) reduced immune function, measured by the ability of the immune system to encapsulate a foreign body, is associated with higher rates of drumming to attract a female (Ahtiainen et al., 2005). These trade-offs represent evolutionary constraints which place limits on life history evolution and stop fitness being maximised past unsustainable levels (Arnold, 1992). Life history trade-offs are common, have been demonstrated in many species and affect many traits. Resources are finite and have to be

allocated between many different processes such as immunity, growth, survival and reproduction. If an organism has to allocate extra resources to the immune system, for example, then other processes may suffer. For example, in birds, high quality plumage is important in attracting a mate and male birds with low parasite resistance may have to allocate more resources to the immune response, thus having fewer resources to allocate to finding a mate (increasing plumage quality). However, reproduction may be more critical to the male than fighting parasites and he may sacrifice his immune function for long enough to attract a mate and reproduce (Hamilton & Zuk, 1982). Here is the trade-off. If the most important thing for an individual is immediate reproduction then immune function may be compromised, as may growth. Further, sometimes the risk of predation outweighs the cost of reproduction. In the tree swallow, Tachycineta bicolor, immune function (measured by the presence of antibodies in blood samples) was found to be reduced in mothers with large brood sizes in comparison to those with fewer offspring and also according to their location (Ardia, 2005). In various frog species a trade-off has been observed between mating calls and increased risk of predation by bats (Tuttle & Ryan, 1981) and male Dollar Sunfish (Lepomis marginatus) will expose themselves to greater predation risk to guard their nests when offspring are present (Winkelman, 1996). In a detailed review of reproduction and survivability, trade-offs were discovered in eleven species of tits which showed a negative correlation between fledging success and survival, but not fecundity as only the surviving offspring directly impacted the survivability of the parents (Dhondt, 2001). These types of trade-offs can also be considered as trade-offs between current and future reproduction, with, for example, investment in growth and/or immune function now likely to increase potential future reproduction.

In parasites there is a trade-off between virulence and transmission with a higher transmission rate being associated with a lowered period of infectivity (Alizon et al., 2008) and in the entomopathogenic nematode Steinernema carpocapsae, there is a trade-off between survival and reproduction that is mediated by the presence, or absence, of symbiotic bacteria of the genus Xenorhabdus with the trade-off not being as apparent in nematodes that did not carry the symbiont, indicating that there is a further cost associated with symbioses (Emilianoff et al., 2007). Fitness is generally measured mathematically, taking a number of quantifiable fitness traits into consideration. For this study, the intrinsic rate of population increase is calculated using the Euler-Lotka equation (Rockwood, 2006) and is used as a primary fitness indicator. This calculation takes into consideration two of the major fitness traits: age at reproduction and reproductive success (Brommer, 2000) and provides a clear measure of life history variation between individuals exposed to different environments.

Maternal effects have been defined as a `direct effect of a parent˘s phenotype on the phenotype of its offspring_ (Bernardo, 1996). Note that this definition includes the effects of both (or either) parents, not just the mother and paternal phenotype can elicit a similar phenotypic change in offspring as the maternal phenotype can. Both the maternal and paternal environment can affect progeny life history, but maternal effects have been more widely studied and have been documented in almost all major phyla (Bernardo, 1996; Rossiter, 1996; R±s±nen & Kruuk, 2007; Simons, 2011) with the maternal environment influencing multiple life-history traits (e.g. fecundity, life-span, reproductive strategy, immune response). Paternal effects are generally less common than maternal effects and occur when offspring phenotype is affected by a non-genetic, male factor such as paternal genotype or environment. Paternal

effects are often much more complex than maternal effects which can be transmitted directly from mother to offspring. Fathers generally have less influence in the fate of their offspring, yet there is evidence of sperm and seminal fluid containing various compounds that affect offspring development. Paternal effects may be of more importance than maternal effects due to the increased susceptibility of the male germline to environmentally induced epigenetic changes (Crean & Bonduriansky, 2014). In the literature, the term paternal effect has been widely used to define genetically based paternal influences on both offspring phenotype and genotype (Wolf & Wade, 2009). Other terminology includes ‘parental effects’ which encompasses both maternal and paternal effects and ‘kin effects’ which includes the effect that sibling phenotypes can have on other siblings, although these are usually a by-product of a maternal effect anyway (Wolf & Wade, 2009). To avoid confusion, here, ‘maternal effect’ is used to describe any effect caused by either parent’s (maternal or paternal) phenotype and/or the effect that the parental environment has on their offspring. Although for the majority of the work conducted will be using the hermaphroditic free-living nematode C. elegans, therefore making any observed effects a consequence of maternal provisioning, the entomopathogenic nematodes Steinernema carpocapsae and Heterorhabditis downesi used to demonstrate maternal effects in a parasitic species reproduce by way of asexual (in H. downesi) and sexual reproduction so the influence of paternal effects is possible.

Environmental stress can reflect both biotic and also abiotic factors such as temperature, population density and resource limitation with the latter being perhaps the most commonly reported maternal effect inducing factor (Bernardo, 1996; Rossiter, 1996) and a variety of reproductive strategies are adopted in response to such changes. Classically, maternal effects are demonstrated in traits such as offspring size and number with size being an important factor in offspring survival (Bownds et al., 2010). For example, Daphnia spp. will opt for a

smaller number of higher quality offspring rather than a larger number of lower quality offspring in response to many environmentally induced stresses. Under crowded conditions, Daphnia will produce fewer, larger offspring that contain more lipids than those that are reared singly. This causes a transgenerational increase in parent and offspring survival probability when food is limited due to higher population size (Cleuvers et al., 1997). Gliwicz and Guisande (1992) found a similar effect in response to food availability with offspring from mothers on a restricted diet producing fewer, larger offspring than those given an unrestricted diet, however here, there was no change observed in the lipid content of the offspring. As well as progeny size adaptations in response to food limitation, Daphnia pulex will also reallocate resources from asexual to sexual egg production in response to maternal food shortage (LaMontagne & McCauley, 2001). In the planktonic rotifer, Synchaeta pectina, maternal temperature influences not only offspring size, but also developmental rate and the size and fecundity of the second generation of offspring with mothers kept at lower temperatures allocating more resources into fewer, larger offspring and those offspring developing into large adults with lower fecundity than offspring that had small hatch size and developed into small adults (Stelzer, 2002).

Some environmentally induced phenotypic changes are adaptive, acting to increase offspring survival and/or reproductive success in a specific environment. As environments are constantly changing, adaptive maternal effects generally represent attempts to maximise fitness across a range of conditions (Simons, 2014) and broadly fit into two categories: phenotypic plasticity and bet-hedging (Crean & Marshall, 2009; Marshall, Bonduriansky & Bussi· re, 2008; Marshall & Uller, 2007; Rossiter, 1991). Adaptive phenotypic plasticity occurs when environmental change is predictable and mothers attempt to match progeny phenotypes to a predicted environment that is within a normally encountered range (Simons,

2011). Bet-hedging, in contrast, works to maximise fitness when conditions are uncertain, with progeny phenotype being varied to suit a range of environments (Phillippi & Seger, 1989). This is sometimes referred to as risk management and is described as the `reduction in temporal variance in fitness at the expense of the arithmetic mean fitness_ (Ripa et al., 2010). This means that individuals should produce offspring that might do better in many conditions when conditions are variable causing an increase in lifetime fitness (or between clutch fitness; geometric mean fitness) but a reduction in immediate fitness (or within clutch fitness; arithmetic mean fitness) (Olofsson et al., 2009). Because bet-hedging strategies increase mean geometric fitness but decrease the mean arithmetic fitness, bet-hedging is sometimes perceived as detrimental with short observation times not revealing long term advantages that might take multiple generations to become clear (Simons, 2011). Bet-hedging and phenotypic plasticity are not mutually exclusive, with some species, especially plants, showing high variability under some conditions (bet-hedging) and plasticity under others (Simons, 2014). Importantly, maternal effects, as described here, are the result of a phenotypic change in offspring that maximises the fitness of the mother and her offspring with no genetic change; offspring genotype remains the same (Wolf & Wade, 2009 ¯ although maternal effects have been shown to contribute to selection for resistance to pathogens in plants ¯ see Wolinska & King, 2009). The key difference between adaptive phenotypic plasticity and bet-hedging is that adaptive phenotypic plasticity results in the expression of phenotypes that maximises fitness in a range of environments and is a consequence of variation in progeny development. Bet-hedging results in progeny phenotypes that are on average sub-optimal across all environments but that avoid complete failure in specific environments (Simons, 2011).

1.2 Mechanisms that produce maternal effects

1.2.1 Resource provisioning

While maternal effects are ubiquitous, there is an overall lack of understanding of the mechanisms that produce them (Uller et al. 2013, although see Wong et al., 1995). What is clear is that at a broad scale, maternal effects are often produced either by changes in offspring provisioning; mothers investing more (or less) resources per capita in response to their experienced or perceived environment (direct maternal effects) or are produced as a result of maternal choice, such as when to lay eggs or oviposition site choice (indirect maternal effects) (Wolf & Wade, 2009). Mothers have a finite amount of resources to allocate to their offspring and so must choose to produce many small offspring or fewer, larger offspring (Bownds et al., 2010). In oviparous species, this is routinely manifested as a trade-off between the quality and quantity of offspring, with egg size and egg number changing in response to the maternal environment (for example Lack (1940) found that birds residing near the equator had smaller clutches of eggs than those in more northerly environments, likely due to fewer available resources in hotter climates). However, it is often difficult to show a direct link between egg size and offspring fitness. For instance, even in well studied avian species, the precise relationship(s) between egg size, offspring size, and offspring fitness remain unclear (Jager et al., 2000) with direct effects of egg quality being confounded by the parental rearing ability and nest site quality. It is however clear that, in general, egg size is linked to offspring quality in birds, with smaller eggs producing slow growing chicks that fledge earlier than usual. Size is critical to survival with small eggs reducing embryo viability and hatchling survival (Wagner & Williams, 2007). This effect has been attributed to increased yolk size (and, therefore, resource provisioning) in developing eggs and also to better quality parenting. To

test these two hypotheses, Bize et al. (2002) looked to see if rearing ability had an effect on offspring quality by distributing eggs between various foster parents, thus removing the confounding factor of rearing quality, and found that hatchling size is dependent on yolk sac size and not incubation efficiency. In a meta-analysis of 5000 studies into the effects of egg size on offspring quality, Krist (2011) found that egg size is positively correlated with many traits that are used as a measure of quality (e.g. hatchling size, survival/mortality and time to fledging). However, the resulting fitness of the offspring is not well studied in birds; further study of offspring phenotype and life-history traits is needed to elucidate the effects of egg size, egg quality and parent rearing ability on offspring fitness. Similar effects are also observed in the fishes. For instance, in the Zebra Fish, Danio rerio, there is a strong correlation between egg size and hatchling size that is negatively affected by increasing maternal temperature (Bownds et al., 2010).

1.2.2 Epigenetics

Aside from direct changes in progeny provisioning, effects on progeny life history can also be produced via epigenetic changes, often in the form of maternal messenger RNAs passed into the unfertilized egg (Wolf & Wade, 2009) or as a result of changes in DNA methylation (Cooney et al., 2002). Such changes subsequently result in large scale differences in gene regulation and in life history (Youngson & Whitelaw, 2008). It is, however, important to note that, as with the types of maternal effect, changes in provisioning and epigenetic effects are not necessarily exclusive. Indeed, most maternal effects are likely to be a consequence of a complex combination of these underlying mechanisms.

Within free-living nematodes, a growing body of research into maternal effects has highlighted a variety of life-history strategy shifts in response to environmental changes (Harvey & Orbidans, 2011; Casanueva et al., 2012; Niu, Zhao & Sun, 2013; Hall et al., 2013). For instance, in model species Caenorhabditis elegans progeny reproduction is reduced in good environments when mothers are resource limited and the likelihood of larvae developing into a dauer larva is reduced under low maternal food conditions (Harvey & Orbidans, 2011). In C. elegans, progeny reproductive strategy has been found to be affected by both maternal (Harvey & Orbidans, 2011) and paternal (Miersch & DØring, 2012) effects, with the maternal environment also known to affect larval development (Harvey & Orbidans, 2011). There are fewer examples of paternal effects in the literature, perhaps because in hermaphroditic species such as C. elegans it is difficult to prevent hermaphrodites from self-fertilising their eggs. Meirsch & DØring (2012) used fog-2 mutants (fog-2 hermaphrodites are sperm defective and hence are true females yet males are unaffected) to demonstrate the effects of male dietary restriction on offspring fat content, finding that males fed a restricted diet produced offspring that had a higher fat content than males fed ad libitum. This higher fat content increased with greater dietary restriction until restriction reached a critical level and fat content dropped. This showed an inverse U-shaped effect (Meirsch & DØring 2012). Mechanisms underlying such changes are not fully understood, but appear to be controlled, at least in part, by RNAi-mediated pathways that result in phenotype disruption (Hall et al., 2013). Similar maternal effects have also been shown in other species of free-living nematode (e.g. Niu, Zhao & Sun, 2013).

1.3 Maternal effects in parasites

Whilst maternal effects are seen in many species, there is a notable absence of explicit consideration of maternal effects in parasites. Given the prevalence of maternal effects in non-parasitic species, it would be surprising if maternal effects were not occurring in parasites. Such an absence in the literature may stem from three, non-exclusive, causes. Firstly, there may be issues of terminology, with patterns of variation that would be regarded as maternal effects if observed in free-living species described using differing terminology when observed in a parasite. Considering the variable terminology used to describe maternal effects in free-living species (i.e. parental effects, environmental effects, kin effects etc.) it would not be surprising if this was true for parasites. A second possibility is that the types of experiments required to detect maternal effects have not been undertaken in parasites. The third possibility is that maternal effects in parasites do not impact on traits of medical or veterinary importance and hence they are not considered to be important. Given the expected signature of maternal effects, it is likely that evidence for maternal effects in parasites would be most obvious in species that infect a wide range of hosts and in parasites with an extended free-living stage such as the entomopathogenic and plant parasitic nematodes.

It has been widely recognised since the mid-20th century that parasite-host interactions are strongly affected by the environment. For example, genotype alone is not enough to predict the ability of barley to resist aphid infestation nor is it enough to predict the effect of bacterial infection in fruit flies where survival is more dependent on environmental temperature than genotype (Wolinska & King, 2009). In a number of parasite species, patterns of variation between populations in various traits are consistent with bet-hedging, with variation

potentially maximizing the chances that at least some individuals successfully infect. For example, Fenton and Hudson (2002) hypothesise that in an environment in which host availability is unpredictable, parasite infectivity should be spread out with some parasites being highly infective to take advantage of immediately available hosts and others being less infective in order to wait for hosts yet to appear. This has been demonstrated by Griffin (2012) in entomopathogenic nematodes with the first infective juveniles emerging from a host being less infective than those emerging later. Perhaps in single-host parasites, maternal effects are not as important due to mothers predicting that a host environment is relatively constant; however, based on the wealth of information about maternal responses in so many species it would be short-sighted to overlook this area of research. Temperature and nutrient availability are the most commonly investigated environmental conditions when looking at host-parasite-environment interaction with most studies focussing on outside host conditions, however it is equally, if not more, important to consider the effect of the within host environment where parasites have to contend with host immune function and other physiological responses to invasion (Wolinska & King, 2009). This is true for plant and animal hosts alike.

1.3.1 Maternal effects in plant parasitic nematodes

The study of maternal effects may be particularly important in plant parasites, for instance, where the fitness and subsequent virulence of infective juveniles is of significance. In plant parasitic nematodes, environmental factors are taken into consideration when looking at hatching success and offspring survival (e.g. Goodell & Ferris, 1989); however, fitness consequences are rarely considered and authors tend to concentrate on determining the direct influence of the environment. Temperature is demonstrated to have an effect on hatch rate in

a number of studies (e.g. Ploeg & Maris, 1999; Starr, 1993) but again fitness cost to progeny is not considered. Some past studies have touched on the idea of maternal effects in the Meloidogyne family, perhaps without realising it, but have not necessarily considered the potential consequences of their findings. For example egg masses of M. incognita grown on tomato are different from those isolated from cotton (Starr, 1993). In this case, the egg masses were more embedded into the tomato roots than they were in cotton, resulting in the cotton derived eggs being lost (i.e. desiccation or detachment from the root) more quickly. Whether or not this affects progeny fitness in unknown; however, this does highlight the fact that there is a difference in progeny (at least at egg stage) produced by mothers in different hosts. Environmental differences such as host species or cultivar, nematode population size host stress and growth conditions also affect egg production rates and sex ratio in Meloidogyne spp. (e.g. Ferris, Schneider & Semenoff, 1984; Holz, Troth & Atkinson, 1999) and Globodera spp. (e.g. Phillips, 1984) and are therefore likely to impact life-history traits in similar ways to how the same conditions affect non-parasitic species. Temperature affects the rate at which RKNs develop and M. hapla has been shown to be able to adapt to freezing conditions after being exposed to cold (Forge, 1990). Host species dramatically affects parasite development and has been shown to impact fecundity, and the size and severity of root galling in a host, however, such changes in development are not always considered when assessing development in pot and field trials. For example, in pot trials to determine the susceptibility of Winter Rapeseed (Brassica namus) to Meloidogyne hapla, Bernard & Montgomerydee (1993) used infective juveniles isolated from stock population reared in peanut plants to cross infect rapeseed crops and then used infected tomato plants as a control population for reference. This meant that the severity of infection in rapeseed was compared to the severity of infection in tomato, a very good host. The conclusion here was that Winter Rapeseed is a host for M. hapla, but the severity of infection may be greatly influenced by the life-history

of previous generations especially given that development in peanut is much quicker than in tomato with moulting occurring much faster in the former than in the latter (Castillo et al., 1973)

1.3.2 Maternal effects in entomopathogenic nematodes

Entomopathogenic nematodes are important biological control agents, partly due to their wide host range (although see Lamb et al. (1996) who believe this has been overestimated in the Steinernematids). They infect multiple host species, mostly in the Coleoptera and Lepidoptera (Diptera, Hymenoptera and Satatoria infections appear to be less common) and play a major role in regulating natural insect populations (Peters, 2010). In these systems, fitness is likely to be a factor that can be manipulated for various benefits; a fitter parasite might be better at controlling a pest problem, whereas a less fit parasite might be better suited to reducing a pest population without destroying it entirely although nematode persistence in an area they may be considered invasive is fairly low with the number of nematodes falling to 1% of the initial application number within four to six weeks and this population size remaining sustainable (Smits, 1996). Temperature greatly contributes to parasite fitness and EPN species vary greatly in their thermal niches which must be considered when selecting a species for biological control in specific climates (Grewal et al., 1994), although developments in genetically modified organisms for biocontrol are promising. A transgenic strain of Heterorhabditis bacteriophora, modified by the insertion of a C. elegans heat shock protein has been developed to better persist in warmer climates (Gaugler et al., 1997). Environmental effects have also been documented in entomopathogenic nematodes with various species changing their reproductive strategy in response to competition, host condition and time of hatching; however, such studies are rare and do not always consider

such strategy shifts as a result, or direct consequence, of a maternal effect. Perhaps the most important type of maternal effect is one that affects the transmission ability (or virulence) of infective juveniles. In Steinernema carpocapsae and Heterorhabditis megidis, host condition and competition have been shown to have an impact on offspring size, number of offspring produced and juvenile infectivity (Therese & Bashey, 2012; Ryder & Griffin, 2003).

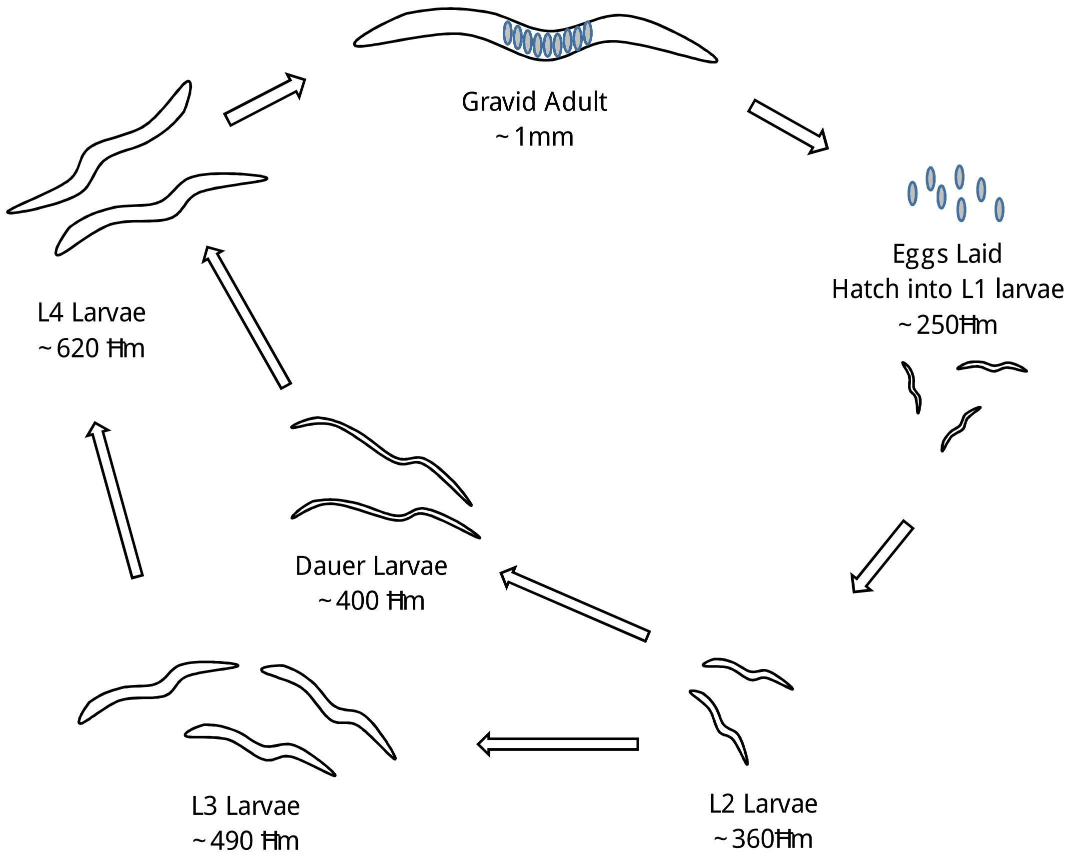
1.3.3 Maternal effects in vertebrate parasites

Maternal effects in vertebrate parasites are harder to identify, likely due to the complexity of the host and outside confounding factors such as the host˘s environment and its effect on host behaviour and physiology. However, there is evidence to suggest that parasite life-history is greatly affected by the environment it experiences and this has consequences for the fitness of subsequent parasite generations. Helminth parasites of fish have been shown to be influenced by the host˘s environment with parasite population size reduced when external temperatures cause increase immune function in the host. This causes offspring to favour intermediate hosts for further reproduction during warmer seasons (Thomas, 2002). The impact of intermediate hosts on parasite fitness introduces a further complication for assessing environmental impact on parasite life history and fitness. The immune response of the host elicits many responses in parasite life history traits. There is a growing body of evidence to show that parasites will adjust their reproductive strategy according to the strength of the host˘s immune system. For instance, if immune response is delayed or ineffective, parasites are likely to slow their growth, live longer and increase lifetime fecundity (Viney & Cable, 2011).

1.4 Caenorhabditis elegans as a model for nematode parasites

While is it important to directly investigate maternal effects in parasites, most parasites of medical and veterinary importance are difficult to culture in the laboratory. Obligate parasites require a host, making the continuous production of many parasitic species costly and labour intensive. For vertebrate parasites, there are also ethical considerations around the use of host animals. These represent a particular concern given the highly factorial nature of the experiments required to detect maternal effects. The use of model organisms is therefore appropriate as they provide an excellent system for preliminary investigations.

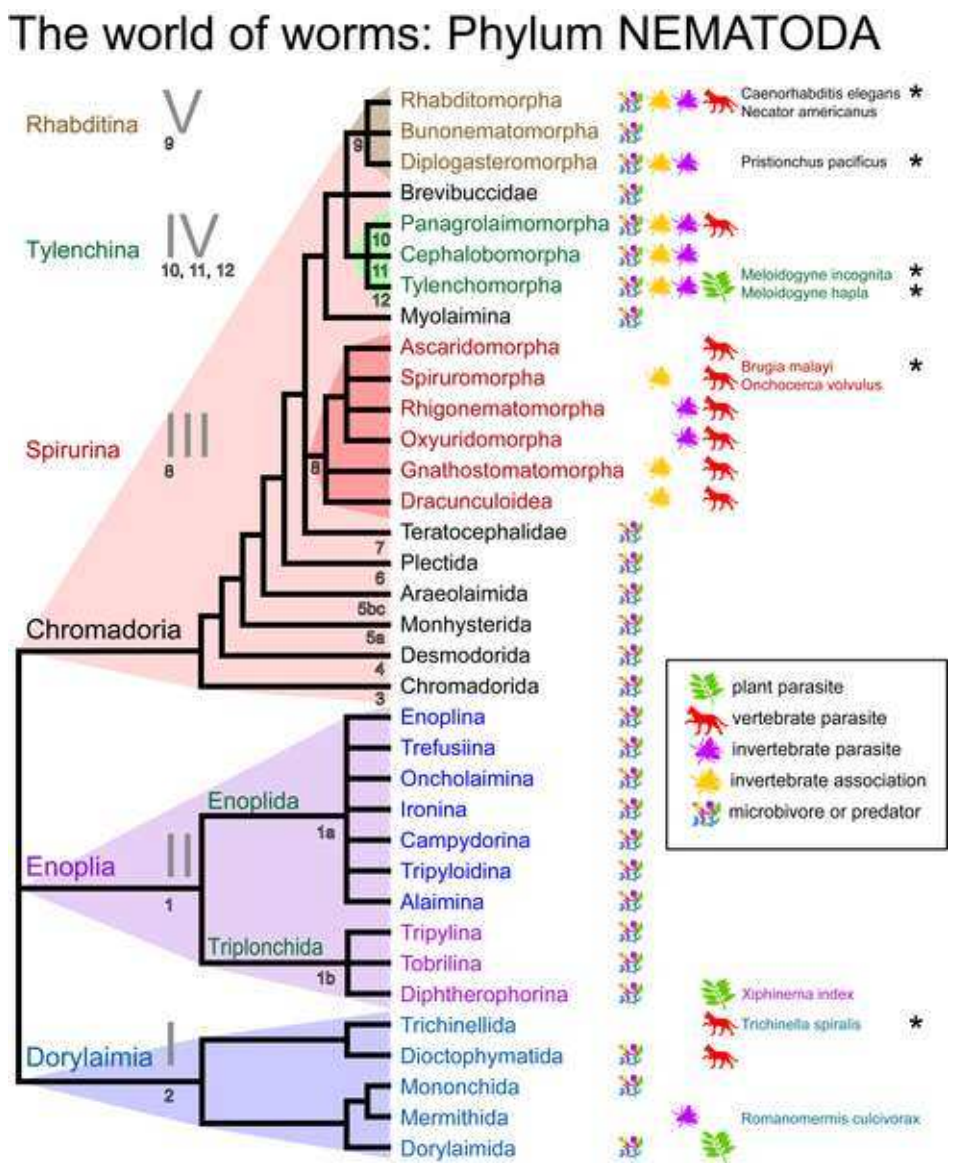
Caenorhabditis elegans is a free-living nematode of the genus Rhabditidae first used to study development and behaviour by Sydney Brenner (Brenner, 1974). Since then, its use had become widespread in studies from population biology to neurology. More recently, there has been more work on the ecology and evolution of the species. This work indicates that C. elegans is associated with rotting vegetable matter and that very large populations can be found in the wild (F¶lix & Braendle, 2010). Worms are easily reared in the lab on agar plates with a bacterial food source (usually Escherichia coli) and have a fast generation time and rapid life cycle (figure 1-1) with fertilisation to maturity occurring in just three days at 25éC (Riddle et al., 1997) and can withstand and remain viable after long term freezing (Sulston & Hodgkin, 1988). The transparency of the worms means that cells are easily observed under differential interference contract (DIC) microscopy and this has made it possible to observe cell division and migration in living animals leading to a complete cell lineage being described by Sulston et al. (1988). Worms can either develop into self-fertilizing hermaphrodites or males (0.2% of a population in the canonical wild type, N2).


Gravid Adult
~1mm
Eggs Laid
Hatch into L1 larvae
~250Ħm
L4 Larvae
~620 Ħm
Dauer Larvae
~400 Ħm
L3 Larvae
~490 Ħm
L2 Larvae
~360Ħm

Further, C. elegans can opt to enter a third larval stage diapause forming dauer larvae in response to various environmental cues (Golden & Riddle, 1984). The dauer larva is a facultatively formed alternative stage three larva that is arrested (meaning it does not need to feed for an extended period) and stress resistant. Dauer larvae form in response to environmental cues such as low food availability in crowded conditions, temperature and in response to nematode pheromones (Golden & Riddle, 1984). Dauer development is therefore likely to be adaptive in that it will allow larvae to disperse and find a more suitable environment for development and reproduction. The dauer larva is highly similar to the free-living infective stages of many parasites and is likely to be a pre-adaptation for the evolution of parasitism (Stasnick et al., 2012). Indeed, some nematode species that are facultative parasites, meaning that they can be completely free-living given the right conditions but will develop into infective dauer-like larvae when a host is required, have lifecycles very similar to C. elegans. An example of this is the facultative intestinal parasite, Parastrongyloidies trichosuri which is able to complete its life cycle without a host but under environmental stresses (similar to those that cause dauer development in C. elegans) will complete the life cycle in a host (Stasiuk et al., 2012).

In 1998, C. elegans was the first organism to have its 100Mb genome fully sequenced (C. elegans sequencing consortium, 1998). The C. elegans genome contains 20, 444 protein-coding genes (Wormbase Release WS245, 2014) with an average gene size of 3kb, which are spread over six chromosomes, linkage groups (LG) I, II, III, IV and V, and X (Corsi et al., 2015). With a fully sequenced genome, C. elegans can be used in fitness assays (measuring changes in phenotypic traits such as fecundity and longevity to predict fitness advantages/disadvantages following various treatments, such as drug exposure, and/or

environmental manipulations, such as temperature change) to predict parasite responses to similar conditions due to shared homology and highly conserved genes throughout the phylum Nematoda. C. elegans also represents an attractive model to understand gene function in parasites as parasite genes of interest can be identified and homologous C. elegans genes can be investigated, understood and the knowledge gained can be transferred to parasite study (Blaxter, 1998). Nematode phylogenies identified a number of clades (figure 1-2). This framework indicates that C. elegans, a member of clade V, is closely related to a number of animal parasites and to the entomopathogenic nematodes (Heterorhabditis spp and Steinernema spp.).


The world of worms: Phylum NEMATODA
Rhabditina
V
9
Tylenchina
IV
10, 11, 12
Spirurina
III
8
Chromadoria
Enoplia
II
1
Enoplida
1a
Triplonchida
1b
Dorylaimia
I
2
Rhabditomorpha
Bunonematomorpha
Diplogasteromorpha
Brevibuccidae
Panagrolaimomorpha
Cephalobomorpha
Tylenchomorpha
Myolaimina
Ascaridomorpha
Spiruromorpha
Rhigonematomorpha
Oxyuridomorpha
Gnathostomatomorpha
Dracunculoidea
Teratocephalidae
Plectida
Araeolaimida
Monhysterida
Desmodorida
Chromadorida
Enoplina
Trefusiina
Oncholaimina
Ironina
Campydorina
Tripyloidina
Alaimina
Tripylina
Tobrilina
Diphtherophorina
Trichinellida
Dioctophymatida
Mononchida
Mermithida
Dorylaimida
9
10
11
12
8
7
6
5bc
5a
4
3
Caenorhabditis elegans *
Necator americanus
Pristionchus pacificus *
Meloidogyne incognita *
Meloidogyne hapla *
Brugia malayi *
Onchocerca volvulus
Xiphinema index
Trichinella spiralis *
Romanomermis culicivorax
plant parasite
vertebrate parasite
invertebrate parasite
invertebrate association
microbivore or predator

In drug discovery studies, C. elegans again proves a useful model, especially when looking for mode of action of novel drugs and for mechanisms of resistance (Riddle et al., 1997). Indeed, experiments using C. elegans have been key to identifying mechanisms of action in all major anthelmintic groups (Gilleard, 2006). Mechanisms of action of a relatively newly identified class of anthelmintics, the Amino Acetonitrile Derivatives (AADs), have also been identified in C. elegans as being involved in disrupting nicotinic acetylcholine receptor (nAChR) alpha subunits and mutations in the gene acr-23 (acetyl choline receptor) were found to confer resistance to the compounds. These identifications informed the discovery of the target gene in the gastrointestinal nematode Haemonchus contortus (a nematode that parasitizes sheep, surviving in the abomasum), Hco-mptl-1 (Haemonchus contortus-monepantel-1; monepantel is the generic name of the AAD used for the study) (Rufener et al., 2009a).

Caenorhabditis elegans has also been extensively used to identify mechanisms of drug resistance. Briefly, this involves mutagenesis followed by selection for resistance to a particular compound followed by identification of a candidate gene (Gilleard, 2006). For example, Driscoll et al. (1989) successfully used C. elegans to identify and characterize mutations that confer benzimidazole resistance and these have been confirmed as being present in Haemonchus contortus with homologous genes conferring a similar mechanism of resistance (Rufener, 2009b). C. elegans is particularly well suited to studies focussing on nAChR affecting drugs because the gene nAChr gene family is fully described in C. elegans and the species has the most extensive and diverse nAChr gene family yet to be described (Brown et al., 2005). nAChRs are important for synaptic signalling in the nervous system and neuromuscular junctions and as such, drugs that targets them cause paralysis. However in many cases, genes identified as responsible for conferring resistance in C. elegans have not

always been identified in resistant parasites (Laing, 2010). Further, mutations identified in C. elegans that confer resistance to a compound in in vitro experiments may not always be relevant for parasite resistance as C. elegans is more robust than many parasite species with similar mutations in homologous parasite genes being found to be lethal and, therefore, redundant in their ability to confer resistance (Gilleard, 2006).

1.5 Specific aims of this study

Given the widespread evidence for the existence of maternal effects it is highly likely they also exist within the parasites. Such phenomena in parasites and their impact on parasite life-history could have wide ranging implications on health and agriculture. It is therefore important that we determine the response and impact of environmental change on parasite fitness traits. For the reason discussed above, C. elegans and the entomopathogenic nematodes represent good candidates for investigating these phenomena as a necessary precursor to undertaking such analyses in parasites of medical, agricultural and veterinary importance. In this study the specific aims are:

1. Confirm the existence of maternal effects in the free-living nematode, Caenorhabditis elegans and further explore the role of maternal temperature in nematode development. Temperature has been shown to affect many life-history traits and is linked to many examples of within and among clutch variation however, much of these effects are seen in response to simple temperature change; experiments are performed to investigate the differences in progeny at

two different temperatures (e.g. 15é vs. 25éC). Whilst this has provided a wealth of information about the direct influence of temperature on offspring development as well as the indirect effects of maternal temperature, these conditions do not reflect the conditions likely to be experienced by wild populations and in this sense only go so far to provide ecological knowledge that can be applied outside of the laboratory. By recreating temperature conditions more similar to outside conditions in the laboratory, temperature that mimics daily temperature fluctuations in a cyclical fashion (i.e. cooler at night and gradually increasing throughout the 'day') and observing reproductive behaviour and development in both adult and progeny, a wider understanding of temperature effects can be gained. Importantly, this will reveal whether previously gained knowledge of constant temperature effects need to be revisited and perhaps highlight the effects of constant temperature on laboratory populations which, if not optimal (especially in wild-type strains), may be a confounding factor in many developmental and behavioural studies.

2. Provide evidence that maternal effects exist in parasites using entomopathogenic nematodes to assess progeny traits and fitness effects, firstly in response to temperature differences as have been demonstrated in C. elegans and the effects that different host species have on progeny phenotype. It is expected that low quality vs. high quality hosts will provide a difference in resource availability either due to difference in lipid or protein composition.

3. Investigate the nematicidal properties of the African Marigold, Tagetes erecta by extracting compounds from the roots, shoots and petal of marigold plants and

treating C. elegans with crude extracts. C. elegans will be used to investigate the effects of the marigold extract on nematode development, longevity and reproduction. This will be used to provide information about the impact of maternal exposure to pesticide-like compounds and determine whether offspring fitness is improved when mothers are reared in the presence of such compounds.

4. Demonstrate the expected important role of maternal effects in parasites by using C. elegans to investigate whether maternal exposure to low concentrations of anthelmintic drugs induces a change in reproductive strategy and results in increased progeny fitness. If mothers are able to produce offspring that are better able to cope with anthelmintic treatment then this could have widespread implications for human and veterinary medicine and may be an influencing factor in parasite resistance. Additionally, if maternal effects can mediate increased parasite fitness in traits such as virulence and longevity, this could be an important factor in host-parasite co-evolution.

Chapter Two

Maternal Effects in Caenorhabditis elegans: Temperature

Abstract

All organisms are exposed to environmental fluctuations with some being predictable and some being unpredictable. Temperature has major effects on the physiology, ecology and fitness of many organisms and temperature stress can either negatively affect fitness, when temperatures reach extremes that are outside of an organism˘s natural range, or can increase fitness via the induction of a hormetic effect when gradual change or short term exposure results in an adaptive response and an increase in fitness, causing a change in life-history traits such as reproductive success. Much is known about the effect of temperature in C. elegans with multiple studies that provide insight into the optimal temperature range of the species and much is known about the geographical distribution of the species with isolates from many climates having been discovered. However, much of this work concentrates on the behavioural and physiological adaptations under constant temperatures. Several studies look at multiple temperatures and provide information about nematode responses to temperatures ranging from low temperatures that result in cold stress (below 12éC) and heat stress (above 26éC) and much is known about the genetic pathways involved in such responses. However, little is known about how nematode behave under variable temperatures. In the wild, temperature is rarely constant and fluctuates daily and seasonally yet laboratory experiments are usually conducted under constant conditions. In most cases this is for good reason, however in order to understand the natural ecology and behaviour of C. elegans, the effects of variable temperature should be investigated. Here, multiple C. elegans isolates, are reared under variable temperature conditions and their reproductive behaviour is compared to worms raised under various constant conditions. In the canonical C. elegans strain, N2, exposure to cyclically varying temperature (intended to reproduce a daily temperature cycle) results in delayed development when compared with worms reared under constant conditions. This difference is not as clear in wild isolates and may be indicative of laboratory adaption in N2.

2.1 Introduction

All organisms are exposed to environmental fluctuations over a range of timescales. Some of these fluctuations are predictable and some are not. Intuitively, we would expect that, for evolutionarily relevant variation, natural selection will have optimised the way organisms respond to this variation in a way that maximises fitness. Essentially, organisms would be expected to tailor their life histories to environmental conditions in order to maximise offspring survival and reproduction (Cleuvers et al., 1997; McNamara & Buchanan, 2005). This could be achieved by behavioural, morphological and/or physiological responses and can result in effects that span generations, with the parental environment known to have a major influence in progeny life-history traits (e.g. fecundity, life-span, reproductive strategy, immune response) in many species (see Bernardo , 1996 for review). However an optimised response might be absent in challenges without an evolutionary or ecological relevance.

Temperature has been demonstrated to have major effects on the physiology, ecology and fitness of many organisms and temperature stress can either negatively affect fitness when temperatures reach extremes that are outside of an organism˜s natural range or induce a hormetic effect when gradual change or short term exposure results in an adaptive response and an increase in fitness, causing a change in life-history traits such as reproductive success. Such effects usually follow an inverse u-shaped pattern with fitness increasing up to a critical point where the stress becomes lethal. These effects have also been labelled as adaptive responses and preconditioning and are seen in responses to other environmental stresses such as low doses of toxic compounds including pesticides (see chapter 4), drugs (chapter 5) and heavy metals (Calabrese et al., 2007). For example, early exposure of short periods (3 hours every 3 days) to a higher than optimal temperature (34éC) results in increased lifespan but

reduced cumulative fecundity in Drosophila melanogaster (Snrensen et al., 2008). In C. elegans, exposure to 30éC for six hours increases lifespan by 12.5% (Yokoyama et al., 2002) and exposure to 35éC for up to two hours extends lifespan, however after any longer exposure, effects become deleterious (Cypser & Johnson, 2002) although this may not be a universal response as most identified lifespan effects are constrained to very specific, narrow experimental conditions (Lagisz et al., 2013) and lifespan effects in C. elegans appear to be genotype-specific (Stastna et al., 2015). It is therefore expected that the thermal preference of an organism should be a temperature, or range of temperatures, that maximise fitness. Any deviation from this thermal niche, however temporary, is likely to have an associated fitness cost (Anderson et al., 2011). Whilst there is a vast quantity of research that explores this, observed fitness effects when any organisms are exposed to constant temperatures, which most laboratory based studies are based upon, are unlikely to be indicative of natural behaviour (Arrighi et al., 2013); outside of the laboratory, temperature would not be constant and therefore one would expect wild individuals to adapt their behaviour and physiological responses in response to a constantly changing environmental temperature. Behavioural and physiological adaptations have been reported in multiple species in response to temperature with lower than optimal environmental temperature (i.e. a temperature below the threshold at which development is unhindered) resulting in a reduced number of larger offspring. C. elegans will move toward temperatures that maximise fitness when exposed to thermal gradients with the intrinsic rate of population increase (r) increasing with temperatures up to a maximum of 23éC (after which r declines steeply) and lifetime reproductive success increasing up to 20éC after which it begins to be hindered (Anderson et al., 2007). Butterfly (Bicyclus anynana) eggs laid at 20éC are larger than eggs laid at 27éC and the larger eggs give rise to larger offspring which develop more quickly and have a greater probability of survival at lower temperatures that their counterparts (Fischer et al., 2003). Indeed,

temperature has been shown to affect size both directly, i.e. individual, or parental, size) and indirectly as a result of maternal, and paternal (see Crean & Bonduriansky, 2014), effects in multiple species (e.g. Rotifers (Stelzer, 2002), Zebra Fish (Bownds et al., 2010), and may be indicative of a bet-hedging strategy in response to environmental change (Simons, 2014). An alternative view, suggested by Angilletta Jr. et al. (2006) from a comparative analysis of studies investigating temperature effects, is that it is unclear whether temperature directly or indirectly affects offspring size due to varied methodology and analysis.

The maternal environment has been shown to directly and indirectly affect progeny fitness. Many maternal effects are adaptive and tend to represent attempts to produce progeny that are suited to their expected environment or change the variation between progeny (bet-hedging). Such effects have been documented in many species in response to various environmental factors such as resource limitation, where C. elegans mothers reared with limited food will produce fewer, larger offspring that do better under low food conditions (Harvey & Orbidans, 2011) and Daphnia showing the same plastic response under similar conditions (Gliwicz & Guisande, 1992) and overcrowding (in Daphnia, the direct effects of crowding result in smaller individuals (f_1 generation) but their offspring (f_2 generation) are bigger and more resistant to starvation (Cleuvers et al., 1997)). Previous research has shown that progeny from mothers exposed to poor conditions are more successful in similar poor conditions; they have a fitness advantage over progeny from parents kept in constant non-stressful conditions (Bashey, 2006; Mugabo et al., 2010).

In C. elegans temperature has been demonstrated to affect multiple life-history traits. For instance, subtle temperature differences have been found to influence lifetime reproductive success, reproductive strategy, and changes in offspring size (measured by egg and L1 size)

have been observed at various temperatures (Harvey & Orbidans, 2011) with fertility dramatically reduced at temperatures of 24-27éC (Poullet et al., 2015). Exposure to high temperature has been shown to affect fitness in individual worms and their offspring. High temperatures can be responsible for changes in behaviour and development with worms exposed to higher than optimal temperatures becoming sterile once the temperature exceeds 27éC (F¶lix & Braendle, 2010). Heat stress begins to occur once the temperature exceeds 26éC, at which the heat shock response is induced and major changes in physiology are observed. For instance, feeding rate is reduced (Jones & Candido, 1999). Heat stress has also been linked with increased longevity in a process thought to be mediated by expression changes in the insulin-like signalling pathway with thermo-tolerence and longevity both affected by similar gene expression (Mu¿oz, 2003) suggesting that a worm˜s ability to avoid stress is negatively linked to life expectancy (Lithgow et al., 1995). Interestingly, whilst hormetic responses to heat stress have been shown, in C. remanei heat stressed parents produce offspring that are less tolerant to heat stress (Sikkink et al., 2014). Much less is known about the effects of very low temperatures (i.e. below 12éC), but interestingly, the same signalling pathways involved in responses to high temperature heat stress are also involved in low temperature heat stress and cold tolerance is improved in individuals that experience cold whilst in the dauer stage of development (Savory et al., 2011). Whilst many individuals will survive at such temperatures, eggs take longer to hatch and the time from hatch to egg-laying is extended. Adult worms take much longer to reach sexual maturity and this makes data collection difficult and often incomparable to data collected at other temperatures partly because of the major physiological changes caused by exposure to extreme temperatures (development halts at temperatures below 8éC (F¶lix & Braendle, 2010) and also because of technical limitations; development is so delayed at low temperatures that worms are often incomparable to those reared at higher temperatures.

Fitness effects of various temperatures have been explored and temperature preference can be investigated by testing temperatures along temperature gradients, also determining the particular temperature at which fitness is maximised (e.g. Anderson et al., 2011). Many studies have investigated the effects of temperature on C. elegans fitness, yet do not always provide insight into the natural behaviour of the worms in response to temperature. Whilst these types of studies provide good information about how nematodes respond to a range of temperatures, they are still not simulating realistic, field-like conditions. If we are to truly gain an understanding of wild population dynamics, we need to simulate field-like conditions in the laboratory. Given that the canonical wild type isolate of C. elegans, N2, spent many years in continuous laboratory culture, this system also potentially allows the investigation of the effects of long term selection in an environment that, in contrast to the wild, is relatively constant. Consistent with this, the fecundities of N2 and CB4856, two of the most laboratory adapted C. elegans isolates, are more sensitive to high temperatures, with brood size decreasing progressively from 20éC to 26éC, than those of many more recent isolates (Petrella 2014), although Harvey and Viney (2007) found that N2 was more tolerant to temperature change than some wild isolates and isolates differ in their thermal tolerance according to the geographical location they were originally isolated from. Additionally, Poullet et al. (2015) found that C. elegans strains isolated from regions with different climatic conditions do not significantly differ in their reduction in fecundity observed from 20éC to 26éC and that many strains follow a similar fecundity decrease as N2. However different Caenorhabditis species (such as C. briggsae and C. tropicalis) show strong reactions to upper thermal limits and tolerance is related to natural climatic conditions (Poullet et al., 2015).

Even less is known about the physiological and evolutionary effects of exposure to variable temperatures, especially in field conditions, not just in nematodes but in many invertebrates where much is known about direct effects of constant temperature. Whilst much is known about the behavioural responses of individuals to temperature and the fitness effects of stressful temperatures, very little is known about adaptation to conditions experienced in the wild. Outside of the laboratory, temperature is not constant. Air temperatures can change dramatically throughout a 24 hour period, and soil temperatures are affected by many factors such as air temperature, humidity and precipitation with the most dramatic differences caused by seasonal change. Little is known about wild population dynamics or behaviour (Barri· re & F¶ix, 2005) however, recent research using wild isolates of C. elegans and other Caenorhabditis species is beginning to shed light on this (Cutter et al., 2006; Barri· re & F¶ix, 2007; Prasad et al., 2010; F¶ix et al., 2013). This greatly increases the need and opportunity to explore adaptive behaviour in wild populations. Are maternal effects, for instance, as common in the wild as they are in the laboratory?

Here, the effects of cyclically varying temperature on multiple C. elegans isolates are investigated and show that the temperature experienced by the parent impacts progeny fitness (measured by lifetime reproductive success and the intrinsic rate of population increase). Comparison of the response of recent wild isolates (JU1931 and JU1941) to responses of the Hawaii strain CB4856 and the canonical wild type N2, both of which have an extensive history in the laboratory, indicates that there is a marked difference in the response to temperature variation between the four strains. We also look at the direct effects of various temperatures on population growth and dauer larvae formation and show that the proportion of dauer larvae in a population is slightly higher when rearing temperature is variable that when temperature is constant.

2.2 Methods & Materials

2.2.1 Worms

N2 and CB4856 were obtained from the Caenorhabditis Genetics Centre. The wild isolates JU1931 and JU1941 (see Volkers et al. 2013 for details of these strains), were obtained from Jan Kammenga (Wageningen University). All isolates were maintained on NGM plates using standard methods (Stiernagle, 2006), with Escherichia coli (OP50 strain) as a food source. Worms were synchronised at the L1 stage prior to each assay by isolating eggs from gravid hermaphrodites by hypochlorite treatment (Stiernagle, 2006) and allowing them to hatch overnight at 20éC on NGM plates without food. Arrested L1s were then transferred to plates with food and allowed to develop to the L4 stage, before use in assays. Within assays, plates were blind coded and treatments were randomised.

2.2.2 Fluctuating temperature in N2

To assess the effect of fluctuating temperatures on N2, worms were incubated at one of four different temperature treatments: a constant temperature of 10, 15 or 20éC, or a variable temperature that cycled between 10éC and 20éC over a 24 hour period. For the variable temperature treatment, a programmable incubator (MLR-351H, Panasonic, Bracknell, UK) was set to increase or decrease incrementally over a 24 hour period. The temperature was set to increase gradually for 12 hours from 10 to 20éC and then decrease gradually for 12 hours back to 10éC. This temperature change repeated for each 24 hour period throughout the course of the experiment.

For this assay, arrested L1s were transferred, individually, to NGM agar plates inoculated with 50ıl of an overnight culture of OP50. Plates were then equally divided among

temperatures (n = 20 per treatment) and worms were allowed to develop until they were gravid. Once egg-laying had commenced, worms were moved daily until reproduction had ceased, with eggs allowed to hatch and develop to approximately the L4 stage at which point the number of progeny were counted. Measures of lifetime reproductive success (LRS) and estimates of the intrinsic rate of increase (r, see below for details) therefore include only progeny that successfully complete development to L4 stage. In order to investigate the possible maternal effects of each temperature treatment, an additional plate containing a larger population of worms (Ca. 500 worms per plate) was maintained at each temperature. These worms were allowed to develop until they were gravid then hypochlorite treated (as above) to release their eggs. Eggs from each temperature were divided onto four separate plates, each of which was placed at a different temperature treatment and allowed to arrest overnight. These arrested L1s were then placed individually (n = 20) onto plates with food and used to determine progeny fecundity (as above). Fecundity was recorded daily and the time taken from being fed to the first day of reproduction noted.

2.2.3 Growing population assays

Assays for the number of worms at food exhaustion and the number of dauer larvae at food exhaustion were performed as previously described (Green and Harvey, 2012; Green et al. 2013). Briefly, this involved initiating growing populations on 55mm diameter `sloppy agar_ plates (2% NGM agar, peptone excluded) with one N2 L4 hermaphrodite and 100ı l of 20% weight/volume suspension of E. coli collected from an overnight culture. All food within an experiment was from the same batch of prepared bacteria and plates that became contaminated or on which populations did not develop were excluded from all analyses. Plates were monitored daily until food exhaustion, defined by the depletion of all food, a stage recognised by the dispersal of all worms from the exhausted area. The total number of

worms on each plate was then counted and the number of dauer larvae calculated (Green and Harvey, 2012; Green et al. 2013).

2.2.4 Variation between isolates

Arrested L1s were prepared as stated previously for the isolates N2, CB4856, JU1931 and JU1941. Because the direct effects of temperature had already been established, multiple worms were grown to L4 stage with plentiful food, under uncrowded conditions at each temperature treatment. As previous observed effects were subtle (possibly because 15 and 20éC are considered normal, non-stressful conditions for C. elegans), the temperatures were altered to range between 10 and 25éC. Thus, one cohort of worms was grown at 17éC and another at the increased variable temperature (cycling between 10 and 25éC). Maternal effects were assessed, as before, by isolating eggs from mothers grown at each temperature treatment and separating them between each treatment; offspring from mothers at each temperature were observed at each temperature for potential fitness consequences of maternal temperature.

2.2.5 Statistical methods

The intrinsic rate of increase, r, was estimated by using the Euler-Lotka equation (taken from Rockwood, 2006):

Where e is the natural log (2.7183), l is the rate of survival and m is the daily fecundity. Data were analysed by Kruskal-Wallis and paired Mann-Whitney tests due to non-normal distributions. Here, the intrinsic rate of increase is calculated (according to the Euler-Lotka equation) to measure progeny fitness and provide a more accurate analysis of lifespan, age at maturity and lifetime fecundity. Lifetime fecundity (LRS) as a stand-alone measure of fitness provides information about the ability of an individual to reproduce, but r additionally provides developmental data alongside reproductive success (see Huey & Berigan, 2001 for comparison). Additionally, this helps consolidate data that are gathered under conditions that produce very different progeny. For example, progeny of C. elegans produced at 10éC take much longer to reproduce than those at 25éC, meaning that classic fitness measures may not always be comparable under such divergent conditions. Using r provides a more standardised measure under these conditions because the extended reproduction time is included in the calculation of r.

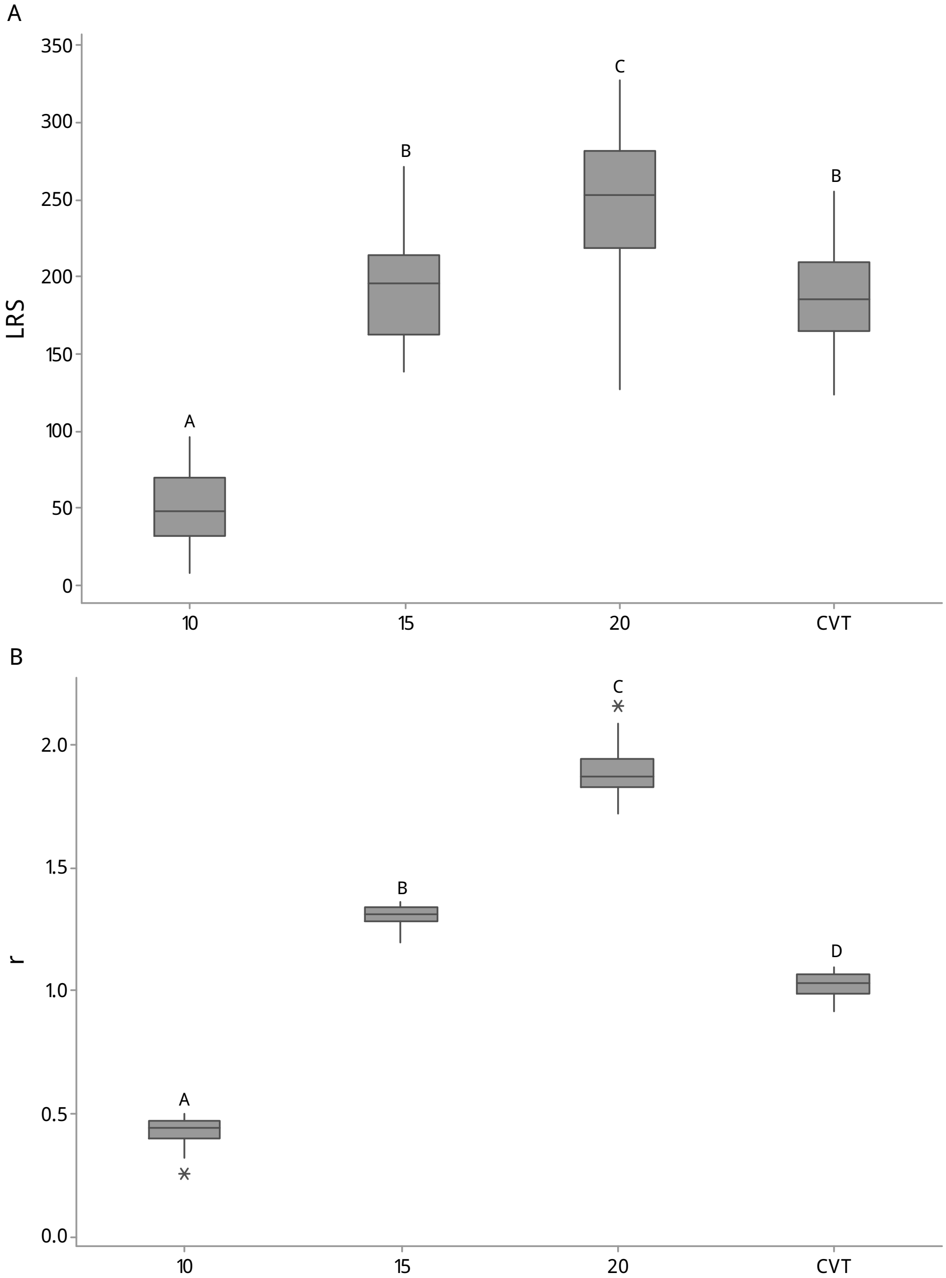
2.3 Results

2.3.1 Direct and indirect individual effects of varying temperatures

Temperature treatment directly affects LRS and r (Fig. 2-1, Kruskal-Wallis test, $H_{(86,2)}$ = 66.23, $p < 0.001$ & $H_{(86,2)}$ = 76.43, $p < 0.001$ for LRS and r, respectively). Comparison of the CVT treatment and the corresponding mean constant temperature treatment (15éC) shows that CVT negatively affects r, but not LRS (Figure. 2-1A vs Figure 2-1B). This indicates that development is slower when worms are exposed to CVT. Analysis of the comparable progeny data (i.e. the progeny from the 20éC parental treatment) shows the same result (Figure 2-2A vs Figure 2-2B). Analysis of the progeny data also reveals indirect effects (Fig. 2-2). Here, maternal effects are observed in LRS when progeny are assayed at all temperatures (Fig. 2-2A, Kruskal-Wallis tests: $H_{86,3}$=17.02, p=0.001; $H_{114,3}$=34.82, p<0.001; $H_{112,3}$=99.33, p<0.001; $H_{115,3}$=42.93, p<0.001 for progeny reared at 10éC, 15éC, 20éC and CVT, respectively). Similarly, maternal effects are observed in r at all progeny assay temperatures (Fig. 2B, Kruskal-Wallis tests: $H_{104,3}$=9.19, p=0.026; $H_{114,3}$=21.52, p<0.001; $H_{104,3}$=20.81, p<0.001; $H_{113,3}$=26.38, p<0.001 for progeny reared at 10éC, 15éC, 20éC and CVT, respectively) except for in progeny from mothers at CVT where LRS is reduced (Mann-Whitney test, W = 1132.0, $p < 0.001$) but r is unaffected (Mann-Whitney test, W = 830.5, p = 0.4) when progeny are reared at CVT compared with those reared at the corresponding mean temperature of 15éC.

These data argue that, in N2, maternal exposure to variable temperature has both direct and indirect effects, and is detrimental to offspring fitness. These effects are most clear in terms of the intrinsic rate of increase. Whilst LRS is a good measure of the ability of offspring to

reproduce successfully it fails to consider the time taken for an individual to reach reproductive maturity, an important aspect of life-history.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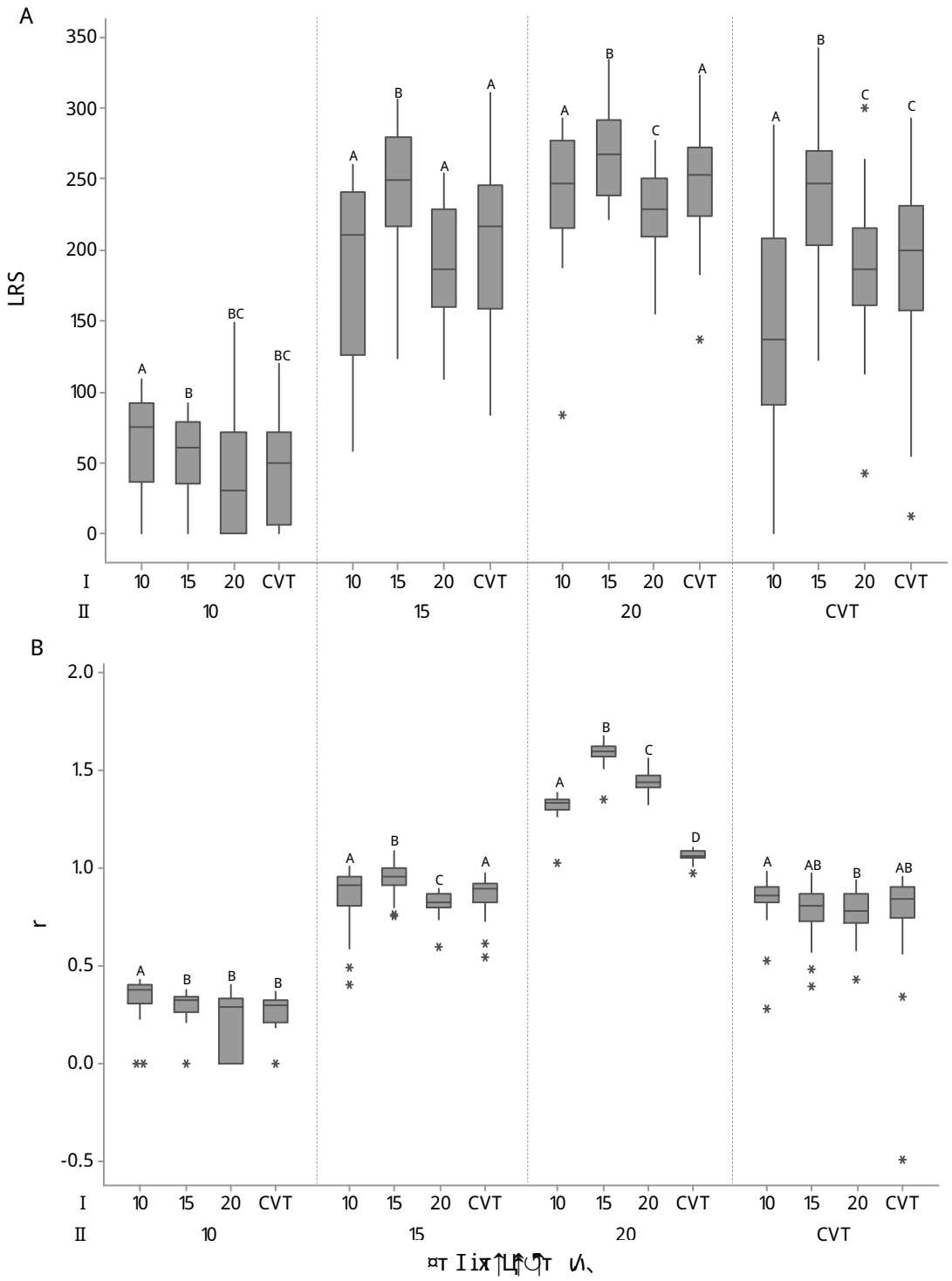
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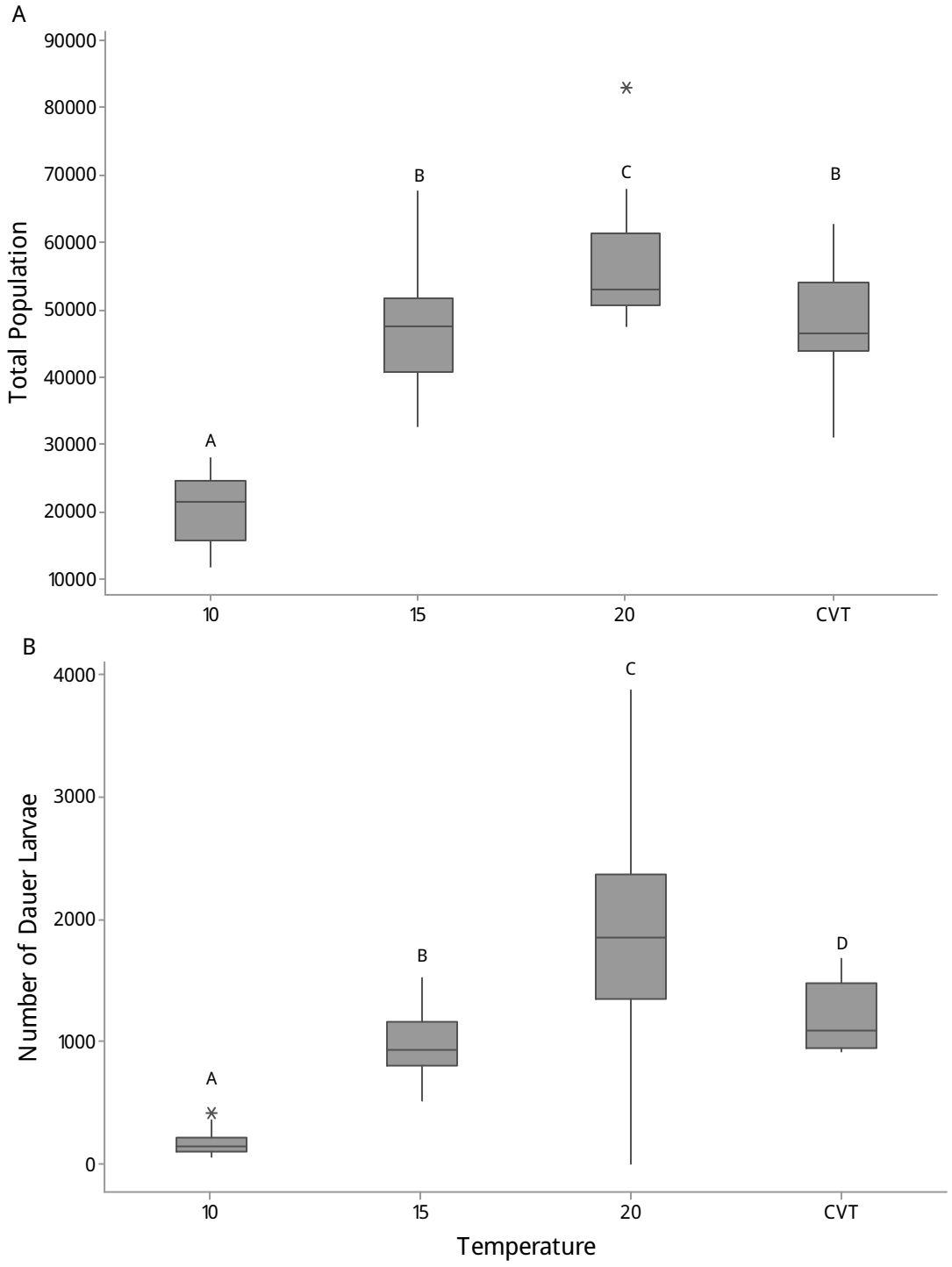
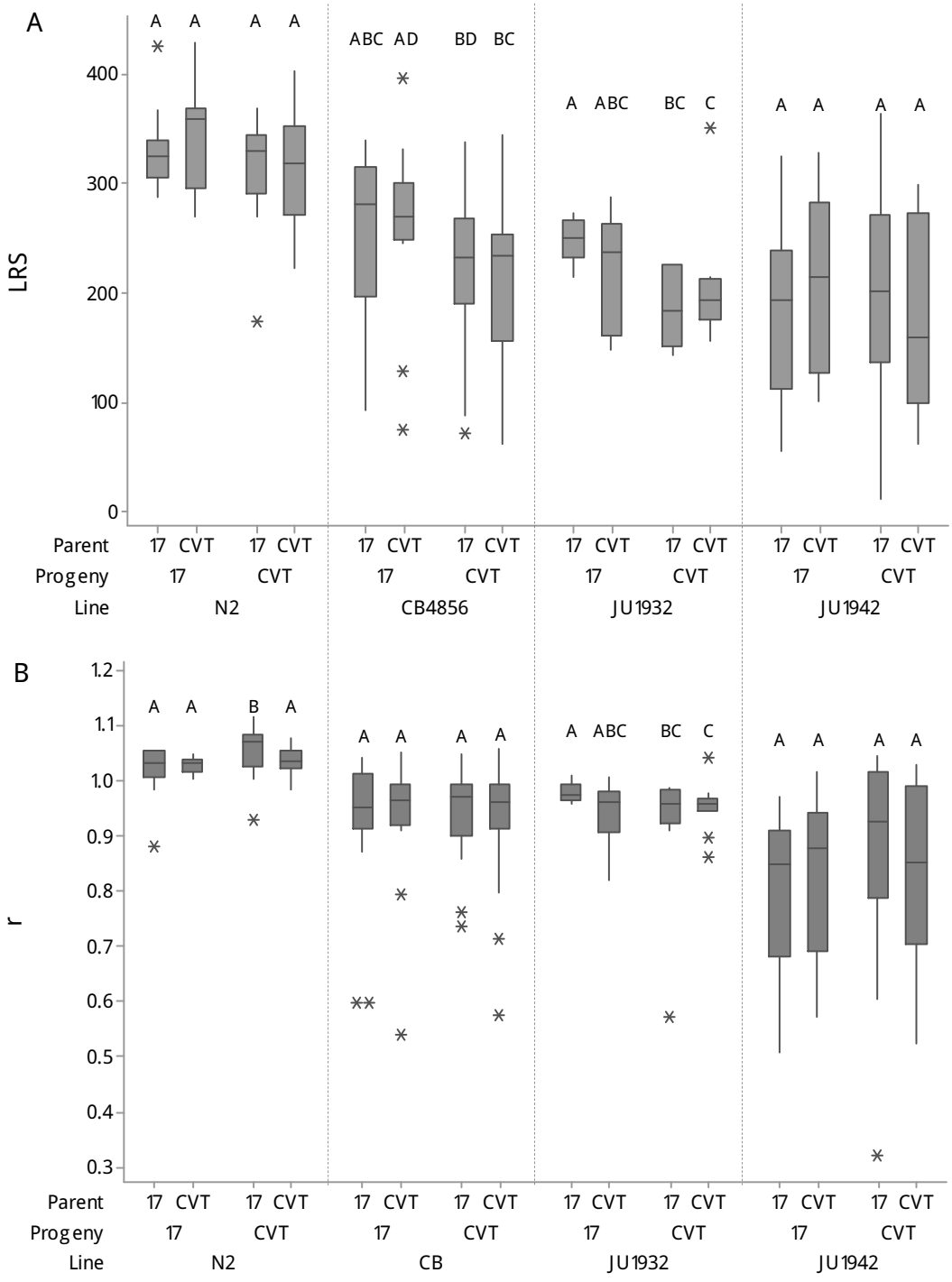
2.3.2 Population level effects of varying temperatures

Analysis of growing populations indicates that, as expected, the number of worms at food exhaustion and the number of dauer larvae at food exhaustion are affected by temperature (Figure 2-3, Kruskal-Wallis tests: $H_{49,3}$=31.24, p<0.001 and $H_{49,3}$=31.6, p<0.001 for the number of worms and the number of dauer larvae at food exhaustion, respectively). Post hoc comparisons indicate that the total number of worms at food exhaustion does not differ between the 15éC and the variable temperature treatments (Figure 2-3A). In contrast, there was a slight increase in the proportion of dauer larvae in the population at the variable temperature (Figure 2-3B).

2.3.3 Intraspecific variation in the effects of varying temperatures

As N2 shows extensive adaptation to laboratory conditions (for instance, N2 is more sensitive to high temperatures than more recently discovered isolates (Petrella, 2014)), it was important to determine whether the effects discussed previously were specific to N2. Investigations into the differences between N2 and wild isolates in response to maternal temperatures that varied across a wider thermal range (10 - 25éC rather than 10 - 20éC) revealed LRS was unaffected by CVT in N2 and line JU1942, but CB4856 and JU1932 were directly negatively affected by CVT with a significantly lower LRS at CVT than at 17éC (Figure 2-4A). In N2, a constant maternal temperature of 17éC results in a significantly higher r estimate when progeny grow at CVT when compared with progeny whose parents grew at CVT (figure 2-4B). The direct effects of CVT can be seen in line JU1932 with r significantly lower in CVT progeny from CVT parents than 17éC from 17éC parents, however this effect is not significantly affected by the maternal temperature.


A
Total Population
90000
80000
70000
60000
50000
40000
30000
20000
10000
A
B
C
B
10
15
20
CVT
B
Number of Dauer Larvae
4000
3000
2000
1000
0
A
B
C
D
10
15
20
CVT
Temperature


A
LRS
400
300
200
100
0
A A A A
ABC AD
BD BC
A ABC
BC C
A A A A
Parent 17 CVT 17 CVT 17 CVT 17 CVT 17 CVT 17 CVT 17 CVT 17 CVT
Progeny 17 CVT 17 CVT 17 CVT 17 CVT
Line N2 CB4856 JU1932 JU1942
B
r
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
A A B A
A A A A
A ABC
BC C
A A A A
Parent 17 CVT 17 CVT 17 CVT 17 CVT 17 CVT 17 CVT 17 CVT 17 CVT
Progeny 17 CVT 17 CVT 17 CVT 17 CVT
Line N2 CB JU1932 JU1942

2.4 Discussion

All organisms adapt in response to changes in the environment. Most responses are plastic with parentsˇ reproductive strategies adapting in ways that maximise offspring fitness. In C. elegans, stress (such as extreme temperature, resource shortage, overcrowding) usually results in a size difference in offspring and/or a change in brood size. Here, analysis reveals that temperature does indeed affect individual fitness (direct response) and progeny fitness (indirect response, maternal effect) but these responses are inconsistent between lines, for example a maternal effect is visible in N2 in response to CVT (r decreases in response to CVT when compared to a constant 17éC) but similar effects are not observed in the other lines. Across two different sets of temperature ranges and across genotypes, a complex pattern of responses is revealed (Table 2-1). These data strongly support both direct and indirect effects in N2 when temperature is cyclically varying between 10éC and 20éC when compared with a constant temperature of 15éC. When the cyclically varying temperature range is extended to include 25éC as the upper limit these effects are not as apparent (Table 2-1).

		Parent		Progeny									
		Direct effect		Direct effect		Maternal Effect 10éC		15/17éC *		20éC		CVTéC	
Experiment	Line	r	LRS	r	LRS	r	LRS	r	LRS	r	LRS	r	LRS
10-20 vs 15éC	N2	Y	N	N	N	N	N	Y	Y	Y	Y	N	Y
10-25 vs 17éC	N2	-	-	Y	N	-	-	N	N	-	-	Y	N
	CB4856	-	-	N	N	-	-	N	N	-	-	N	N
	JU1932	-	-	Y	Y	-	-	N	N	-	-	N	N
	JU1942	-	-	N	N	-	-	N	N	-	-	N	N

[illegible]

Using the intrinsic rate of increase, r, as a measure of fitness in place of a simple fecundity count provides more accuracy when analysing data. Lifetime reproductive success (or lifetime fecundity), LRS, gives us a good idea of the ability of an individual to reproduce; however, r also takes into account the age of the mother at the time of reproduction, therefore providing developmental data alongside reproductive success (see Huey & Berigan, 2001 for comparison). Analysing the data in this way has provided a greater understanding of the effects of temperature to parents and their offspring. Additionally, because development time is considered here, the dramatic difference between development at 10éC and 25éC are accounted for.

Interestingly, fluctuating temperature seems to have little effect on population growth dynamics; however, it would be prudent to repeat these population assays with a bigger sample size. Whilst population size was significantly different between temperatures (as expected), population size was not different between the 15éC and CVT treatments leading to the possible conclusion that average temperature is more important for population dynamics than exposure to extreme temperatures. Having said that, there was a slight increase in the proportion of dauer larvae recorded in the variable temperature population perhaps indicating that exposure to non-constant temperatures is stressful. These populations experienced the extreme low of 10éC, a temperature at which development is much delayed. Additionally, whilst population size alone is a good indicator of fitness and growth it does not provide insight into the developmental rates of each population. While population size may be very similar is it possible that the two populations are different from each other in maturity, male to hermaphrodite ratio or individual larva size.

A simple form of predictable variation is that associated with the circadian clock. Circadian rhythms are a feature of both animals and plants and represent ways that allow organisms to deal with the predictable changes associated with the day/night cycle and directly affected by both light and temperature cycles. In plants, circadian clocks maintain timing of physical (e.g. leaf movement) and physiological processes over a range of temperatures and in Arabidopsis thaliana, genes responsible for temperature compensation follow circadian rhythms and are either up or down regulated in a consistent pattern as temperature increases (Gould et al., 2006). These genes continue to follow the circadian rhythm even under free-running (i.e. constant light and temperature) conditions (Eckardt, 2006). C. elegans has been shown to have a circadian clock that is entrained by both light and temperature and temperature sensitive genes that follow a 24 hour expression cycle have been identified to be specifically involved in reproductive development. These studies have revealed that processes such as mating, fertilization and egg-laying are likely under circadian control (van der Linden et al., 2010).

Many temperature-related studies in C. elegans use the strain, N2 (Bristol), which has been kept under laboratory conditions for several decades and is now widely considered to have adapted to these conditions (i.e. constant temperature, abundant foodŭ). Having said that, such experiments have provided the scientific community with a wealth of information about temperature sensitivity in nematodes and how temperature impacts life-history traits. More recent studies have shown that the ecology of wild C. elegans populations is important when studying life-history traits. Wild isolates respond very differently to N2 under laboratory conditions and here, we have attempted to address this and have also demonstrated a clear difference in the response of wild isolates when compared to N2.

Further, because the C. elegans genome is fully sequenced, it has been possible to identify allele-specific temperature sensitivity (O'Rourke et al., 2011). A vast array of gene information is now available for C. elegans and life-history affecting genes have been identified. However, there are many more genes yet to be characterised and the study of wild isolates and responses to field conditions may reveal the function of some of these genes. Further investigation into circadian responses in C. elegans may reveal clearer links between gene regulation and behavioural responses to temperature and light levels (and a combination of these). In C. elegans, temperature has been shown to be a large part of circadian responses with temperature cycles affecting rhythmic gene expression (van der Linden et al., 2010) and response to osmotic stress (Kippert et al., 2002).

Isolates of C. elegans are found throughout the world, in various geographical locations and climates, yet there are no distinct geographically related phenotypes (Hodgkin and Doniach, 1997) unlike C. briggsae which shows clear genetic differences between temperate and tropical strains (Cutter et al, 2006). In this species, temperature has a much more dramatic effect on the fecundity of an individual and is directly related to the latitude at which a strain was isolated (Prasad et al., 2010). In this instance, C. briggsae might prove a better candidate for studying the adaptive effects of temperature and is therefore likely to reveal more obvious maternal effects. Further, whilst fecundity (measured by LRS) and the intrinsic rate of increase, r, are appropriate measures of fitness here, a measurement of offspring size, something which has been demonstrated in multiple species to change in response to environmental stress, may be an important indication of the effects of CVT and would be a logical next step.

Chapter Three

Maternal Effects in Entomopathogenic Nematodes

Abstract

Maternal effects are well documented in the free-living nematode Caenorhabditis elegans yet have not been systematically demonstrated in parasites. Parasite life-histories are difficult to investigate as parasites are notoriously difficult to rear in the laboratory for practical and ethical reasons. Entomopathogenic nematodes (EPNs) represent a potential model for parasite study as they are easily reared, require insect hosts that are easily obtainable and take up much less space than the hosts of mammalian parasites, for example. EPNs are obligate parasites of insects that have a free-living stage, the infective juvenile (IJ) - but complete their life cycle inside an insect host. In an attempt to provide evidence of maternal effects in parasites EPNs, Heterorhabditis downesi and Steinernema carpocapsae fitness effects were investigated in response to two major environmental factors: temperature and host species. Rearing temperature and rearing host species both impacted on progeny development and reproduction, with higher temperature (25éC vs 20éC) being associated with increased rate of development. Host species has a direct effect on emerging infective juveniles and the maternal host species affects the rate of population growth as well as lifetime fecundity. The maternal environment was more important for progeny development than the direct progeny environment, with matching parent and progeny environments giving the greatest fitness advantage for offspring. Infective juvenile size was also affected by both temperature and host insect species (Galleria mellonella vs Tenebrio molitor). These data confirm that maternal effects are present in parasites and highlight the associated fitness consequences of changes in the maternal environment.

3.1 Introduction

Entomopathogenic nematodes (EPNs) of the genera Heterorhabditis and Steinernema, are obligate parasites of insects that share similar life-histories, probably due to convergent evolution, (Blaxter et al., 1998; Boff et al., 2000). EPNs complete their life cycle (figure 3-1) inside an insect host and have a free-living infective stage, the infective juvenile (IJs). IJs, developmentally arrested dauer larvae similar to Caenorhabditis elegans L1 larvae, enter the host insect, releasing pathogenic, symbiotic bacteria into the insect haemocoel which kill the insect within 24-48 hours and provide a food source for the EPN population (Stock & Goodrich-Blair, 2012). These symbiotic bacteria (Xenorhabdus spp. or Photorhabdus spp. for Steinernema spp. and Heterorhabditis spp., respectively) are carried in the gut of the IJ (Steinernematids have an additional bacterial vesicle in which bacteria are carried) (Adams & Nguyen, 2002). When the IJ enters the host insect, the bacteria are released (Heterorhabditids regurgitate and Steinernematids defecate the bacteria) and their proliferation along with the production of various toxins and enzymes breaks down the insect tissues creating a nutrient rich 'soup' and an ideal environment for the nematodes (Forst et al., 1997). Once infection has occurred and feeding commences, IJs resume development into sexually mature adults. Heterohabditid IJs develop into hermaphroditic adults, which then produce males and females in the second generation. In contrast, Steinernematid IJs directly develop into male and female adults. When food availability becomes low, egg laying ceases and juveniles hatch inside the mother, an event termed Endotokia matricida (endo- [Greek] = inside, -tokia [Greek] = birth, matri- [Latin] = mother, -cida from caedere [Latin] = kill).). As the number of juveniles inside the mother increases, their movement disturbs and breaks apart internal tissues. Bacterial cells are ingested and retained by the IJ once the mother's digestive system is destroyed. IJs are likely formed due to lack of food in the uterus prior to the destruction of the gonad. Unlike C. elegans dauer larvae, there is little evidence that

nematode pheromone is involved in IJ development (Johnigk & Ehlers, 1999; Noguez et al., 2012).

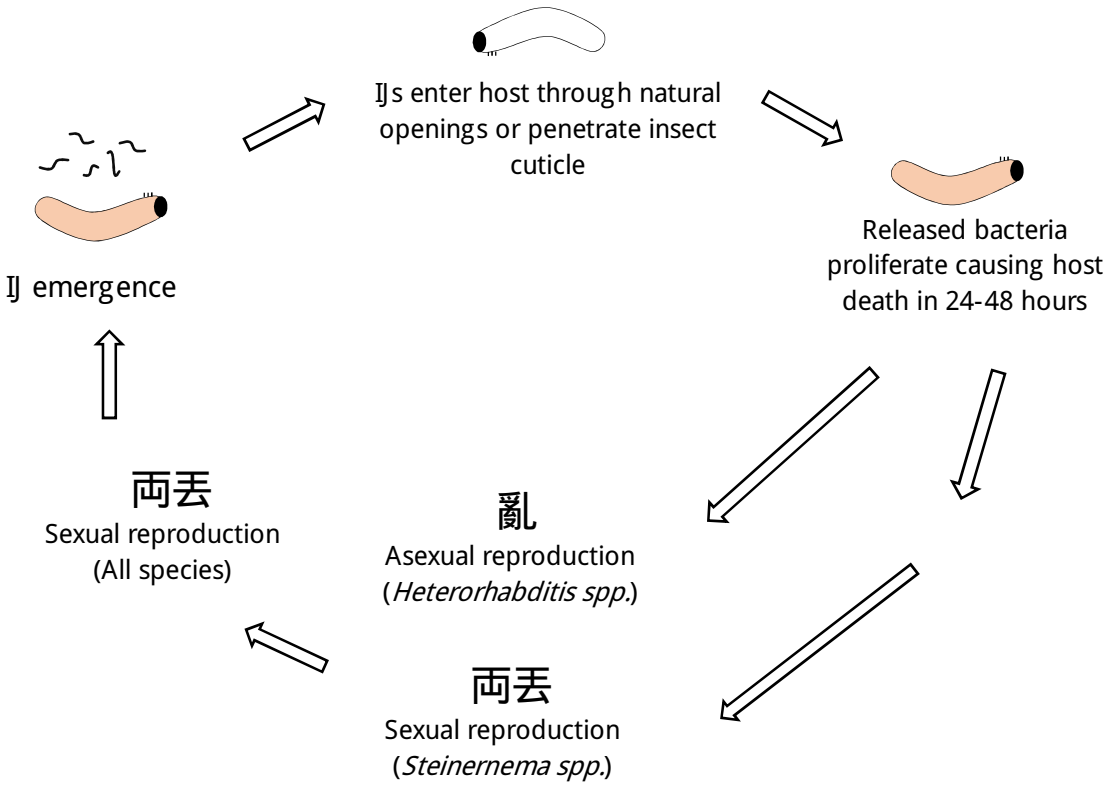


Figure 3-1. Life cycle of entomopathogenic nematodes Steinternema spp. and Heterorhabditis spp. Adapted from Ffrench et al., 2003.

Reproduction continues until resources are depleted or crowding occurs (Zenner et al., 2014) causing emergence of a new generation of IJs which leave the host, disperse and search for a new host to colonise (Forst and Clark, 2002). This stage of the life cycle is analogous to the dauer larva stage in the C. elegans life cycle, with dauer larvae developing as an alternative to the third stage larvae (L3) in response to environmental stresses such as resource limitation and/or crowding/high population density (Golden & Riddle, 1984; Harvey et al., 2008).). Like EPN IJs, dauer larvae are able to survive for an extended period of time without feeding and disperse from their host in search of food or, in the case of EPN IJs, a new host. EPNs use two main foraging strategies (although these strategies are more likely to represent a continuum rather than an `either/or when trying to locate a host) with some species being particularly mobile and able to search and locate relatively sedentary hosts (cruising strategy) (Lewis et al., 2006). Others have the ability to nictate (raise their bodies from the ground, using their tails to anchor themselves) and appear to use an `ambush strategy_ to attach themselves to passing insects (Grewal et al., 1993; Lewis et al., 2006). This is a phenomenon which also occurs in C. elegans dauer larvae which have been observed nictating on growing fungal colonies on contaminated agar plates (Lee et al., 2013).

Maternal effects are ubiquitous in free-living organisms (Bernardo, 1996; Rossiter, 1996). Their importance is well understood and the mechanisms that produce maternal effects have been widely investigated and discussed (see Chapter 1). If maternal effects are so prevalent in free-living species then they likely exist in parasites. In particular, maternal effects observed in C. elegans should be reproducible in parasitic nematode species due to shared homology, as is reflected in highly conserved genes throughout the Phylum Nematoda. Moreover, Caenorhabditis spp., Heterorhabditis spp. and Steinernema spp. are all members of the same taxonomic order, the Rhabditida (Blaxter, 1998; Adams & Nguyen, 2002). There

is growing interest in the use of EPNs for ecological and environmental modelling and they potentially represent a useful tool for investigating the ecological dynamics of soil communities, population dynamics and various aspects of parasite evolutionary biology (Campos-Herrera et al., 2012). Gaining an understanding of maternal effects in this context would be very informative, especially in relation to environmental fluctuations that affect fitness. This would be of particular interest for organisms used for biological control (e.g. entomopathogenic nematodes) as their efficacy may be affected, even improved, by maternal effects that arise from various environmental factors. Previous dauer effects in C. elegans show that dauer larvae develop as a result of food limitation caused by crowding, temperature stress and in response to dauer pheromone (Golden & Riddle, 1984). This enables larvae to survive in a developmentally arrested state until optimal conditions are found or restored. In H. bacteriophora a similar pheromone has been identified that inhibits IJ (analogous to dauer larvae) recovery in low food conditions, further establishing the similarities between free-living parasite infective stages and C. elegans dauer larvae (Noguez et al., 2012). IJ emergence has been shown to be linked with the increased concentration of ammonia inside a host when IJ population density increases with emergence occurring sooner when ammonia concentration is higher. However alongside increased ammonia concentration is higher population density and as such the hastened emergence may be due to resource limitation (San-Blas et al., 2008). Organisms often display behavioural responses to harsh environmental conditions and most parasites are protected from temperature extremes by existing inside a host (for example, H. bacteriophora, S. carpocapsae, S. glaseri and S. feltiae infective juveniles can survive freezing when inside G. mellonella cadavers (Lewis & Shapiro-Ilan, 2002)), as with mammalian parasites, or adopt other survival mechanisms (e.g. root knot nematodes Meloidogyne spp. adults survive inside the host plant but their eggs, laid on the outside of the root are protected by gelatinous matrices (Curtis et al., 2009)). EPNs

have an extended period in which the infective juveniles reside in the soil in search of a new host. This means they are exposed to fluctuations in environmental conditions both inside and outside the host (Brown & Gaugler, 1997) and have adapted a number of coping mechanisms to deal with environmental unpredictability. Phased infectivity of IJs may be adaptive and indicative of bet-hedging. By delaying the infectivity of IJs emerging from host insects, the likelihood of some IJs infecting a new host is increased even in the event that there are no hosts available immediately upon emergence from the host cadaver. Similar methods of delayed development have been observed in root knot nematodes with Meloidogyne chitwoodi showing delayed hatching during periods of low host availability (Curtis et al. 2009). This also highlights the importance of good quality lipid reserves as infectivity declines over time as lipids are depleted (Ryder & Griffin, 2003).

In an attempt to provide evidence of maternal (or indeed, paternal) effects in parasites, highly inbred lines of the entomopathogenic nematodes Heterorhabditis downesi and Steinernema carpocapsae were reared in varying environments and the effects on life-history traits were investigated. Two major environmental factors were investigated: temperature and host species. EPNs are primarily soil dwelling organisms and are therefore exposed to an environment that is under constant change in its biotic and abiotic characteristics (Stuart et al., 2015). Temperature change can be dramatic in the soil and is subject to both daily and seasonal changes. High and low temperatures have been shown to have dramatic consequences for nematode life-history and maternal effects have been identified in response to temperature changes resulting in both bet-hedging strategies and phenotypic plasticity (Anderson et al., 2011; Arrighi et al., 2013; Harvey & Orbidans, 2011; Harvey & Viney, 2007; LaMontagne & McCauley, 2007). EPN have been isolated from many climates and they are able to survive under sub-arctic, temperate and tropical conditions due to survival

mechanisms such as diapause (the IJ stage) and quiescence (Glazer, 1996). Further, the insect host can provide some protection from extreme temperatures (Brown & Gaugler, 1995) and at less than optimal temperatures, IJs will remain in the host for as long as 50 days, although this can result in failure to emerge if the cadaver dries to the point of desiccation (Brown & Gaugler, 1997). Understanding the effects of temperature on nematode development is important for determining the fate of IJs used for biological control under various environmental conditions (Brown & Gaugler, 1997). Temperature greatly affects EPN development with few IJs forming at 15éC and reproduction ceasing entirely at 31éC in S. carpocapsae (Han et al., 1993). Their optimal temperature range is between 19 and 27éC but there are dramatic differences in growth rates at the extremes of this range (Han et al., 1993). Temperature can also affect the growth and survival of the symbiotic bacteria which will potentially affect the quality of the food ingested by the EPNs. This has been shown to be detrimental to IJ longevity and infectivity (Boff et al., 2000) and raises questions about host-environment interactions and how they relate to host-parasite interactions.

EPNs have broad host ranges making them ideal for use biological control systems (Griffin, 2012). Generalists like S. feltiae and S. carpocapsae have the broadest host ranges and widest geographical distributions although laboratory host range tests do not always accurately reflect natural host ranges (Peters, 2010). While EPNs may be the natural enemy of many pest species, it is likely that insects have evolved survival mechanisms to avoid attack from native EPN species but may not be as good at resisting invasion from foreign species (Gaugler et al., 1997). It is here that an understanding of host effects is crucial. In the EPN system, the environment inside the host is probably at least as important, if not more so, than the external environment. Parasite development may be negatively affected by high population density within a host with sex ratio, size, fecundity and survival being affected by crowded conditions

(KoppenhØfer & Kaya, 1995; Ryder & Griffin, 2003; Alsaiyah et al, 2009). Host condition can affect bacterial proliferation within the host, which in turn can affect the parasites. Host age and diet are also likely to influence within-host conditions. For example, bacteria grow more rapidly in older Tobacco Hornworm (Manduca sexta) larvae than in younger larvae and insects that have a high-lipid diet are more susceptible to EPN infection (Miranda et al., 2013).

It is important to note that, in EPNs, whilst it is the maternal environmental change that is potentially mediating changes in offspring quality, EPN undergo multiple generations of reproduction (typically 3 to 4) within the host. The infective juveniles emerging from the host are not the direct offspring of the first IJ, or cohort of IJs, that infect a host. Therefore the term :maternal effect˘ does not apply to effects expected for EPN in-vivo models in the same way it does for in vitro experiments on C. elegans, for example, and must be interpreted accordingly. However, there are still likely to be transgenerational effects that are causing trait changes in offspring. To avoid the use of additional, potentially confusing terminology, the term :maternal effect˘ is used in this chapter in the context of an EPN in vivo model.

Here, the effects of maternal temperature and host insect species on offspring development are demonstrated. Inbred lines of H. downesi and S. carpocapsae are initially reared (parental generation) under two different conditions (differing either by temperature (20éC or 25éC) or by host species (Galleria mellonella or Tenebrio molitor) and the progeny isolated from these parents are divided between the two conditions in order to compare the effect of the parental rearing environment on the development of the offspring. Rearing temperature directly affects development and lifetime fecundity with maternal rearing temperature affecting the same

traits in progeny. Additionally, the maternal host affects the same life-history traits and progeny fate is shaped by the maternal host environment.

3.2 Methodology

3.2.1 Nematodes and insects

Inbred laboratory cultures of H. downesi strain K122 (Griffin, Moore & Downes, 1991) and S. carpocapsae strain US-S-25 (Koppert Ltd.; Berkel en Rodenrijs, The Netherlands) were obtained from Christine Griffin (Maynooth University). Lines were maintained in the laboratory in final instar Galleria mellonella (Lepidoptera: Pyralidae) (Bedding & Ackhurst, 1975) and further inbred over five and seven generations for H. downesi and S. carpocapsae, respectively. Inbreeding was performed by injecting single IJs of H. downesi or pairs of IJs of S. carpocapsae using a 25ga syringe needle (Fisher Scientific, 10299100), harvesting the emerging IJs using modified White traps (Kaya & Stock, 1997) and repeating this procedure with the emerged IJs. IJs were stored in tap water at 9éC for at least one week before use to increase infectivity and IJs used in each experiment originated from the same insect cadaver used for culture. G. mellonella (waxworm) larvae and Tenebrio molitor (mealworm) larvae were purchased as required from Live Foods Direct (www.livefoodsdirect.co.uk, UK). Larvae were stored at room temperature and used within a week of purchase.

3.2.2 In vitro analysis of nematode development: lipid agar

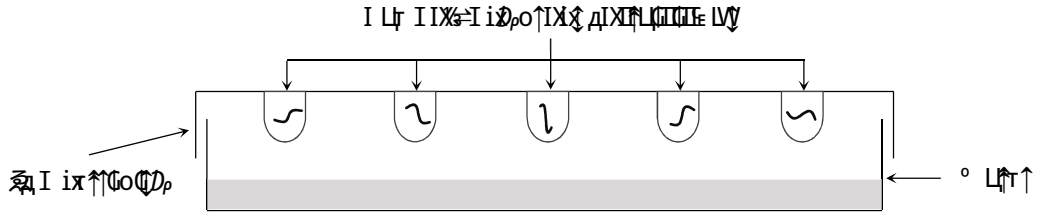
Initial investigations into developmental changes were attempted in vitro using lipid agar plates. These were created by preparing nutrient agar (Sigma, 70148) with the addition of 5g yeast extract, 890ml H_2O, 10ml 0.2g/ml $MgCl_2$6H_2O, 4ml corn oil, 96ml corn syrup mix (7ml corn syrup in 89ml H_2O) (Stock & Goodrich-Blair, 2012), intended to recreate a viscous, haemolymph-like environment. Forty lipid agar plates were inoculated with 100ıl P. luminescens and allowed to grow at room temperature overnight. To isolate P. luminescens,

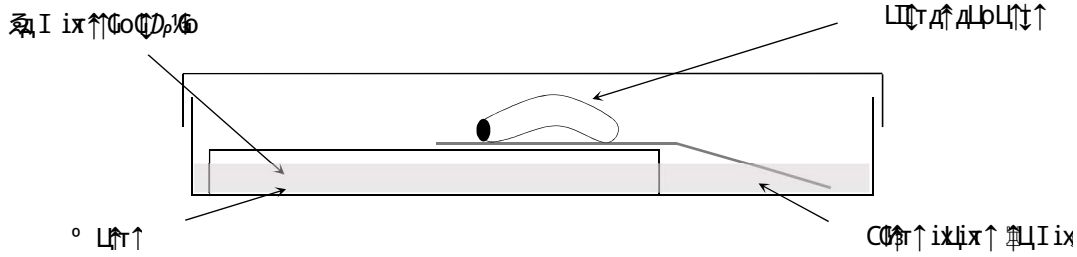
Approx. 1,000 Infective juveniles of Steinernema carpocapsae (containing Photorhabdus luminescens bacteria) in 1ml tap water suspension were placed in separate 1.5ml reaction tubes and centrifuged at 14,000 rpm for one minute to precipitate. The pellet of nematodes was re-suspended in 1ml of 0.1% 4 Mm Hyamine (Sigma Aldrich, 51126) and incubated for 30 minutes for surface-sterilization of IJs. IJs were then centrifuged at 14,000 rpm for one minute and re-suspended in 1ml of sterile Ringer˘s solution to wash off remaining Hyamine. This was repeated three times. IJs were then ground up to release bacteria in 1ml of sterile Ringer˘s solution using a sterile plastic reaction tube pestle (Argos; Elgin, USA). P. luminescens suspension was streaked onto five replicate MacConkey agar plates using a sterile inoculation loop. Plates were incubated at 30 degrees centigrade for five days and colonies were picked from each plate using a sterile inoculation loop and maintained in liquid culture in MacConkey broth at 30éC for use in experiments. To confirm isolation of the entomopathogenic bacteria, 5ul of each broth culture were injected into final instar wax moth larvae using a micro-syringe (supplier) (5 replicates for each bacterial culture) and insects were incubated at 22éC for five days to confirm death as well as colouration of insects indicating successful infection (Stock & Goodrich-Blair, 2012). Twenty-four hours after the plates were inoculated, a single H. downesi IJ was transferred onto each plate. Before transfer, IJs were surface-sterilised in a 0.1% hyamine solution for 15 minutes and washed 3x in sterile dH_2O. Plates were then incubated at either 20éC or 30éC (20 plates at each temperature) and monitored daily for nematode development and reproduction.

3.2.3 In vitro analysis of nematode development: hanging drops

In vitro hanging drop assays were attempted according to (Stock & Goodrich-Blair, 2012). Haemolymph was collected from G. mellonella larvae by piercing the larvae between two

thoracic segments with a 25ga syringe needle (Fisher Scientific, 10299100). Haemolymph was collected in a 1.5ml Eppendorf tube with 50 larvae producing approximately 1ml of haemolymph. Single, 40ı l drops of haemolymph were pippeted onto 5cm petri dish lids (5 per plate) and 10ı l of protein free insect culture medium (serum free medium, Fisher Scientific, 10308153) was added to each drop to prevent melanisation caused by the oxidation of the haemolymph. Single H. downesi IJs were picked into each drop using a platinum wire (Fisher Scientific: 10724381). The base of each petri dish was then filled with approximately 0.5cm of tap water to prevent the drops drying out and the lids were then inverted and place on top of the bases (Figure 3-2). No bacteria were added to the drops as IJs were expected to inoculate drops with the bacteria they carried. Plates were place inside air tight containers and incubated at 20éC. Hanging drops were monitored daily for one week to assess nematode development. Haemolymph collection was also attempted from T. molitor larvae, however the thickness of the exoskeleton made penetration difficult and the volume of haemolymph collected was insufficient for performing assays.



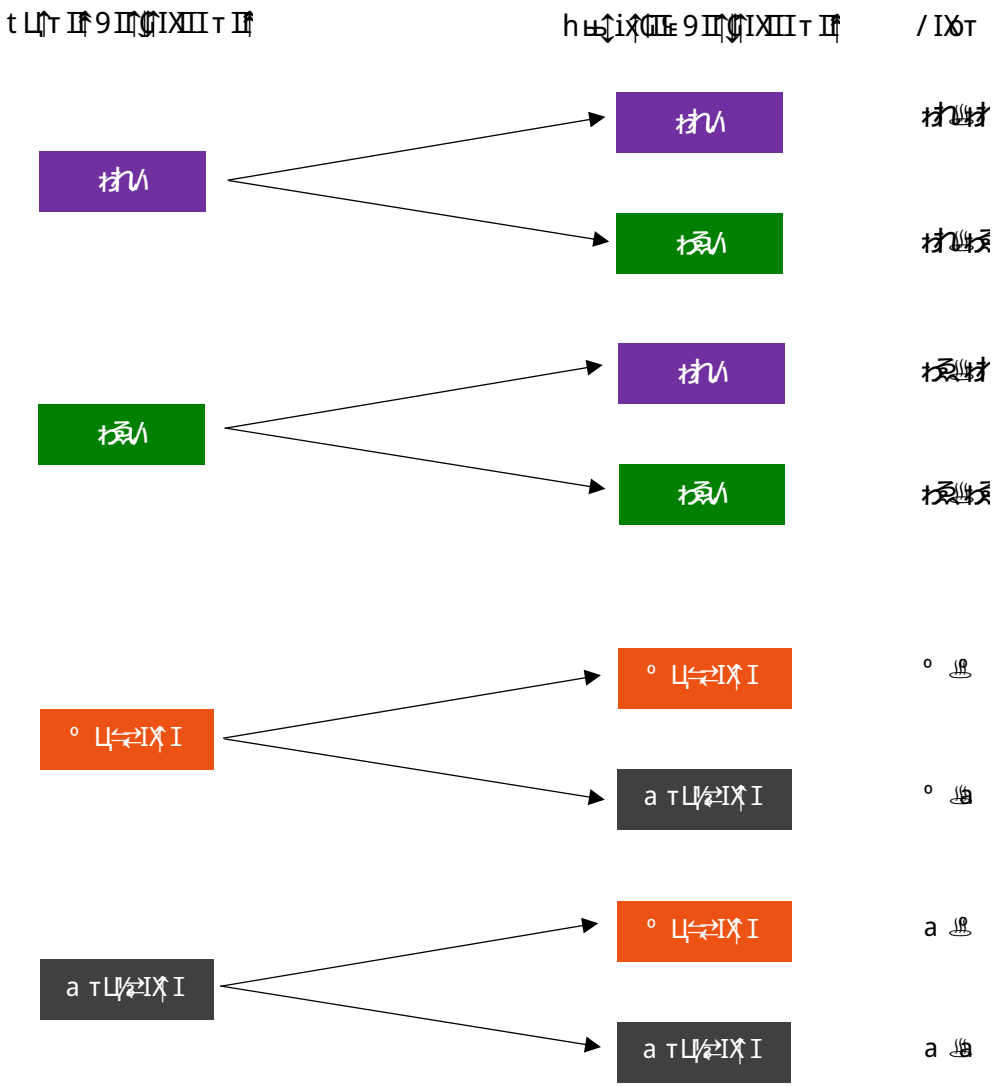


3.2.4 The effects of maternal temperature on nematode emergence and population size

Initial infections were carried out by inoculating G. mellonella larvae with H. downesi or S. carpocapsae IJs. To do this, a single G. mellonella larva was placed into each well of a 24 well plate containing a circle of Whatman filter paper (Fisher Scientific, 11374270). Approximately forty IJs in 40µl H_2O were pipetted onto each piece of filter paper. IJ number in the stock suspension was adjusted by counting the number of IJs in 20µl and diluting as required. Forty-eight wax moth larvae were inoculated for each nematode species. Of these, twenty-four were incubated at 20ºC and 24 at 25ºC. Larvae were monitored daily for signs of successful infection, characterised initially by the death of the insect followed by a colour change of the cadaver indicating bacterial infection (red in the case of H. downesi and cream in the case of S. carpocapsae). Ten days after the initial inoculations, successfully infected cadavers were transferred onto modified White traps (Figure 3-3) (adapted from Kaya & Stock, 1997), placed back into the incubator and monitored daily for emergence. Emerging IJs were collected daily. To do this, the water was removed from the white trap and the White trap was rinsed twice with clean tap water to ensure all IJs were collected. Emerged IJs were stored for 10 days at 9ºC before being used for the second stage of the experiment. The H. downesi inoculations resulted in too few emerging IJs at 20ºC so the rest of the experiment was performed using S. carpocapsae IJs collected on the fourth day of emergence and stored at 9ºC. The IJs stored at 20 and 25ºC did not survive past four days.

For the second part of this experiment, 60 G. mellonella larvae were inoculated, as before but this time with only S. carpocapsae IJs due to poor survival of H. downesi at 25ºC, for each treatment. IJs collected from 20ºC cadavers were used to inoculate 60 larvae to be incubated

at 20éC and 60 to be incubated at 25éC. IJs collected from 25éC were used to inoculate 60 larvae to be incubated at 20éC and 60 to be incubated at 25éC (figure 3-4). For each of the 60 larvae in each treatment, 24 were intended for recording emergence in White traps , 24 were intended for dissection to monitor weekly nematode development inside the host and 12 were used to replace any failed inoculations and keep the number of White traps as close to n=24 as possible. Larvae were incubated at their respective temperatures and monitored daily for successful inoculation. To ensure larvae were transferred to white traps at the optimal time (i.e. population size was large enough and infective juveniles were being produced), dissections were performed at 7 days after infection, using separately infected G. mellonella larvae using a pepsin enzymatic digestion. Cadavers were cut in half and placed into 50ml screw top tubes with 15ml pepsin solution (Maule Æ et al., 1993 in Stock & Goodrich-Blair, 2012). Tubes were placed in a shaking incubator for 2 hours at 37éC (120rpm) and then diluted with 15ml H_2O. 1ml of the solution was removed and the number of adult and juvenile nematodes counted. The day seven and ten dissections revealed that population levels were not high enough for emergence to commence. Dissections performed on day fourteen revealed large numbers of juveniles and therefore experimental cadavers were transferred to white traps on day fourteen post-inoculation. White traps were monitored daily for emergence. Emerged IJs were collected and counted daily for ten days. To determine whether size affects fecundity in EPN populations, parental IJs (i.e. those that emerged from the initial round of inoculations (figure 3-4)) were photographed and measured. Additionally, to ensure observed effects were not due to the size of the insect, each G. mellonella larva was weighed to ensure any effects observed were not due to host weight.



3.2.5 The effects of maternal host species on nematode emergence

Initial infections were set up by inoculating G. mellonella and T. molitor larvae with H. downesi or S. carpocapsae larvae. Insect larvae were place individually into wells of a 24 well plate (Corning, 351147) containing a circle of filter paper. Approximately 50 IJs in 40ı l H_2O were pipetted onto each piece of filter paper. 24 G. mellonella and 24 T. molitor larvae were inoculated for each nematode species and all larvae were incubated at 20éC. Larvae were monitored daily for signs of successful infection, as before. Ten days after the initial inoculations, successfully infected cadavers were transferred onto modified White traps, placed back into the incubator and monitored daily for emergence. Emerging IJs were collected daily, as before, and stored at 9éC for 10 days before being used for the second stage of the experiment. The S. carpocapsae inoculations were unsuccessful in T. molitor larvae so the rest of the experiment was performed using H. downesi IJs collected on the fifth day of emergence.

For the second part of this experiment, 60 G. mellonella or T. molitor larvae were inoculated, as before, for each treatment. For this round of inoculations, only emerged H. downesi IJs were used due to the S. carpocapsae parents failing to reproduce. Parental IJs collected from G. mellonella cadavers were used to inoculate 60 G. mellonella larvae and 60 T. molitor larvae. Parental IJs collected from T. molitor larvae were used to inoculate 60 T. molitor larvae and 60 G. mellonella larvae (figure 3-4). Larvae were incubated at 20éC and monitored daily for successful inoculation. To ensure larvae were transferred to white traps at the optimal time, dissections were performed as before. Dissections performed on day twelve revealed large numbers of juveniles and therefore cadavers were transferred to white traps on day fourteen post-inoculation. White traps were monitored daily for emergence. Emerged IJs

were counted daily as before. To determine whether IJ size affects progeny fecundity in EPN populations, the IJs that emerged from the first sets of inoculated larvae (i.e. the parent generation) were photographed using Olympus microscope (Olympus IX 83, Olympus, UK) and measured using Cellsens Dimension (Olympus, UK).

3.2.6 Statistical analyses

Analyses were carried out using Minitab ÷ 17.2.1.0. One-way ANOVA and t-Tests were performed where data were normally distributed (confirmed by Anderson-Darling tests with a significance of p ħ 0.05) or where transformations were possible. Otherwise Kruskal-Wallis and Mann-Whitney tests were used. Regression analyses were performed to determine if there was a relationship between host size (measured by weight before inoculation) and nematode developmental traits. Data here were normally distributed (Anderson-Darling, p ħ 0.05). Lifetime reproductive success (LRS) was calculated as the total number of IJs that emerged from a single cadaver over ten days. The intrinsic rate of increase of the population (r) was calculated using the Euler-Lotka equation as described in Chapter 2.

3.3 Results

3.3.1 In vitro rearing techniques

The lipid agar plates and the hanging drops assays did not result in sufficient nematode development to assess maternal effects. Approximately 80% of the IJs that were placed on lipid agar plates did not grow into reproducing adults. Of these half appeared to have left the plate, a common problem seen in free-living nematodes such as C. elegans when resources are lacking. Very few nematodes (approximately 20%) developed past the IJ stage and those that developed and didn't leave the plate burrowed into the agar making it difficult to locate them and any offspring. The remaining nematodes died. The hanging drops were equally unsuccessful. Many of the drops melanised despite being treated with serum free medium, others dried or fell into the water in the bottom of the Petri dish. Due to these issues it was decided that all subsequent assays would be performed in vivo.

3.3.2 Nematode life-history is affected by maternal temperature

To assess the effects of maternal temperature on nematode development, G. mellonella cadavers infected with S. carpocapsae IJs were incubated at two different temperatures and emerging offspring from these cadavers were assessed for differences in development at the two temperatures. Analysis of the size of IJs indicated that worms emerging from cadavers incubated at 25éC were significantly smaller (Mann-Whitney test, W=3919.0, $p < 0.001$) than those that emerged from 20éC cadavers (figure 3-5). There was a trend for faster development (r) and increased LRS for progeny whose parents were reared at 25éC compared with progeny whose parents were reared at 20éC. 25éC offspring that came from 25éC parents (treatment 25/25) developed significantly faster (One-way ANOVA, $f_{(3, 84)} = 7.11$, $p < 0.001$) than the

25éC offspring from the 20éC parents (treatment 20/25, figure 3-6A). Maternal temperature was important for progeny lifetime reproductive success (LRS) (figure 3-6B). Parent IJs that emerged from 25éC cadavers also went on to produce significantly more offspring than those that emerged from 20éC cadavers (Mann-Whitney, $p = 0.019$ & $p < 0.001$ for progeny rearing temperatures of 20é and 25éC respectively; figure 3-6 and table 3-1). Regression analyses (figure 3-7) revealed that the weight of the G. mellonella larvae bore no significant relationship to either r ($p = 0.107$, $r^2 = 2.99$) or LRS ($p = 0.64$, $r^2 = 0.24$). Emergence patterns showed that maternal temperature does not cause a change in emergence phases with only the temperature directly experienced by the final, emerging IJs having an effect on emergence patterns (figure 3-8).

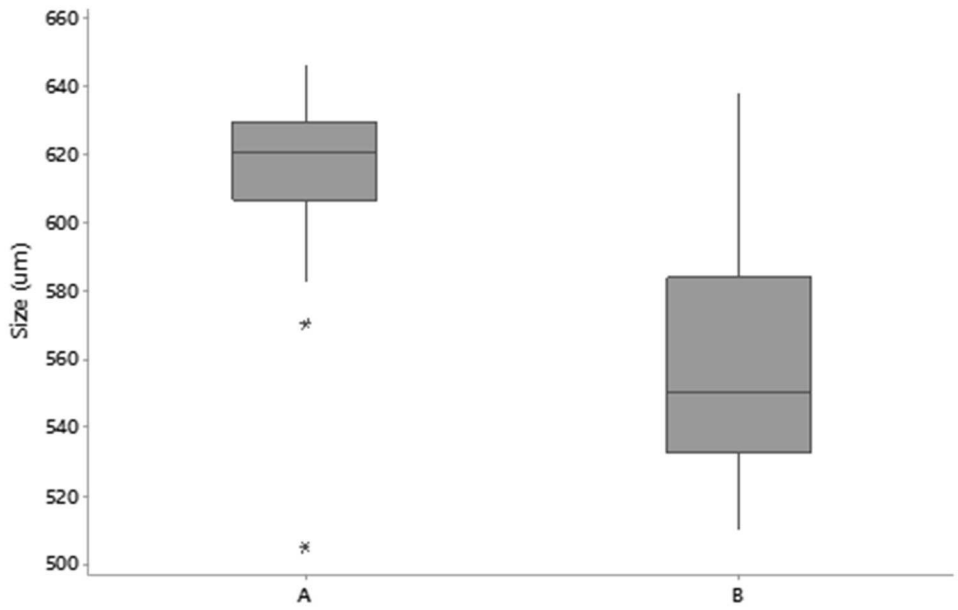
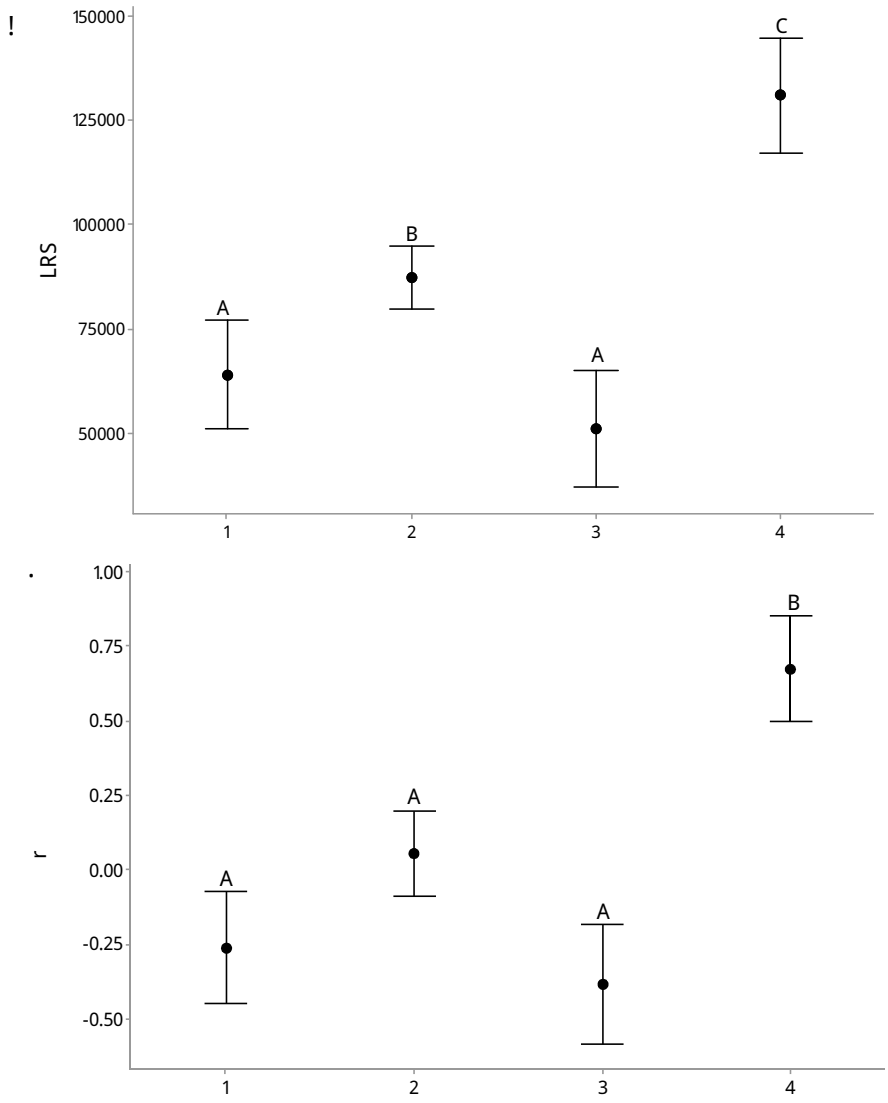
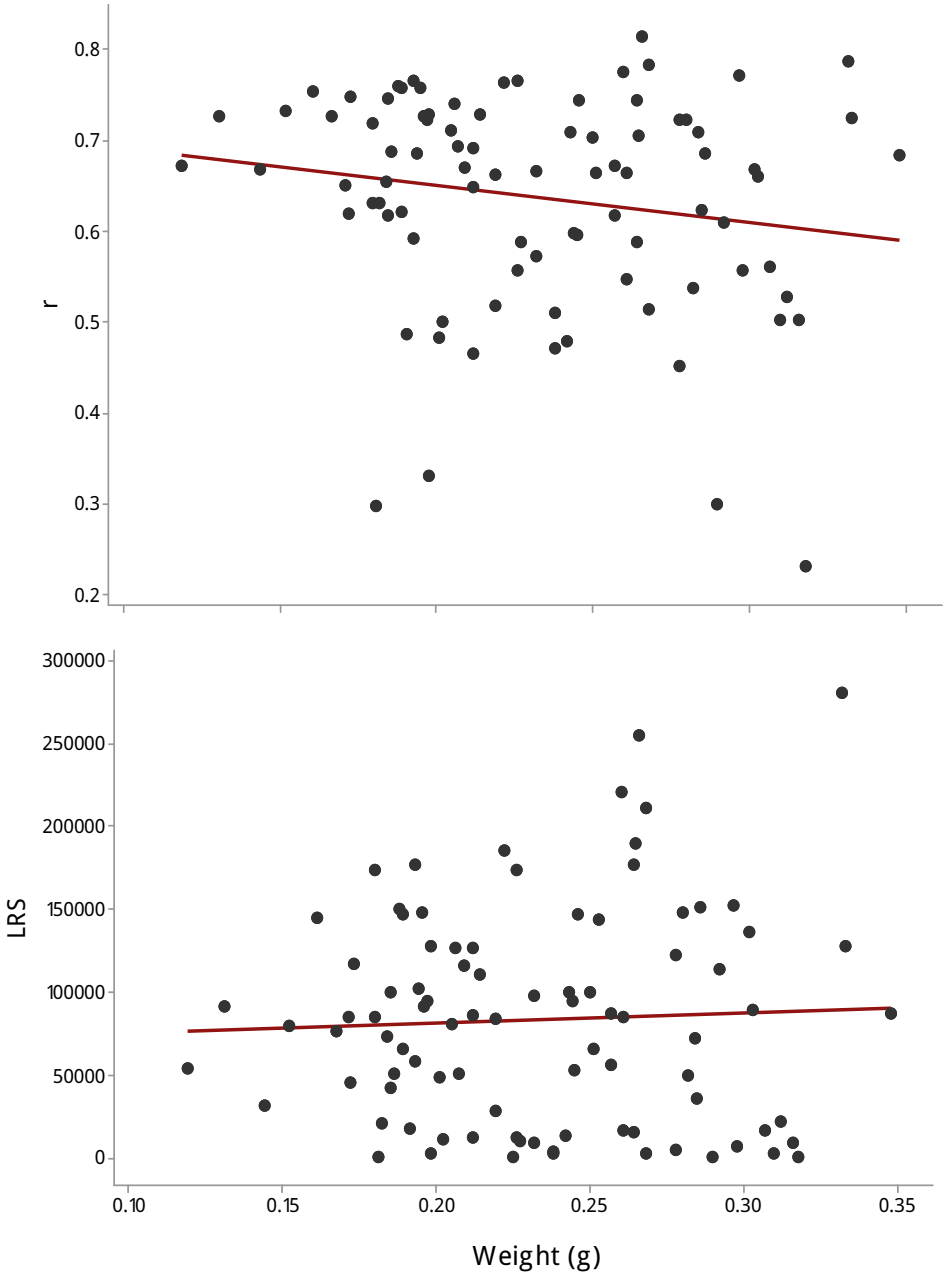


Figure 3-5. Size of parental *S. carpocapsae* IJs that emerged from *G. mellonella* cadavers incubated at 20°C (A) and 25°C (B).


!
150000
125000
100000
75000
50000
LRS
A
B
A
C
1
2
3
4
.
1.00
0.75
0.50
0.25
0.00
-0.25
-0.50
r
A
A
A
B
1
2
3
4

Direct Effect		Maternal Effect			
Temperature	**IJ size ± se (µm)**	**Parental Temperature**	**Progeny Temperature**	**LRS ± se**	***r* ± se**
20°C	615.55 ± 11.09[a]	20°C	20°C	64094 ± 3983	0.607 ± 0.027[a]
		25°C	20°C	87422 ± 3717.86	0.651 ± 0.026[a]
25°C	557.98 ± 11.05[b]	20°C	25°C	49112 ± 3709.37	0.579 ± 0.027[b]
		25°C	25°C	130858 ± 6153.91	0.706 ± 0.03[c]
Host	**IJ size ± se (µm)**	**Parental Host**	**Progeny Host**	**LRS ± se**	***r* ± se**
G. melonella	751.94 ± 11.86	*G. melonella*	*G. melonella*	63942.88 ± 4386.79	0.565 ± 0.03
		T. molitor	*G. melonella*	75493.69 ± 4884.61	0.589 ± 0.03
T. molitor	707.89 ± 13.51	*G. melonella*	*T. molitor*	35457.76 ± 1851.14	0.408 ± 0.01
		T. molitor	*T. molitor*	54106.73 ± 4183.58	0.435 ± 0.03

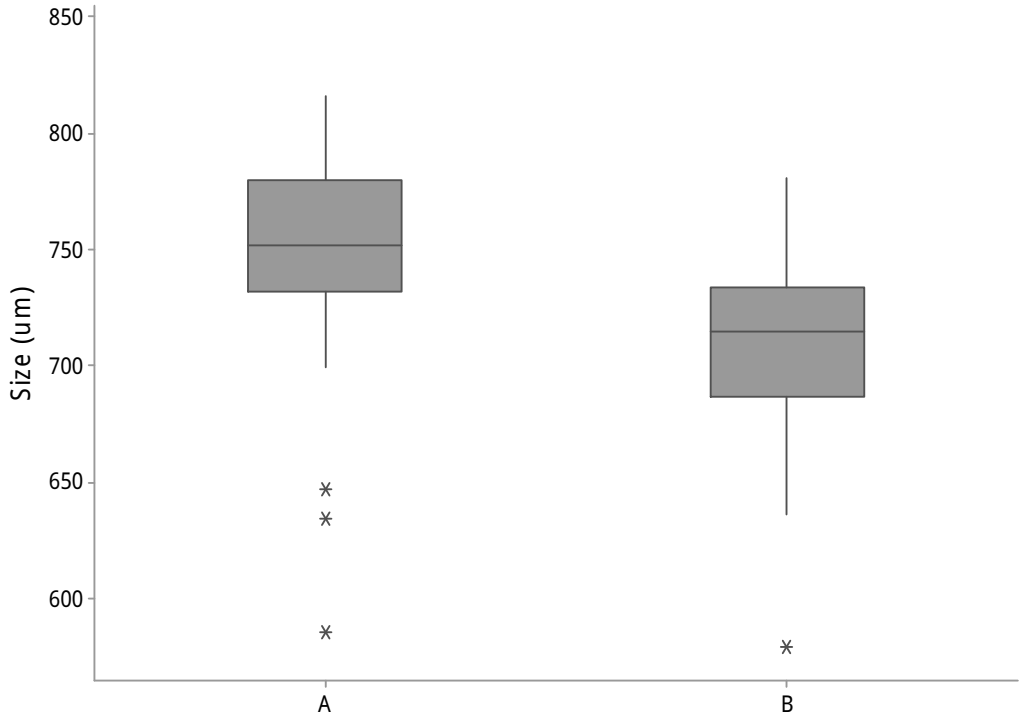
Table 3-1. Maternal length and progeny lifetime reproductive success (LRS) and intrinsic rate of increase (*r*) in nematodes reared either at two different temperatures or within two different host species. Superscript letter indicate significant differences between progeny groups that differ in parental treatments with groups that do not share a superscript letter differing significantly (by pairwise comparison: Temperature LRS $p<0.05$ by Mann-Whitney test; Temperature *r*, $p<0.01$; Host difference LRS and *r*, $p<0.001$ by 2 sample t-test). *Steinernema carpocapsae* and *Heterorhabditis downesi* are used for temperature and host different assays, respectively.

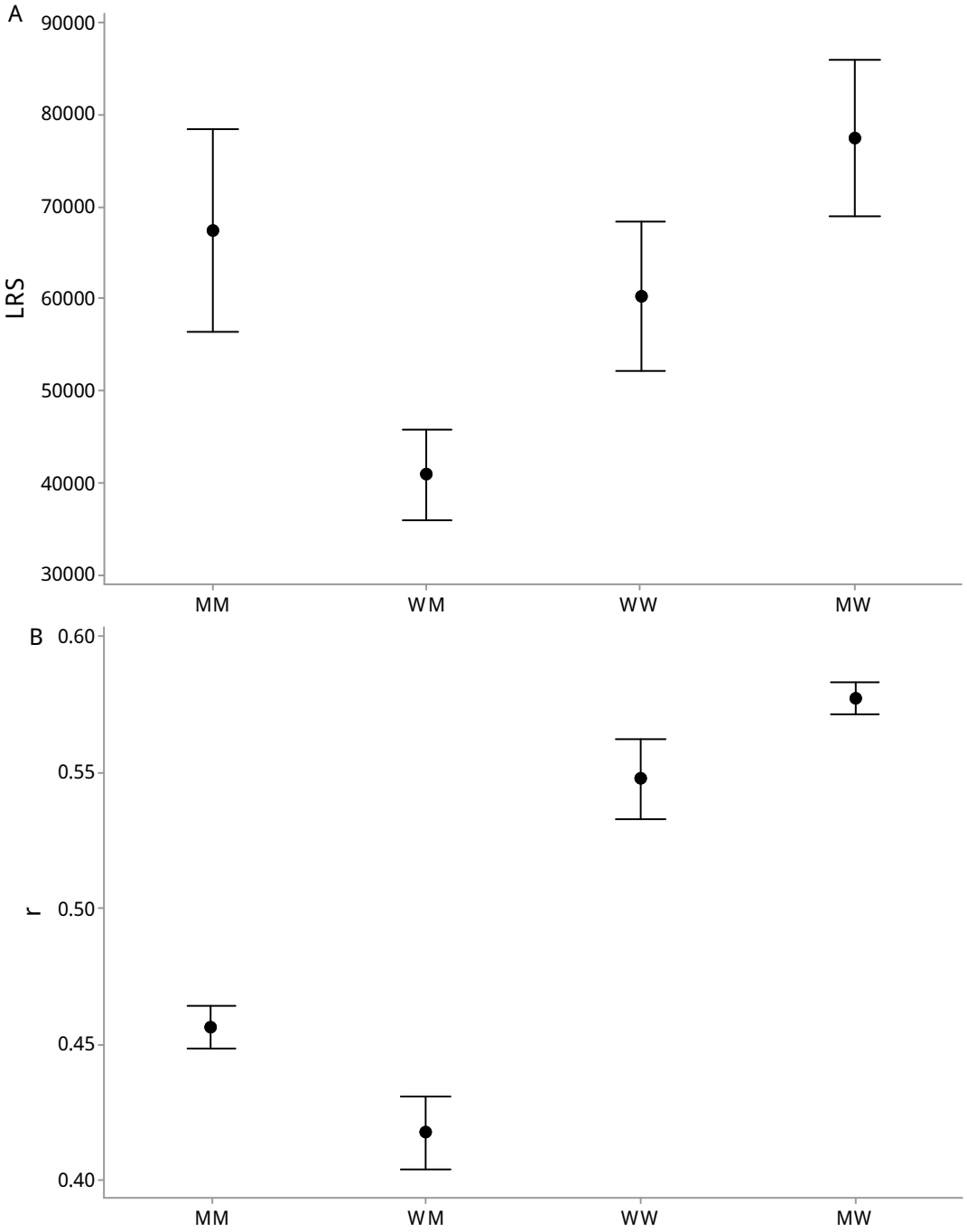

r
0.8
0.7
0.6
0.5
0.4
0.3
0.2
LRS
300000
250000
200000
150000
100000
50000
0
0.10
0.15
0.20
0.25
0.30
0.35
Weight (g)

A
B
C
D
Daily Emergence
Day of Emergence
0
10000
20000
30000
40000
50000
60000
2000
4000
6000
8000
12000
14000
16000
1
2
3
4
5
6
7
8
9
10

3.3.3 Host species affects nematode development

H. downesi IJs that emerged from T. molitor cadavers were significantly smaller (figure 3-9, table 3-1) than those that emerged from G. mellonella cadavers (Mann-Whitney test, $p < 0.001$, $W = 4757.0$). Nematodes reared in G. mellonella larvae developed faster and had increased fecundity as measured by r and LRS when compared to those reared in T. molitor larvae (figure 3-10; table 3-1). Further, parental host did not seem to matter if the second (progeny) host was G. mellonella. However, parental host had a significant effect on progeny that developed in a T. molitor cadaver with progeny that emerged from T. molitor cadavers developing more quickly, as measured by r (2 sample t-test, $p=0.042$, $t_{(18)}=2.19$) and producing more offspring, measured by LRS (2 sample t-test, $p= 0.02$, $t_{(26)}=2.48$) than progeny that emerged from G. mellonella larvae. Host species did affect emergence with G. mellonella IJs emerging in two distinct phases and T. molitor IJs only having one phase. Maternal temperature did not affect emergence pattern (figure 3-8).


850
800
750
700
650
600
Size (um)
A
B


A
LRS
90000
80000
70000
60000
50000
40000
30000
MM
WM
WW
MW
B
r
0.60
0.55
0.50
0.45
0.40
MM
WM
WW
MW

3.4 Discussion

EPNs are able to withstand extreme temperatures and have adapted mechanisms to survive cold and freezing. For example, H. zealandica IJs can survive at -32éC due to a protective sheath (Wharton & Surrey, 1994). EPNs can also survive heat stress within limits, with several EPN species showing great homology with C. elegans heat shock proteins involved in survival at higher than optimal temperatures (Lithgow et al., 1995; Cysper & Johnson, 2002). In this study, temperature affected both development time and lifetime reproductive success in S. carpocapsae, with faster nematode development the higher temperature of 25éC compared with 20éC. This is consistent with how temperature affects C. elegans, which develops from an egg to gravid adult in 72 hours at 20éC, but in only 48 hours at 25éC. Above this temperature, development begins to be hindered due to heat stress (F¶lix & Braendle, 2010). Here, in EPNs, there was a trend for faster development (r) and greater fecundity (LRS) for progeny of parents reared at 25éC compared with progeny from parents reared at 20éC. Indeed, the most similar groups were those whose parents were reared at the same temperature, suggesting that maternal temperature may be more important than the temperature the progeny experience directly. If 25éC is a more optimal temperature for maturation, growth and development than 20éC, this may also explain why there is a size difference between the parents reared at 20éC and 25éC. Over the course of IJ emergence from an insect host, Steinernema carpocapsae IJ size decreases, with the earliest emerging IJs being larger and more virulent than later emerging IJs (Therese & Bashey, 2012). However, the size of emerging IJs is not affected by in-host population size, which might be expected to differ between hosts at different temperatures if population growth within the host is faster at the higher temperature (Therese & Bashey, 2012), as was found in the present study. Therefore, size difference in the S. carpocapsae IJs reared at different temperatures in

the present study is therefore not likely to be caused by crowding, but may be a direct response to environmental temperature experienced in the host insect. This effect of temperature may, in turn, contribute to a maternal effect influencing the offspring of these IJs.

Here, data show that G. mellonella was a better host than T. molitor overall, with both LRS and r highest when G. mellonella was the second host (Table 3-1). The first insect host (G. mellonella or T. molitor) did not significantly affect progeny fitness when G. melonella was the second insect host. However, when T. molitor was the second insect host, the first insect host did have a significant effect, with a change of host from G. melonella (first host) to T. molitor (second host) being detrimental to fecundity and development (figure 3-6). Host species also affected IJ size, with parental IJs from T. molitor cadavers being significantly smaller than IJs that emerged from G. mellonella cadavers.

IJs are non-feeding dauer larvae, similar to C. elegans dauer larvae, and therefore require enough stored energy reserves to survive for long enough to reach and infect a new host that represents a new resource for growth and development. Fatty acids in insect haemolymph have been shown to be the source of IJ lipid stores and host lipid content affects IJs lipid content, for example Steinernema glaseri IJs were found to have a higher lipid store content when reared in the natural host P. japonica compared with those reared in the factitious host, G. mellonella (Gaugler, Lewis & Stuart, 1997). Lipid stores may also influence life history traits. For instance, larger worms are likely to survive longer due to the potential increase in lipid storage and infectivity of IJs may vary as a direct consequence of the quality of lipids stored by the IJ (Hatab et al., 1998). If lipid quantity and/or quality is lower in one of the host insects, thus creating a difference in food availability between the two host insects, then

life-history traits, such as growth, longevity, virulence and fecundity, are likely to be affected. This may explain why parental IJ size was significantly different between host species. This difference is further reflected in the differing fecundities and developmental timing of IJs in each insect host. Analysis of the fatty acid composition of each host species (and perhaps other commonly used insects for EPN production and/or target pest species) would help to determine if this may have been a factor here.

Emerging IJs differ in their infectivity according to the population density in the original host with crowding causing a delay in maximum infectivity (Ryder & Griffin, 2003). This may be an interesting factor to investigate further. Keeping in mind the environmental factors that have been demonstrated to impact development here, different levels of crowding could have been a confounding factor due to, for instance, populations growing faster (i.e. the intrinsic rate of increase, r, is higher). Having said that, the emergence trends over time show clear effects of the maternal environment even in the first emergence phase where crowding is unlikely. Here, emergence patterns showed that progeny temperature treatments were important for emergence with parental temperature not affecting emergence in either species or under any conditions. Host species did affect emergence with G. mellonella IJs emerging in two distinct phases and T. molitor IJs only having one phase. However the emergence patterns were not affected by the parental host and are therefore unlikely to be the cause of any maternal effect. Phased emergence and infectivity may be an important factor in ensuring a lower risk of overcrowding as it enables some IJs to infect hosts found in the immediately surrounding area whereas others are able to migrate further (Griffin, 2012).

The maternal environment can affect many life-history traits and maternal effects have been identified in a vast number of organisms. Here, the parental rearing environment has been shown to affect progeny fitness in S. carpocapsae and H. downesi. This suggests that maternal or paternal (or more broadly, transgenerational) effects could also be of importance in other parasites. The external environment as represented here by temperature can impact parasite fitness, implying that the host, while offering some protection from the external environment, cannot entirely protect a parasite from environmental effects, although this is likely only of particular relevance for parasites of ectotherms that are subject to external temperature. Mammalian parasites are not as likely to be affected by external temperature due to the ability of the host to regulate internal temperature. Similar effects of host lipids have been found in both EPNs and parasitoids with fatty acid composition of the host being reflected in the fatty acid composition of the parasite, in both cases and having an effect on parasite fitness (Thomson & Barlow, 1974). This highlights the importance of further studies of parasites which investigate the impact of environmental change and the potential for altered life-history strategies that increase parasite fitness and the impact this has to the virulence of infective stages. In EPN, an important consideration in this regard is whether or not these effects can change the quality of EPN IJs used for biological control. If the conditions in which IJs (or their parents) are reared contribute to the fitness of the IJs, the possibility of increasing virulence can be considered. If fitness can be increased by selecting the correct rearing conditions, then EPN effectiveness could be increased dramatically. For instance, it might be possible to increase fitness by using optimal rearing temperatures to induce desired maternal effects. Various studies have presented methods of in vitro mass culturing to maximise the number of IJs produced and a number of different culturing parameters are recommended, which also take into consideration the ideal growing conditions for the symbiotic bacteria, Xenorhabdus and Photorhabdus (Ferreira & Malan, 2014). For example Buecher and Popiel

(1989) recommend a medium containing tryptic soy, yeast extract and cholesterol with an incubation temperature of 25éC whereas Bedding (1981) recommends the inclusion of pig kidney in the growth medium. However, nematodes produced in liquid culture have been shown to be less virulent (measured by insect mortality) than those raise in vivo (Gaugler & Georgis, 1991). In vitro culturing is designed to produce mass quantities of nematodes and as such population density/crowding and resource limitation, as well as the potential of increased concentrations of ammonia and pheromone, are likely to be factors that affect the fitness of the produced IJs. Maternal effects have been demonstrated in response to most of these factors and it is therefore possible that the decrease in virulence noticed by Gaugler and Georgis (1991) are mediated by maternal responses to the in vitro environment. Additionally, it is possible that growth medium (and indeed, host species (or haemolymph)) directly affects proliferation of the colonising bacteria Xenorhabdus spp. and Photorhabdus spp., which in turn may indirectly affect nematode growth, development and, ultimately, fitness. This is another potential form of resource limitation caused by a difference in food quality.

Here, the influence of the maternal environment on progeny reproductive success is clear, as is the difference in population increase, as estimated by r and the direct effects of the environment on IJ size. It is important to consider that the estimation of r here is different to the estimation used in the previous chapter. In chapter two, r was calculated for C. elegans using an assay that involved moving a single worm on a daily basis and directly measuring daily fecundity. Here, due to the in vivo assays used, it was impossible to directly measure one worm˘s daily fecundity (egg production) and therefore the calculation is based on overall fecundity for multiple individuals within a host as reflected by IJ emergence. Additionally, in C. elegans age is counted from the time the arrested L1 is placed onto a plate with food whereas for EPNs, age is calculated from the time the IJ enters the host. However, the data

for r presented here still capture the effect of the maternal environmental differences as r is still measuring the overall population increase.

In order to expand the knowledge gained here, it would be interesting to determine how the same environments affect other traits such as survival, persistence and infectivity; all traits that relate to the efficacy of parasites and manipulation of which can lead to increased productivity in biological control agents. A deeper understanding of behavioural ecology is needed in order to further exploit the role of EPNs as biological control agents (Griffin, 2012). If parasites can be reared to be more suitable for the climate they are to be used in, then this may be another way to increase the effectiveness of EPN biological control methods. EPN species vary in their longevity, persistence in the soil and foraging behaviour and it could be commercially and ecologically beneficial to be able to manipulate production of EPNs that live longer, can survive stress better and are able to change their foraging behaviour according to. These data confirm the existence of maternal influence on progeny phenotype and suggest that environmental conditions contribute to parasite fitness. This is an important discovery for parasitology as nematode parasites (and indeed, free-living nematodes) are similar to one another. Maternal effects caused by temperature and resource availability have been demonstrated in free-living nematodes and now have been observed in two parasitic species. Finally, it will be important to establish the presence of these effects in nematodes outside of the Rhabditida as parasitism has evolved separately multiple times in the Nematoda (Blaxter et al., 1998). However, it is highly likely that similar responses will be observed in other parasitic nematodes and it is important, not just for biological control, but also for nematodes of importance for human and veterinary medicine and agriculture, that these effects are investigated further.

Chapter Four

Maternal Effects in Caenorhabditis elegans: Plant Extracts

Abstract

Chemical pesticides have been used increasingly since the mid-20th Century to increase agricultural productivity and chemicals such as organophosphates, organochlorides and carbamates successfully control insects, fungi, weeds and rodents, as well as both human and veterinary disease vectors. Of the agricultural pests, control of plant parasitic nematodes is particularly important due to their devastating impact on an extremely wide range of crops. Of these, the most economically important plant parasites are the root knot nematodes (Meloidogyne spp.) which are responsible for US $100 billion of crop damage every year. The control of root knot nematodes is difficult due to their being protected from the environment by surviving inside the plant root, however they have a free-living infective stage that is primarily found in the soil and is therefore exposed to the environment. Pesticides such as Methyl bromide have been effective at controlling root knot infections, however these are being phased out and new, environmentally friendlier control methods are needed. The African Marigold (Tagetes erecta) has been shown to control nematode populations when planted alongside crop plants and produces various compounds with potentially nematicidal properties. Here, extracts of marigold roots, shoots and flowers are prepared and their efficacy tested against the free-living nematode, Caenorhabditis elegans. Marigold crude extract reduces lifespan and fecundity of C. elegans at a concentration of 2% (v/v). Fractionation of the crude extract reveals that ethanol- and chloroform-soluble compounds also have nematicidal effects with dramatic reductions in lifespan and the cessation of reproduction, however, no maternal effects were observed at the tested concentrations. Data confirm that compounds produced in marigold petals have nematicidal properties and suggests that marigold extract, once refined, could be a viable pest treatment option worth further investigation.

4.1 Introduction

Chemical pesticides have been used since the middle of the 20th Century to increase agricultural productivity, keep up with growing demand for food production and ensure food security (Carvalho, 2006). Their usage increased dramatically with the discovery of DDT (Dichlorodiphenyltrichloroethane) in 1939 (Turasov, 2002) and chemicals such as organophosphates, organochlorides and carbamates have been used increasingly to successfully control insects, fungi, weeds and rodents in an attempt to intensify farming and keep up with increasing demand as well as to control disease vectors, both human and veterinary (Casida & Quistad, 1998).

Of the agricultural pests, control of plant parasitic nematodes is particularly important due to their devastating impact on an extremely wide range of crops. Plant parasitic nematodes are found worldwide and parasitize virtually every plant species (Moens et al., 2009). Perhaps the most economically important members of the plant parasites are from the Meloidogyne (Greek ¯ apple shaped female) genus, also known as the root-knot nematodes (RKN). They are responsible for huge annual economic losses causing up to US $80 billion of crop damage every year (Nicol et al., 2011). RKN are obligate parasites and they feed on modified cells within the plant root (Moens et al., 2009). This severely impacts plant physiology and in a plant considered to be a good host (e.g. tobacco, tomato) the worms can complete several reproductive cycles leading to severe crop damage and loss. Above ground symptoms include wilting and yellowing and below ground symptoms are the characteristic root galls, or knots, from which they get their common name. Ultimately infection leads to reduced yield, either directly from the stunted growth or damaged crop (especially in potatoes), or indirectly from increased susceptibility to pathogens (Manzanilla-Lopez & Starr, 2009).

Until recently, the most effective pesticide used for the control of RKN was Methyl Bromide (or Bromomethane) a broad-range soil fumigant which, as well as nematodes, also kills fungi and weeds (Zasada et al., 2010). However, due to environmental concerns, the use of Methyl Bromide, as well as many other major pesticides, has been outlawed leading to the need for new, `environmentally friendly_ methods of pest control (Zasada et al., 2010). Whilst biological control methods are being increasingly used to control a wide variety of pests (e.g. aphids (Schmidt et al., 2003); mites, whitefly (Messelink et al., 2008) and weeds (Messersmith & Adkins, 1995)), nematodes, especially RKN, pose more of a problem. Biological control methods, (e.g. parasitoid wasps (Beckage & Gelman, 2004) and entomopathogenic nematodes (Hazir et al., 2004)), work well for many below ground pests, but are not particularly effective against Meloidogyne spp. infections due to the parasites location within the host. Some progress has been made using fungi (Mankau, 1980) and bacteria that are pathogenic to nematodes, e.g. Pasteuria penetrans can be used in the control of M. incognita (Chen et al., 1996), but not all biocontrol species are specific and can be pathogenic to many other, sometimes beneficial, non-target soil organisms.

In resource-poor areas, where chemical pesticides are often too expensive to acquire, farmers rely on other methods of pest control by way of utilising long fallow periods, cover crops, antagonistic and trap crops, resistant crops and cheaper chemical control (Coyne et al., 2009). Using cover crops, that is planting non-host plants or antagonistic plants that either alongside (intercropping) or before and after a cash crop, can be an effective way of reducing nematode populations in the soil (Viaene & Abawi, 1998; Desaeger & Rao, 2000). Plants that are among the most effective to be used as a cover crop in the treatment of RKN infested fields are of the Asteraceae family, in particular the Marigolds (Tagetes spp.). The African marigold (Tagetes erecta) can effectively reduce the populations of multiple RKN species (Hooks et al., 2002) including the most important (in terms of their having the widest host ranges) species, M. incognita and M. hapla. Marigolds

are poor hosts to RKN and are considered resistant to infection (Tsay et al., 2004; Motsinger, 1997 (although see Belair (1992) who used T. patula as a stock plant to increase nematode numbers for pot trials) due to their roots producing nematicidal compounds such as polythienyls and thiophenes (Marotti et al. 2010). Indeed, Ploeg (2000) used marigolds to suppress RKN populations by amending soil in pot trials with marigold roots and shoots (i.e. mixed into the soil) leading to increased yield of tomatoes and melons and a decrease in the number and sizes of galls on the plant roots. Marigolds have also been shown to be effective at reducing nematode numbers when used as a companion crop with various cash crops such as tomato (e.g. Hackney & Dickerson, 1974), carrot (e.g. Huang, 1984), soybean (Adekunle, 2011) and aubergine (Dhanger et al., 1996) and when used as a green manure, or soil amendment, to treat the soil with before planting. By incorporating marigolds into the soil, before planting, yields have been shown to be improved when compared to crops grown in mono-culture (El-Hamawi et al., 2004; Akhtar & Malik, 2000) Another way of using cover crops is to use a trap species, a plant that is a more attractive host plant than the crop, to reduce the risk (although not entirely eliminate) to crop destruction (Melakeberhan et al., 2006; Belair & Benoir, 1996).

It is likely that the compound -terthienyl is the most active compound found in Marigold plants (Bakker et al., 1978). This is a phototoxic compound shown to have in vitro nematicidal properties, enhanced with exposure to UV light (Bakker et al., 1978). While the effects of -terthienyl have been investigated thoroughly, it has not become a commercially available nematicide, due to its high toxicity to other, non-target organisms, especially fish (Nivsarkar et al., 2001). It would, however, be interesting to investigate further the nematicidal properties of marigolds and determine if other, potentially less environmentally harsh, compounds found within the plant also have nematicidal properties.

To confirm the nematicidal effect of compounds produced in marigold plants, here, extracts are produced from the three major parts of the African Marigold, Tagetes erecta (cv. Crackerjack): The roots, shoots (stems and leaves) and flowers (petals) as all parts of the plant have been found to contain similar compounds in differing concentrations (Marotti et al., 2010). The model organism Caenorhabditis elegans is used to test the potentiality of each of the extracts nematicidal effect. Due to homology, if a compound is toxic to C. elegans it is likely to have the same effect on other nematodes (Hashmi et al., 2001). As such, C. elegans has been used as a model to investigate nematicidal effects of many compounds, including in anthelmintic drugs (see Chapter 5).

Finally, once the nematicidal effect of the marigold extracts have been confirmed it is important to establish whether treatment can induce a maternal effect. If nematodes living in soil are subjected to a stressful environment (i.e. the introduction of a toxic compound), it is possible that mothers, predicting a similar environment for their offspring, will shift their reproductive strategy in order to maximise the fitness of their offspring (Bernardo, 1996). In the case of Meloidogyne spp., because mothers are protected from most soil treatments due to them residing inside the root system, they are more are likely to survive. This does, however, mean that a mother may be more likely to pick up environmental cues from the soil and the plant, thus being ideally placed to increase her progeniesˇ ability to survive. Maternal effects have been shown in nematodes in response to multiple environmental cues such as temperature and resource limitation (Bernardo, 1996; Rossiter, 1996; Harvey & Orbidans, 2011). Responses to toxic compounds are complex (Leung et al., 2008; Williams & Dusenbery, 2009), however they have been shown to have dramatic effects on life-history affecting population numbers for multiple generations (Lopes et al., 2008). In RKN, maternal effects have not been explicitly demonstrated. However, there are many clues throughout the literature that point towards their existence, as discussed in chapter 1. RKN have been shown to be different according to the host they grow in and the temperature they

are grown at (Noe, 1991), the climate the plant is growing in (e.g. Barker et al, 1976), the amounts of nutrients the plants receive and the amount of rainfall a crop is subject to (although Niblack et al, (1986) found that population density was not affected by the environment at all). They are also capable of adapting to cold stress (Forge, 1990). Offspring are more likely to develop as males if they are from stressed plants, especially water stressed and pathogen infected plants. This response to stress has been manipulated by Snyder et al. (2006) who discovered that by over-pruning tomato plants, a continuous production of males could be significantly increased. Life-history strategy changes have been documented in some, but not all, RKN species and different species show different mechanisms and responses (Evans & Perry, 2009) although these have not necessarily been attributed to a maternal effect (although see chapter 1 for concerns about differing terminology in the literature).

C. elegans is an ideal candidate species to investigate the potential maternal response to the plant extracts as multiple maternal effects have been demonstrated in response to a wide range of environmental factors. C. elegans is widely used in drug discovery studies to determine if a drug has an anthelmintic property (Holden-Dye & Walker, 2007). Unlike parasites which are difficult to grow, isolate and maintain as well as being reliant on a host, C. elegans is easily cultured in vitro and due to its genetic and physiological homology with other nematodes it is an ideal model species; once effects are confirmed they can be further tested in target species.

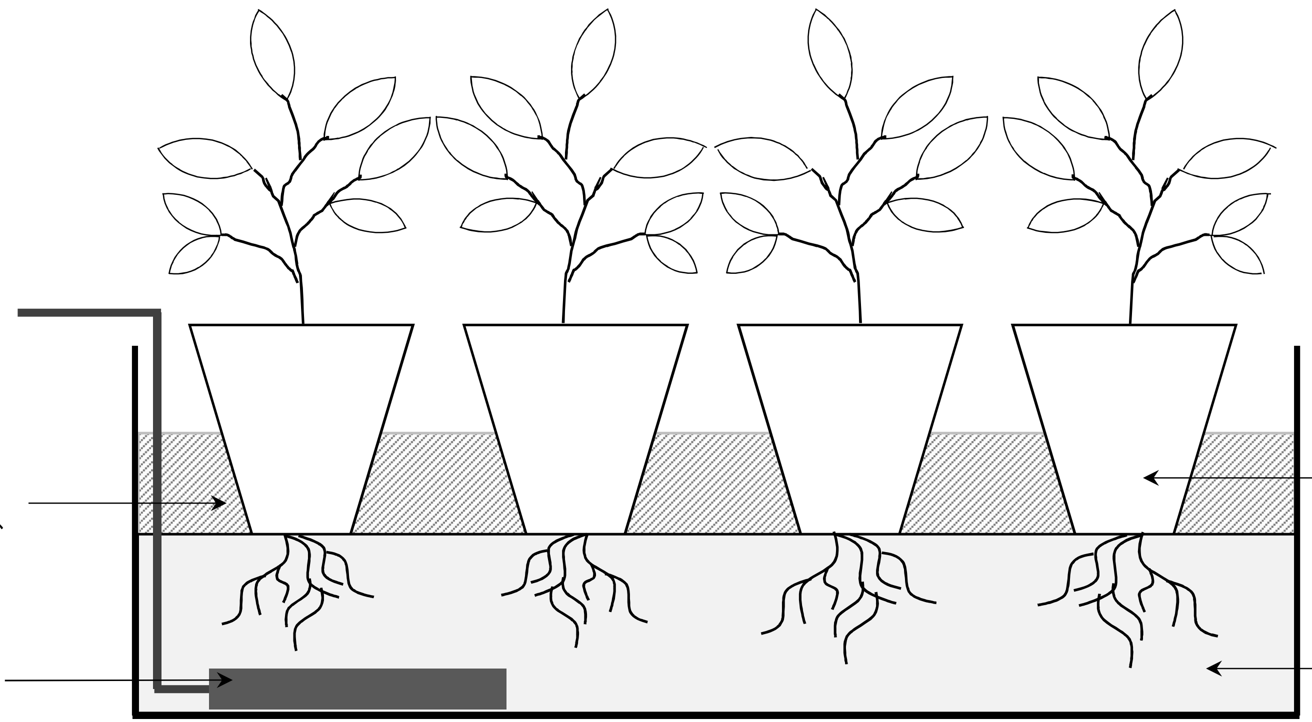
4.2 Materials & Methods

4.2.1 Worms

C. elegans isolate N2, obtained from the Caenorhabditis genetics centre, was used for experiments. Synchronised nematode populations were produced, as previously described (Chapter 2), by allowing eggs isolated from hypochlorite treated hermaphrodites to hatch on NGM agar plates without food and develop at 20éC overnight.

4.2.2 Marigold Extract

African marigold, Tagetes erecta (cv. Crackerjack), seeds were placed into pots containing a 3:1 mix of compost and sand and allowed to grow for 6 weeks until they were large enough to handle. Seedlings were then transplanted into polystyrene cups filled with perlite and placed into a hydroponic raft floatation system (Figure 4-1) containing nutrient solution prepared as per Lambert et al. (1992) for the exception of the micronutrients which were replaced with commercial solution, Supervit (Hesi Plantenvoeding BV, Netherlands) at a concentration of approximately 100ìl per 4.5 litres. Plants were allowed to grow to flowering, at which point the plants were removed and separated into three parts: roots, shoots, and flower heads. The remaining plant material discarded. All plant parts were placed in a drying oven for 12 hours at 70éC and then ground to a powder with a mortar and pestle. Once dried, the ground plant material was placed into glass bottles with ethanol and left for 3 days on a shaker at room temperature. After 3 days the supernatant was strained through muslin and remaining plant material discarded. Samples were centrifuged at 10000rpm for 10 minutes, supernatant retained, filtered through a 0.4ìm Nalgene syringe filter, and stored at 5éC.



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4.2.3 Initial Toxicity Determination

To determine whether any plant part was toxic, 200 arrested L1 larvae were placed in 100ul solutions of M9 containing 1% and 5% v/v concentrations of flower, shoot or root extract and incubated overnight (16 hours) at 20éC whilst shaking at 180 rpm. Worms were the centrifuged to form a worm pellet, the supernatant was discarded and the pellet re-suspended in M9 buffer. This wash procedure was repeated three times before the worms were pipetted onto NGM plates with 100 ≈l Escherichia coli strain OP50 and monitored for growth and reproduction.

4.2.4 Bacterial Toxicity Test

To ensure the bacteria were growing normally in the presence of the petal extract, bacterial growth over time was recorded by spectrophotometry. Two flasks of LB broth containing marigold extract at a concentration of 1% v/v were inoculated with 100ul of growing E.coli (OP50) and Pseudomonas fluorescens cultures. Four further flasks of broth were inoculated with each type of bacteria, two containing 10 ≈l of the bactericide sodium azide (0.1M), and one containing just broth as a negative control which was used as a blank. Absorbance was recorded every hour for four hours at OD600.

4.2.4 Toxicology Assay with Crude Petal Extract

Approximately 200 synchronised L1 larvae were placed into 1ml Eppendorf tubes containing 100ı l M9 solution [3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1ml 1M $MgSO_4$, H_2O to 1L] (Stiernagle, 2006) and petal extract at concentrations 0.2%, 0.3% and 0.4% (v/v), ethanol controls at concentrations 2%, 3% and 4% (v/v) and an M9 control. The tubes were incubated at 20éC for 24 hours whilst shaking at 180 rpm. The solutions were then transferred by pipette onto NGM agar plates until all liquid had evaporated. 30 individual L1s per treatment were picked onto individual

NGM agar plates with 100ı l OP50 and allowed to grow to gravid adult stage. Once reproduction began, adults were moved onto new plates daily and fecundity was scored. Life span was also recorded for all animals.

4.2.4 Fractionation of Crude Petal Extract

African Marigolds were grown, collected and prepared as in section 4.2.2. Following filtering, the petal extract was transferred to a round bottomed flask and fractionated in sequence using water, ethanol, chloroform and glycerol. Each solvent was sequentially evaporated under pressure using a rotary evaporator. Extraction in ethanol left a yellow sticky substance which was reconstituted in chloroform. This produced a brown liquid which was removed, leaving a paler yellow substance. This was then dissolved in ethanol and poured into a 15ml Eppendorf tube. The solution was centrifuged at 6000rpm for 5 minutes and the supernatant retained as the ethanol fraction. The remaining substance was insoluble in all standard solvents except for glycerol. The chloroform and ethanol fractions were evaporated under nitrogen gas and stored at 5éC until use. The glycerol fraction remained as a 50% w/v solution. No fractions were soluble in water.

4.2.6 Maternal effect assay with fractionated petal extracts

Approximately 200 synchronised L1 larvae were placed into 1ml Eppendorf tubes containing 950ul M9 solution plus 50ul of 60% (w/v) stock solution (in DMSO) of each petal extract so the final volume in each tube was 0.3% (w/v) extract and 2% (v/v) DMSO (DMSO is not toxic to C. elegans at 2%, Dengg & van Meel, 2004). The tubes were incubated at 20éC for 24 hours whilst shaking at 180 rpm. Tubes were centrifuged at 3000 rpm to pellet the worms and washed 4x with M9 before being pipetted onto NGM agar plates with 50ul OP50 and allowed to grow until gravid. After 72 hours it was clear that the worms in the chloroform and ethanol extracts were sterile

indicating that 0.3% extract is high enough to inhibit reproduction. To test the maternal effect the assay was restarted with 0.2% extract and left to develop, as before. Once gravid, adults were hypochlorite treated and eggs allowed to hatch and arrest overnight. Arrested L1s from each treatment (2% DMSO control, 0.2% chloroform extract, 0.2% ethanol extract and 0.2% glycerol extract) were divided into four groups and exposed to the four different treatments, (2% DMSO control, 0.2% chloroform extract, 0.2% ethanol extract and 0.2% glycerol extract) for 24 hours, as before. Following treatment 30 individual L1s per treatment were picked onto individual NGM agar plates with 100ı l OP50 and allowed to grow to gravid adult stage. Once reproduction began, adults were moved onto new plates daily and fecundity was scored. Life span was also recorded for all animals, with animals recorded as dead when they failed to respond to being touched with a platinum wire.

4.2.7 Statistical Analyses

Lifetime reproductive success (LRS) was calculated as the total number of progeny produced over a mother˘s lifetime. Analyses were performed using Minitab÷ 17.2.1.0. Differences in LRS were analysed by pairwise Mann-Whitney tests due to non-normal distributions and lifespan differences were analysed by Kaplan-Meier non-parametric distribution analyses.

4.3 Results

4.3.1 Petal extract

All worms treated with root and shoot extract developed into gravid adults. Worms treated with 5% v/v petal extract died prematurely, some appearing to have `melted_ with internal organs not developing properly and some worms having an empty space where the gonad should be, thus no data are reported here due to lack of reproduction. Worms treated with 1% v/v petal extract survived long enough to reproduce. For this reason, it was decided to discard the shoot and root extracts and continue investigating the effects of the petal extract.

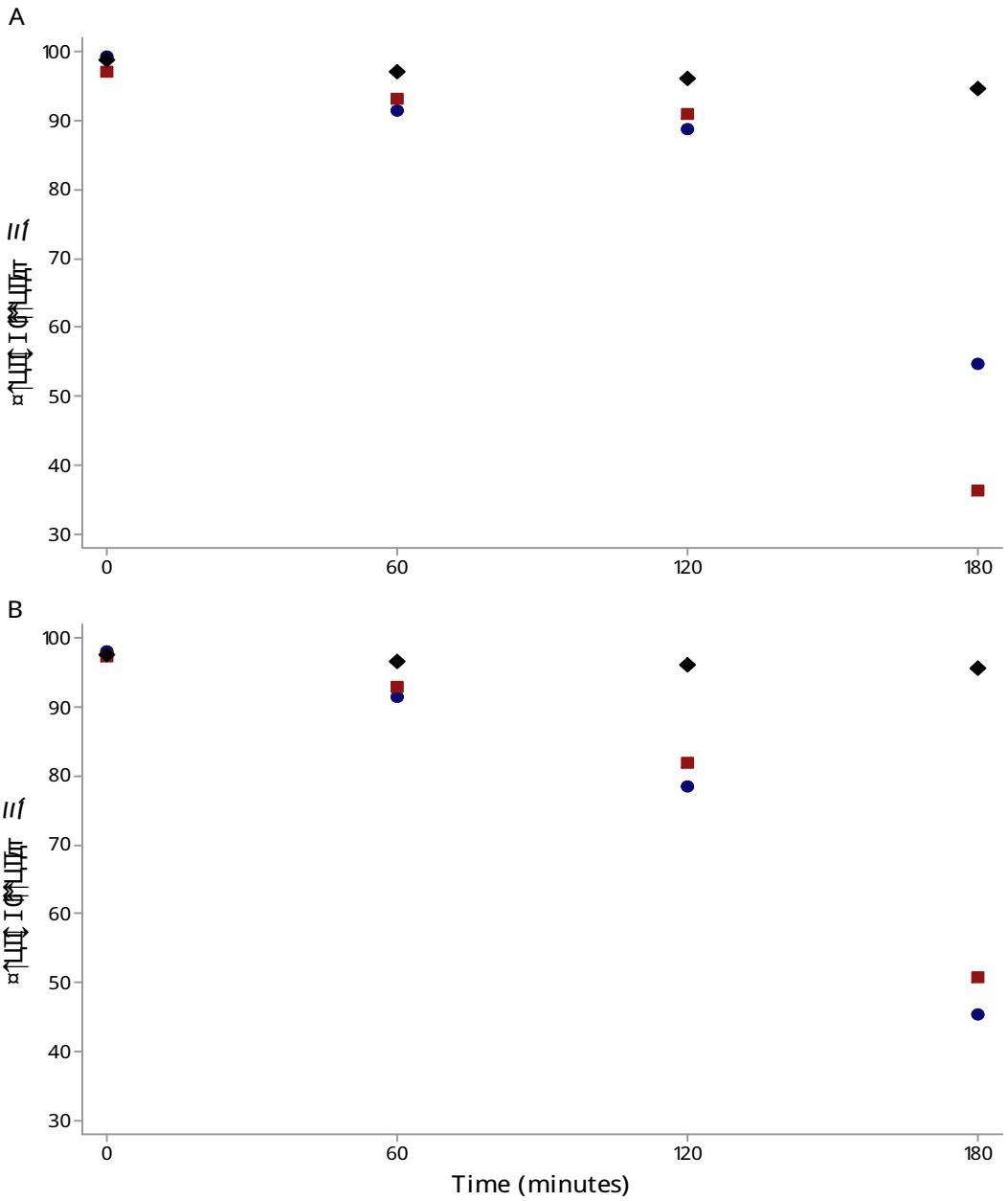
4.3.2 Bacterial growth is unaffected by crude marigold extract

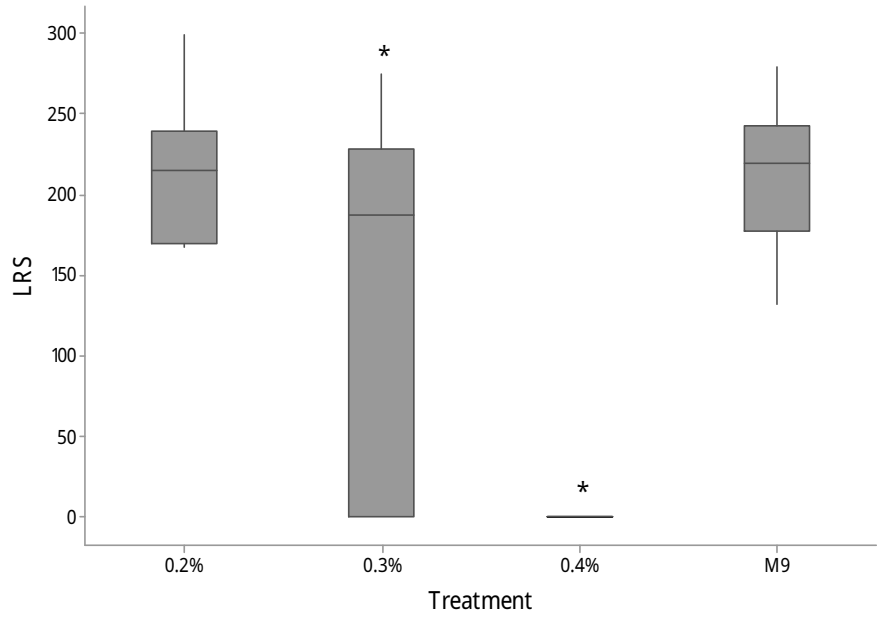
Before any C. elegans fecundity or lifespan assays could be undertaken, it was important to ensure that bacterial growth was unaffected by the plant extract. As C. elegans is primarily a bacterial feeding nematode, any change in bacterial growth could impact worm development and would potentially confound any result. No change was seen in the rate of growth of E. coli or P. fluorescens when compared to the control broth (figure 4-2) and the sodium azide broth, which completely inhibited growth. Both bacteria were therefore able to grow successfully in the presence of the plant extract, meaning that food quality should not be negatively affected during assays using the marigold extract.

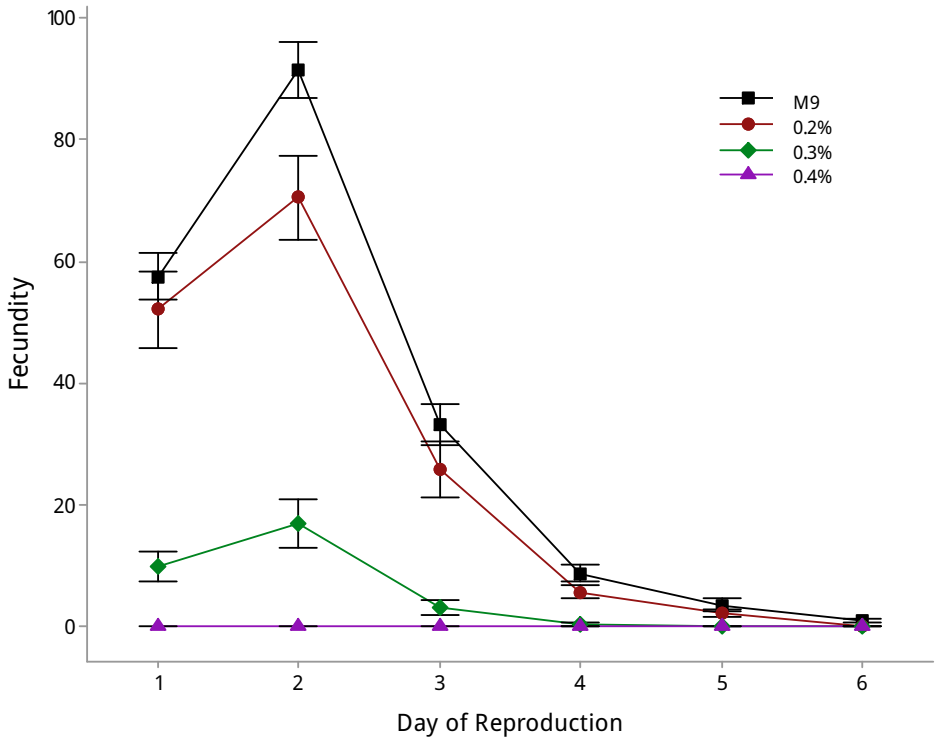
4.4.2 Lifespan and fecundity is limited by exposure to crude marigold extract

To determine whether exposure to marigold crude extract has an effect on lifetime reproductive success (LRS), fecundity was recorded daily for each worm and analysed by Mann-Whitney test.

LRS was found to be reduced at a concentrations of 0.2% and 0.3% extract ($p = 0.0036$ and <0.001, respectively) and reproduction ceased entirely at 0.4% (figure 4-3). Reproductive schedule was not altered by any treatment (except 0.4% where reproduction ceased entirely) with daily fecundity only being significantly reduced (Mann-Whitney test, $p< 0.001$) following the 0.3% treatment on days 1 ¯ 3 (figure 4-4). Lifespan was also monitored and it was shown, by Kaplan-Meier analysis, to be significantly reduced at 0.3% and 0.4% concentrations ($p=0.041$, $p<0.001$, respectively), but not at 0.2% ($p=0.325$) when compared to the ethanol and M9 (negative) controls (figure 4-5).


A
100
90
80
70
60
50
40
30
0
60
120
180
B
100
90
80
70
60
50
40
30
0
60
120
180
Time (minutes)

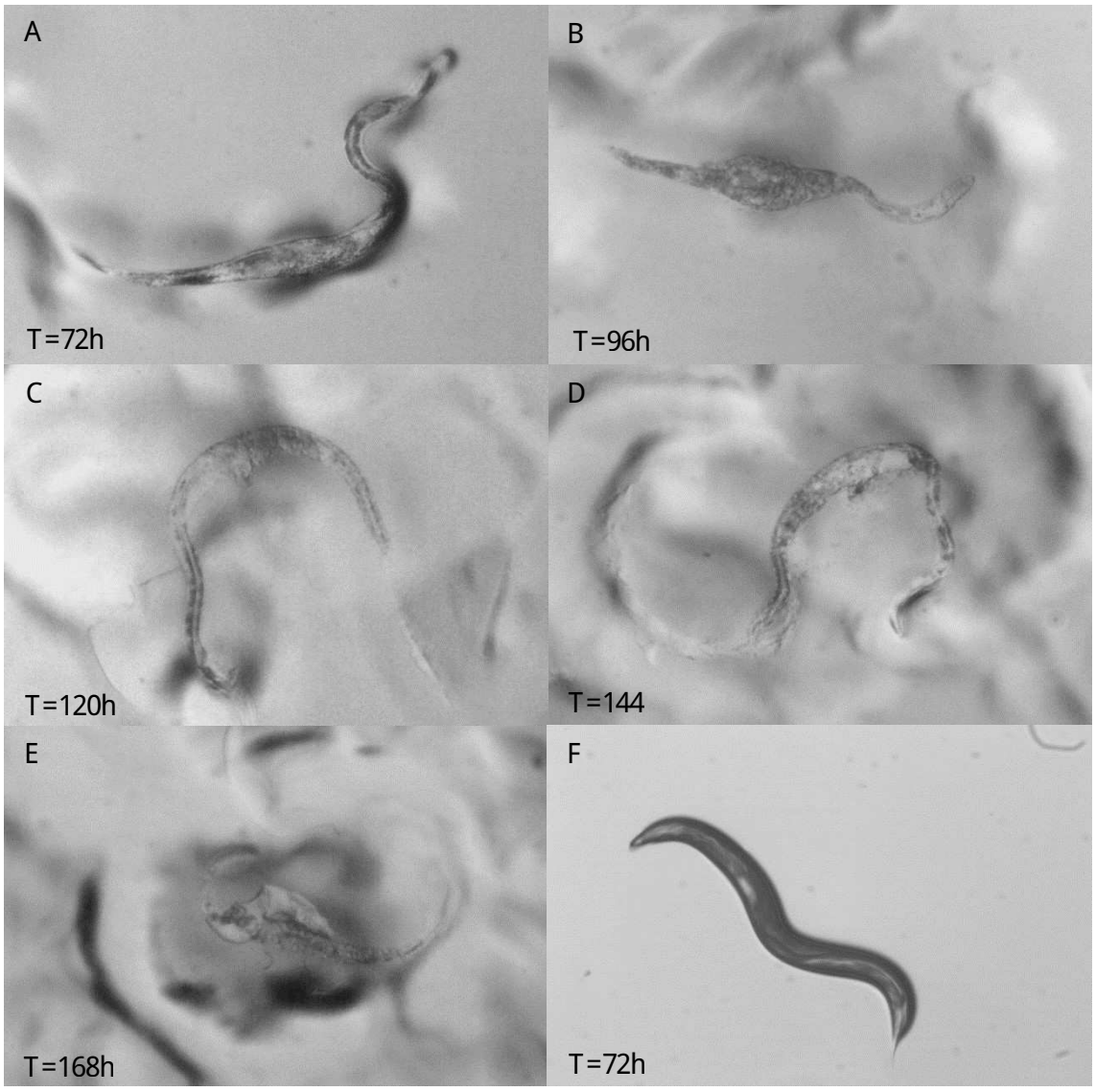

300
250
200
150
100
50
0
LRS
*
*
0.2%
0.3%
0.4%
M9
Treatment


100
80
60
40
20
0
Fecundity
1
2
3
4
5
6
Day of Reproduction
M9
0.2%
0.3%
0.4%

A
B
C
D
Survival (%)
Lifespan
0
20
40
60
80
100
0
5
10
15
20
25

4.4.3 Maternal effects in response to fractionated marigold extract

Initially, worms were exposed to three separate marigold extract fractions at a concentration of 3% v/v. At this concentration, all worms failed to reproduce when exposed to the chloroform and ethanol fractions, suggesting that there are active nematicidal compounds within both fractions. Due to the lack of reproduction at this concentration, a new assay was performed with a concentration of 2% v/v. Here, worms exposed to the glycerol fraction showed no reaction to the treatment, worms exposed to chloroform fraction produced very few offspring and the worms exposed to the ethanol fraction did not reproduce at all. The worms that experienced exposure to the ethanol fraction exhibited a similar phenotype to those exposed to the crude extract; their gonads appeared not to develop and had a melted appearance (see figure 4-6). Due to the low numbers of progeny produced by the chloroform exposed worms and complete lack of offspring from the ethanol exposed worms, it was not possible to determine if a maternal effect existed after maternal exposure to marigold petal extracts. Lifespan was recorded for each of the treatments and neither the chloroform nor glycerol treated worms showed a decrease in lifespan. The ethanol extract treatment did, as expected, reduce lifespan dramatically although some worms did survive for longer than might be expected with regards to their phenotype. The ethanol fraction was also more effective at 0.2% and 0.3% v/v (figure 4-7) than the same concentrations of the crude extract suggesting that whatever compound was responsible for reducing lifespan so dramatically at 0.4% v/v in the crude extract is potentially present at a greater concentration in the ethanol fraction of the extract.


A
T=72h
B
T=96h
C
T=120h
D
T=144
E
T=168h
F
T=72h

A
B
C
D
M9
EtOH
Chl
Gly
Survival (%)
Day
0
20
40
60
80
100
5
10
15

4.5 Discussion

The aim of this chapter was to determine whether there was a maternal effect in response to exposure to a toxic compound within the maternal environment. Due to the established and well known effects of marigolds and their ability to suppress nematode population growth when used as a cover crop, it was expected that a crude root extract would produce enough of a toxic effect to induce a phenotypic change in the nematodes. The product that resulted from extracting a crude solution from the marigold roots had no physiological effect on larval or adult stages. This lack of activity may have been due to many factors such as the hydroponic growing method or the extraction method (for instance, if the original extraction had have been performed using petroleum ether instead of ethanol (Nivsarkar, 2001)). The petal extract did have a nematicidal effect, both as a crude extract and later as a fractionated extract. This was interesting as petal compounds have not been previously explored as potential nematicidal compounds, although ethanol extracts from T. patula have been demonstrated to be toxic to the tick Rhipicephalus sanguineus (Politi et al., 2012). Marotti et al. (2010) drew attention to the fact that compounds such as -terthienyl were present in marigold flowers but in much lower concentrations than in the roots. Further, compounds that are produced in the petals are not ecologically relevant as their product would not normally end up being expressed into the soil and therefore would not be responsible for the suppressive effects of growing marigolds as a cover or companion crop. This does not, however, mean that further investigation into the properties of marigold petals should be abandoned. Marotti et al. (2010) go on to suggest that various Tagetes plants could be used as a green manure as a method of nematode suppression due to the occurrence of thiophenes in all parts of the plant. Many compounds have been discovered in various Tagetes species and in T. erecta and T. patula (French marigold). In particular, compounds such as pyrethrins (Hitmi et al., 2000) lutein esters (carotenoids) (Harikumar et al., 2008) polythienyls and thiophenes have been isolated and their nematicidal (and sometimes broader pesticidal) use and properties have been

investigated. For example, Dharmagadda et al. (2005) looked at various compounds in marigold essential oil for their potential use for the control of malaria vectors. Marigolds, therefore, and indeed many Asteraceae are clearly prime candidates for further exploration of novel pesticides, nematicides and acaricides. Here, the primary interest was whether or not we need to be concerned about maternal effects in response to such compounds. The physiological effects of the petal extracts used were clear; crude extract reduced fecundity and life-span and the first attempt at fractionation of the extract resulted in a compound that was capable of reducing life-span and stopping reproduction altogether.

The lack of reproduction meant that it was impossible to investigate the maternal effect. This was unfortunate as determining whether maternal exposure to any toxic compound has an influence on offspring fitness is very important. If mothers, protected from the outside environment, are able to sense a pesticide and consequentially alter their reproductive strategy then the implications for nematode control are potentially devastating for growers due to the potential for the development of fitter parasites. Any effective nematicide will reduce overall nematode numbers in an infested field, however, if mothers survive that treatment their future offspring may be different in terms of their ability to survive further treatment. Bet-hedging strategies have been documented in most RKN species in response environmental stress and result in a difference in gall number, fecundity and offspring size (Evans & Perry, 2009); similar responses to those of C. elegans. Whether or not this might contribute to resistance mechanisms is unknown and complex, but if maternal effects can affect an organisms ability to survive in other stressful situations (i.e. extreme temperature change, starvation etc.) then they may be caused by other factors, such as exposure to a toxic compound.

Chapter Five

Maternal Effects in Caenorhabditis elegans: Anthelmintic Drugs

Abstract

Anthelmintic drugs are used to treat diseases caused by parasitic roundworms, tapeworms and flukes (i.e. anti ¯ helminth) that infect humans, livestock and domestic pets. The World Health Organisation estimates that soil born helminth diseases infect over 3.5 billion people in sub-tropical regions. Current control methods utilise mass drug treatments and focus on reducing infection intensity and transmission rates in order to reduce and prevent mortality rather than on complete eradication. Broad spectrum anthelmintics such as Albendazole and Pyrantel are effective against many nematode parasites, but resistance to both drugs is widespread in many parasite populations, particularly in livestock. Doses of anthelmintic drugs intended to control rather than eradicate parasites can affect parasite fitness and alter life-history. Important life-history traits that determine virulence and transmission are fecundity and body size; bigger worms can produce more eggs and human health interventions have led to altered life-history. Thus, the evolution of larger, more fecund parasites is entirely possible and indeed, probable. Here, the effects of low dose maternal exposure to the common anthelmintic drugs, Albendazole and Pyrantel on offspring traits are investigated. Direct exposure to both drugs results in reduced reproductive success, lifespan and delayed development and well as reduced food consumption. Maternal exposure to Pyrantel results in offspring that are better able to survive when treated with higher drug doses and have greater reproductive success. These data suggest that low dose treatment results in mothers altering progeny to be better suited to survive under stressful conditions and, therefore, contributing to the increase of progeny fitness. If these effects, shown here in C. elegans, are also found to exist in parasites then the implications for mass drug administration could be severe and must be taken into consideration.

5.1 Introduction

Anthelmintic drugs are used to treat diseases caused by parasitic roundworms (nematodes), tapeworms and flukes (i.e. anti ¯ helminth). Such parasites infect humans, livestock and crops (see Chapter 4) and are of great importance throughout the world. The World Health Organisation (WHO) estimates that soil born helminth diseases infect over 3.5 billion people in sub-tropical regions (WHO, 2012), the most common being Ascariasis (giant roundworm, Ascaris lumbricoides), Trichuriasis (whipworm, Trichuris trichiura), Necatoriasis (hookworm, Necator americanus. Also caused by Ancylostoma duodenale), Schistosomiasis (Schistosoma spp.) and Lymphatic Filariasis/Elephantiasis (Wuchereria bancrofti, Brugia malayi & B. timori) (Lustigman, 2012; WHO, 2012). Current methods of control utilise mass drug treatments and focus on reducing infection intensity and transmission rates in order to reduce and prevent morbidity rather than complete eradication (Vercruysse, 2011). Treatment regime is commonly by annual or bi-annual mass drug administration programmes (WHO, 2012). Unfortunately, it is difficult to accurately assess the success and effects of mass drug treatment as most results are confounded by differing methodologies, an issue the WHO is working on correcting with a view to establishing standard operating procedures for mass drug treatments (see Vercruysse et al., 2011 for suggested standard operating procedures). Broad spectrum anthelmintics are effective against nearly all important gastrointestinal nematodes (filarial nematodes, however, pose a greater problem), treating both mature and immature life stages and targeting multiple sites such as ion channels, enzymes, structural proteins and transport molecules (Kohler, 2001). For helminth-caused diseases the commonly used drugs for mass treatment are benzimidazoles (such as Albendazole) and Avermectins (such as Ivermectin) which are used in combination to treat multiple diseases (lymphatic

filariasis, schistosomiasis, onchocerciasis and soil-transmitted helminthiasis) at once rather than targeting a single disease with multiple treatments (WHO, 2006).

Albendazole (ABZ) is a member of the benzimidazole group of anthelmintics which bind to and inhibit the polymerisation of ß-tubulin and the production of micro-tubules leading to slowed growth, decreased locomotion and, eventually death of the organism (Driscoll, 1989). This class of compounds are extremely effective anthelmintics because they have highly selective toxicity to helminths, binding to helminth tubulins with much stronger affinity to mammalian tubulins, which in turn means they have a low host toxicity. The effects of benzimidazoles have been extensively studied in Haemonchus contortus, the only nematode in which the downstream effects of benzimidazoles have been fully characterised, with effects such as tissue disintegration and DNA fragmentation seen 12 hours post-treatment (Jasmer et al., 2000). Such effects appear to be concentrated in the intestinal cells (Jasmer et al., 2000).

In veterinary medicine, similar drugs are used to control disease in livestock, but in pets (i.e. dogs and cats) the most commonly used de-wormers are the tetrahydrapirimidines (such as Levamisole and Pyrantel), of which the most commonly used drug is Pyrantel due to widespread resistance to Levamisole. Pyrantel, first discovered by Austin et al. in 1996 (Austin et al., 1966 in Aubrey et al., 1970), is a tetrahydropyrimidine and is in the same class of anthelmintic drugs as Levamisole (Imidothiazoles) although more effective at eliminating parasite infection within a host (Martin, 1997). These are depolarizing neuromuscular blocking agents (Aubrey, 1970) that work by binding to nicotinic acetylcholine (nACh) receptors in somatic muscle cells causing rapid depolarization and, subsequently, paralysis which leads to expulsion from the host (Kohler, 2001).

Most helminths of veterinary importance have been found to be resistant to benzimidazoles (e.g. Albendazole), many are also resistant to the Imidothiazoles (e.g. Pyrantel) (Sangster, 1999) and resistance to Ivermectin and Moxidectin has been observed in cattle in multiple countries (Geurden et al., 2015). Generally, if a population is resistant to one drug in a class it has an increased chance of becoming resistant to other drugs in the same class due to drugs in the same family having similar modes of action and sharing target sites within the parasite (Sangster, 1999). For instance, Hu et al. (2009) found that a newly described anthelmintic, Tribendimidine, had the same mode of action as Pyrantel and Levamisole and C. elegans mutants resistant to Levamisole were also resistance to Tribendimidine.

Resistance to benzimidazoles is commonly caused by mutations in ϕ-tubulin genes (Geary, 1999). Analysis in C. elegans revealed a mutation in the gene ben-1 (benzimidazole-sensitivity) which encodes a ϕ-tubulin and is itself redundant but part of a wider family of ϕ-tubulin encoding genes. Tubulins isolated from multiple resistant species show decreased affinity to the drug (Driscoll et al., 1989). However, whilst this may be one mechanism of resistance it is not likely to be the most common which is instead likely to be a deletion of a dispensable drug sensitive ϕ-tubulin gene in the same family in favour of an insensitive gene such as tub-1 (Geary, 1999). In C. elegans, Levamisole (and therefore Pyrantel) resistance, first noticed in hookworms and schistosomes in 2001, is caused by a lack of levamisole receptors ¯ one of a pair of nACh receptors found at neuromuscular junctions (Kohler, 2001).

Many factors affect resistance development in parasites. Importantly, the reproductive potential of an individual (i.e. number of eggs/female) is a key determinant (Geary, 1999). Subtle effects, such as a decreased rate of pharyngeal pumping, have been reported in C.

elegans in response to benzimidazole treatment (compared with more obvious phenotypes such as decreased motility in parasites) (Geary, 1999). The subsequent reduction in ATP production caused by the lack of food ingested is likely to cause a starvation response and may result in a maternal effect comparable to those observed in response to food shortage. The same may be true for responses to Pyrantel which due to its paralysing effect will cause a decrease in the activity of pharyngeal muscles and an inability to feed (Kohler, 2001). Important life-history traits used as determinants of virulence are fecundity and body size; bigger worms can produce more eggs and that human health interventions have led to altered life-history and the evolution of larger, more fecund parasites is entirely possible (Lynch, 2008) and indeed, probable. In terms of parasites, lifetime reproductive success (i.e. lifetime fecundity) may be considered the most important trait, but if size is to be considered (as in Lynch (2008) who showed that size is positively correlated with fecundity), timing is also crucial. Therefore, the intrinsic rate of increase, which takes into consideration the age of the mother, is also an important factor to consider when looking at life-history traits and potential bet-hedging strategies.

C. elegans is used as a model species in multiple fields and has aided in many important discoveries in neuroscience, developmental biology, cell biology, aging and biomedical and environmental toxicology (Leung, 2008). C. elegans has not been hugely successful as a model for drug discovery, perhaps because it has a higher tolerance for many compounds (Simpkin & Coles, 1981; O'Reilly et al., 2014), but has been valuable for research on the pharmacology of drugs and can be used to detect the potency of drugs and make predictions about the effects in parasites, however it must be noted that C. elegans is often able to tolerate much more environmental stress than parasites (Geary et al., 1999). Ho et al. (1994) found that drugs are more likely to enter the worms via trans-cuticular diffusion than via oral

ingestion, however this method of uptake should not be ignored. Concerns have been raised about the use of C. elegans as a model parasite due to the major differences in lifecycle, however it is generally agreed that parasitic species are as different to each other as they are to C. elegans and most agree that it is a useful tool in both genetic and physiological studies (Holden-Dye & Walker, 2007). Life-history traits have been affected in response to treatment with anthelmintics such as Emodepside, a paralysing agent similar to Pyrantel, which has been shown to be detrimental to developmental rate and egg laying behaviour in C. elegans (Bull et al., 2007), yet the extent of the paralysis is dependent on the larval stage of the worm with adults being more susceptible than L4 larvae, perhaps due to a change in cuticle permeability. Much variation has been demonstrated in C. elegans response to drugs depending on the larval stage and the drug tested. Indeed, the efficacy of many compounds has been shown to vary greatly between species, larval stage and in vitro vs. in vivo assays (Bull et al., 2007). It is important to note that benzimidazoles and nAChr agonists are much less potent against C. elegans than they are against parasitic nematodes (Geary et al., 1999), but this does not detract from the potential knowledge gained about anthelmintic effects using C. elegans as a model parasite.

With evidence of altered life-history in response to drugs similar to Pyrantel (Bull et al., 2007) the potential for maternally mediated changes in offspring are clear. Maternal effects have been observed in C. elegans (chapter 2) and in entomopathogenic nematodes (chapter 3) demonstrating that offspring phenotype is affected by the maternal environment in parasites under various conditions. It is unlikely that the environmental stress caused by drug treatment will not affect parasite reproductive strategy and offspring phenotype is likely to be affected by the toxic compounds the mother is exposed to in an effort for her to maximise offspring fitness in the same way other environmental stresses affect such traits. Maternal effects in

free-living organisms, like C. elegans, in response to environmental factors such as resource limitation, crowing and temperature, are ecologically important and can provide insight into how such organism behave outside of the laboratory. However, the same effects demonstrated in parasites might have serious implications for health management throughout the world. With mass drug treatment strategies being utilised to reduce rather than eradicate parasite load, mechanisms that produce fitter offspring must be understood. Here, the aim is to investigate the fitness effects of low and high dosages of Pyrantel and Albendazole and determine if and how life-history traits are affected. Further the effects of maternal exposure to low doses of each drug is explored and the impact on progeny phenotype, measured by the ability to reproduce and the rate of population increase, are explored and the importance of their consideration in human and veterinary drug treatment programmes is considered.

5.2 Methodology

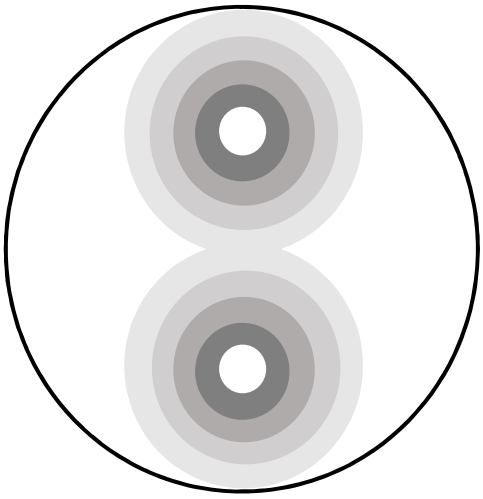
5.2.1 Determination of drug toxicity to Escherichia coli (OP50)

Before any growth assays were performed the effects of the anthelmintics on bacterial growth were assessed. If bacteria are affected by the drug this could confound the analysis of any effects observed in nematodes. NGM agar plates were inoculated with 100µl of E. coli OP50 which was spread across the plate to create a bacterial lawn. These plates were left to dry on the bench. Once the plates had dried, two 5mm holes were cut from the agar (figure 5-1) on opposite sides of the plate. The holes at the top of the plate were filled with 50µl of either 10mM Pyrantel or 10mM Albendazole (in DMSO vehicle diluted in H_2O so the final DMSO concentration was 1% v/v) (ten of each) and the holes at the bottom of the plate were filled with 50µl of a control (either pure H_2O or 1% v/v DMSO in H_2O) (five of each). In total, forty plates were inoculated. The agar absorbs the liquid creating a concentration gradient, the highest concentration being at the edge of the hole. All plates were then incubated overnight at 37ºC to allow the bacteria to grow. The following day the plates were removed from the incubator and assessed for any inhibition of bacterial growth.

5.2.2 Determination of developmental effects in response to low dose treatment

Synchronised nematode populations were produced, as previously described (Chapter 2), by allowing eggs isolated from hypochlorite treated hermaphrodites to hatch on NGM agar plates without food and develop at 20ºC overnight. 100µl of 10mM Albendazole and Pyrantel in DMSO and DMSO control were added to freshly poured NGM agar plates (containing 10ml agar so the concentration on each plate was either 0.01mM drug plus 1% DMSO or just

DMSO) and allowed to absorb overnight. The following day each plate was seeded with 50ı l OP50 and left to grow for 24 hours. In total there were 20 plates for each treatment; 0.01mM albendazole, 0.01mM pyrantel, 1% DMSO and just bacteria (80 plates total). A single arrested L1 was placed on each plate and incubated at 20éC for the duration of the assay. Each day, growth was assessed and the number of worms that reproduced was recorded. Reproducing worms were moved onto new plates containing the same drug and food amount.



5.2.3 Measuring food consumption in response to anthelmintic treatment

Arrested L1s, prepared as before, were pipetted into 1.5ml Eppendorf tubes (approx. 100 worms/tube) containing 1ml S-medium [1L S Basal [5.85g NaCl, 1g K_2HPO_4, 6g KH_2PO_4, 1ml cholesterol (5mg/ml in ethanol), H_2O to 1L,], 10ml 1M Potassium Citrate[20g citric acid monohydrate, 293.5g tri-potassium citrate monohydrate, H_2O to 1L], 10ml trace metals solution [1.86g disodium EDTA , 0.69g $FeSO_4$ Θ7 H_2O, 0.2g $MnCl_2$ Θ4 H_2O, 0.29g $ZnSO_4$ Θ 7 H_2O, 0.025g $CuSO_4$ Θ7 H_2O, H_2O to 1L], 3ml 1M $CaCl_2$, 3ml 1M $MgSO_4$]. To increase the solubility of the albendazole, cholesterol PEG-600 (Sigma, C-1145) was used in place of the usual cholesterol in the same quantity (Laing, 2010) and drug concentrations as follows: 25ı g/ml and 50ı g/ml in DMSO, plus one tube of S-medium and 1% DMSO as a control. Tubes were incubated for 24 hours at 25éC whilst shaking at 180rpm. After 24 hours, tubes were centrifuged at 3000rpm for three minutes to pellet the L1s. The supernatant was discarded and the worms were washed 3X in S-medium before being used for part two of the assay.

For part two, pre-treated L1s were divided between further drug treatments as demonstrated in figure 5-2. Worms (approx. 100 L1s), drugs (10ul 3mg/ml stock to make low concentrations 25ug/ml and 50ug/ml and high concentration 300ug/ml) and food (20ul 2% w/v OP50) were pipetted into wells containing S-medium in a 96-well plate which was then placed inside a plate reader (Fluostar Omega) for 72 hours (Elvin et al., 2011). The plate reader incubated the plates at 25éC and was programmed to shake the plate constantly at 500rpm. Absorbance readings were taken every hour over the 72 hour period to determine the amount of bacteria consumed over time. The absorbance was blanked against wells

containing S-medium and wells containing S-medium, 2% w/v OP50 and each drug concentration. Absorbance readings at 600 OD were used for analysis.

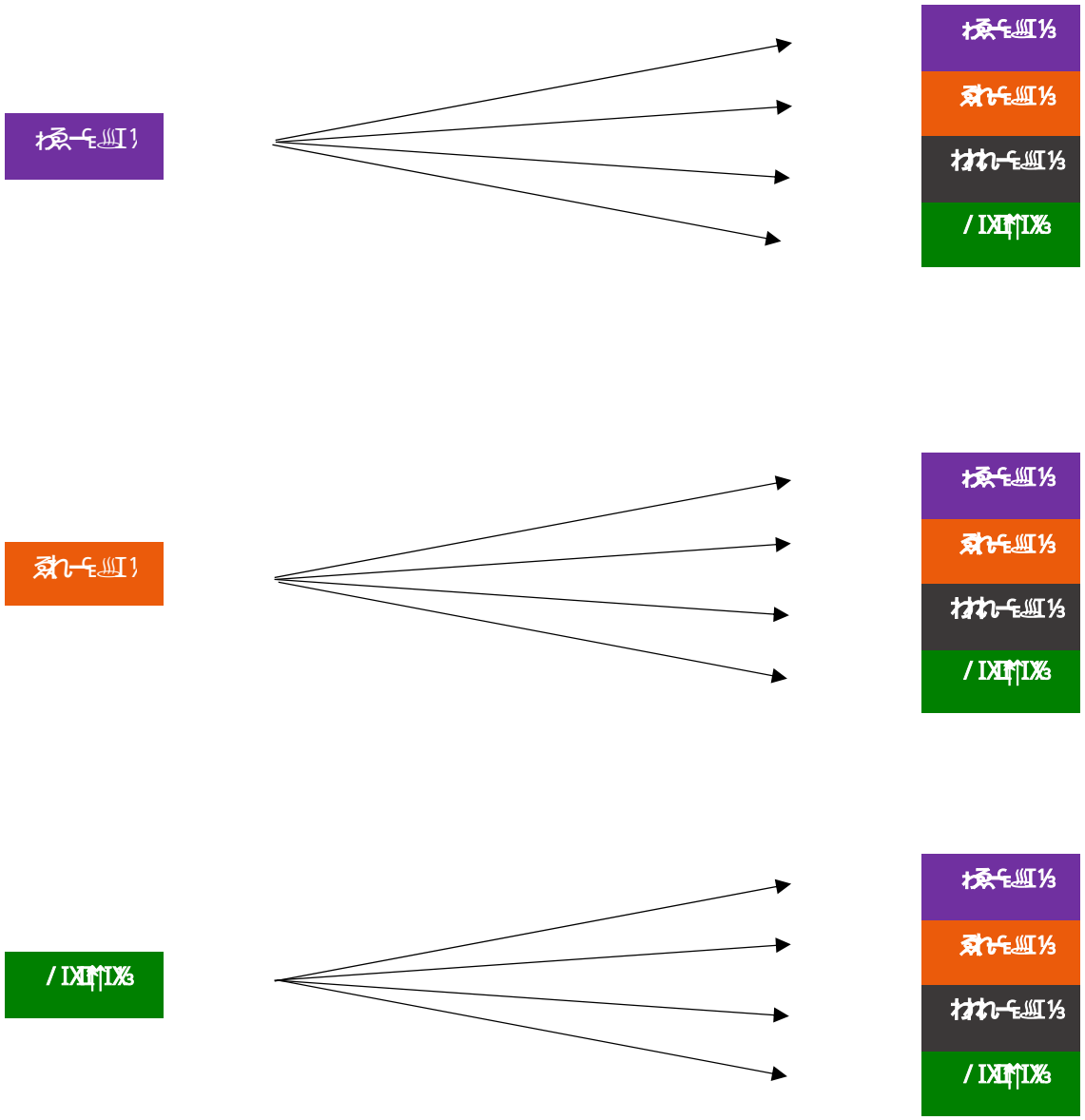
5.2.4 The effects of maternal exposure to low drug doses on offspring fitness.

L1 larvae were prepared and arrested as before and pipetted (approximately 1000 L1s in 100µl of S-medium) into 50ml screw top tubes containing either 300µg/ml albendazole (1.13µM) or pyrantel (0.504µM) (75µl of 20mg/ml stock in DMSO), 50µg/ml albendazole (188.4nM) or pyrantel (84.1nM) (12.5µl 20mg/ml stock in DMSO + 62.5µl DMSO) or 75µl DMSO as a control as well as 100µl of 50% w/v OP50. Tubes were topped up to 5ml with S-medium and then incubated at 20ºC for 48 hours constantly shaking at 180rpm. After 48 hours, the tubes contents were transferred to 15ml screw top tubes and diluted to 15ml with s-buffer. These were then centrifuged at 4000rpm for three minutes to pellet the worms. The supernatant was discarded and pellet retained and washed twice with M9 buffer to remove traces of the drugs and food. Worms were then re-suspended in 100µl sterile M9 and pipetted onto NGM agar plates seeded with 100µl food. Plates were then transferred to a 20ºC incubator and worms allowed to develop to gravidity. Once worms were reproducing (approximately 24 hours later), they were hypochlorite treated (as previously described) and their eggs allowed to hatch in the absence of food and arrest overnight. These arrested L1s were then divided between drug treatments (as in section 5.2.3) so that each set of progeny was exposed to each treatment and control (figure 5-2). These progeny treatments were identical to those experienced by the parent worms as described above and incubated for 48 hours at 20ºC whilst shaking at 180rpm. After 48 hours, the worms were pelleted, washed and transferred to NGM agar plates. This time, once the transferring liquid had been absorbed by the agar, individual worms were transferred onto NGM plates (30 plates per treatment),

containing 50ı l OP50 and incubated at 20éC. Plates were monitored daily and lifespan and fecundity was measured. Reproducing worms were moved daily to count daily fecundity. All plates were randomly coded. During the assay, some worms displayed an inability to lay their eggs which resulted in eggs hatching inside the mother (termed bag of worms). These worms were discarded from analysis.

5.2.4 Statistical Analysis

Data were analysed using Minitab÷ 17.2.1. Data were analysed using non-parametric tests due to non-normal distributions. Where transformations were possible, data were Johnson transformed (Johnson, 1949) and analysed by ANOVA. For the food consumption assays, absorbance readings were corrected against blanks containing buffer and drugs (no worms or food).



5.3 Results

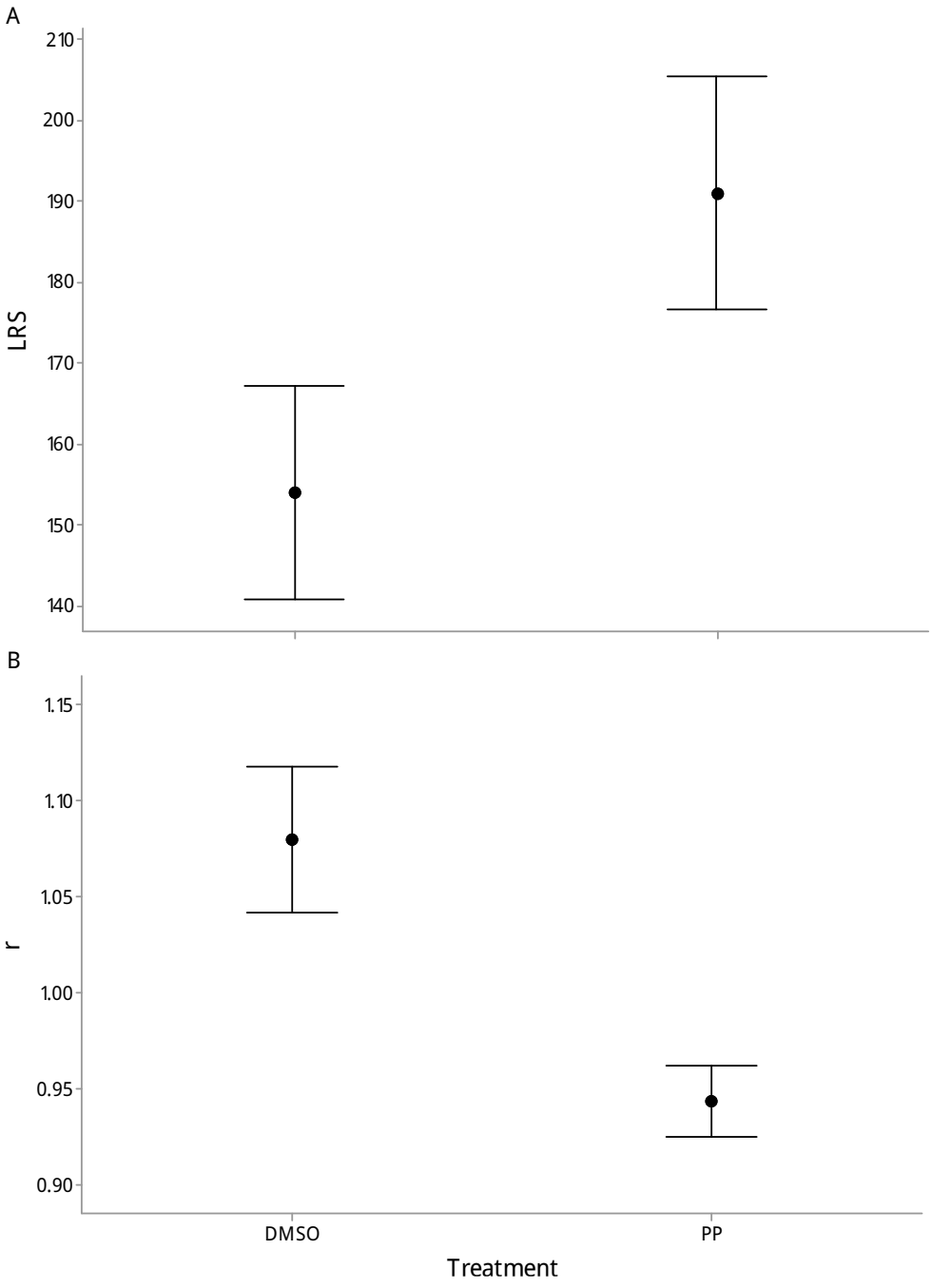
5.3.1 Bacterial growth is unaffected by drug treatment

To ensure developmental changes in C. elegans could not be attributed to negative bacterial growth effects, E. coli was grown on NGM agar plates in the presence of pyrantel, albendazole and DMSO vehicle. Bacterial growth was assessed visually by determining if an inhibition zone had developed around the treatment area (figure 5-1). No treatments resulted in the inhibition of bacterial growth indicating that bacteria will grow unhindered and food will not be limited by the presence of either drug.

5.3.2 Low dose drug exposure negatively affects C. elegans development

To assess the effects of low dosages of anthelmintics on the development of C. elegans, L1 larvae were allowed to develop on NGM agar plates containing pyrantel, albendazole or DMSO. The worms on the albendazole plates mostly failed to reproduce with only 20% of the worms laying any eggs. Of these, only a single worm produced a significant number of eggs (139). The other worms produced between 1 and 3 eggs, none of which hatched. It is important to note that the albendazole was fairly insoluble in the agar and therefore failed to soak into the agar. This meant that some of the drug solution remained on the agar surface and it is therefore likely that the concentration on albendazole was considerably higher than intended and unevenly distributed rather than forming a concentration gradient across the surface of the agar. For this reason, it was decided to continue all further assays in liquid culture. Both the pyrantel and DMSO treated worms developed into reproducing adults. Interestingly, lifetime reproductive success (LRS) was significantly higher ($F_{(1,37)}$=5.99

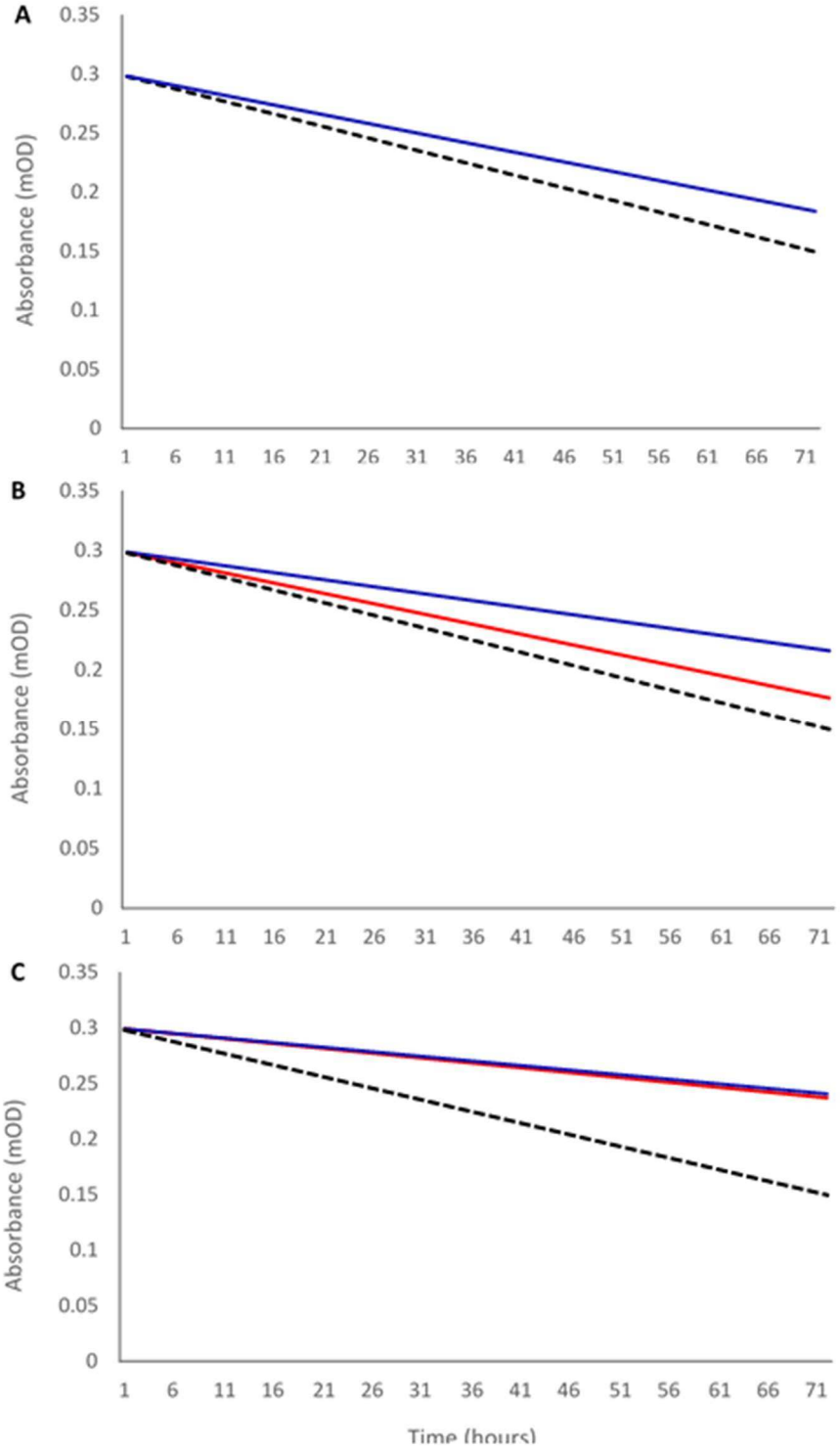
p=0.019 by one-way ANOVA) in the pyrantel treatment than in the DMSO control (figure 5-3A). However, the intrinsic rate of increase (r) was significantly lower ($F_{(1,37)}$=11.52, p=0.002 by one-way ANOVA; Johnson transformed) in the pyrantel treated worms, indicating that development time is increased in response to pyrantel treatment with worms taking longer to become mature and reproduce (figure 5-3 B).


A
LRS
210
200
190
180
170
160
150
140
B
r
1.15
1.10
1.05
1.00
0.95
0.90
DMSO
PP
Treatment

5.3.3 Pyrantel and albendazole reduce food ingestion and affect development

The results from the plate reader were not as clear as was hoped due to multiple factors, discussed below. Therefore, only one repetition was completed. The albendazole readings were not clear enough to interpret. Following the 72 hour treatment, the opacity of the drug increased and it had come out of solution, forming a thick precipitate. In all wells, most obviously in the highest dose treatment wells, this precipitate had collected in the base of the wells and trapped a lot of the bacteria. Paralysed worms were observed to be resting on the top of this precipitate. Few larvae had developed and of those that had, none had grown past the L4 larval stage, characterised by a transparent semi-circular area where the vulva is yet to develop. This suggested that most had been unable to feed.

The readings from the pyrantel treatments were clearer. Here, a linear change was observed in all treatments with the negative control showing the greatest level of food consumption (figure 5-4). Any exposure to pyrantel, either directly or indirectly (i.e. maternally) resulted in a reduction in food consumption, measured by the percentage decrease from the initial to the final absorbance readings (table 5-1) when compared to the negative control. In the highest progeny treatments, 300 ı g/ml pyrantel, there was a 20% decrease in food consumption as might be expected and these worms consumed the least amount of food. In all treatments, maternal exposure to the drug led to an even greater decrease in consumption than the direct treatments. It is important to note that all measurements are based on a median of n=2. Therefore, whilst these results raise interesting questions, the lack of replication makes statistical analysis impossible. For these reasons, it was decided that this method was an unreliable way to detect changes in food consumption and further assays were not performed


A
B
C
Absorbance (mOD)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1
6
11
16
21
26
31
36
41
46
51
56
61
66
71
Time (hours)

Maternal Treatment	Offspring Treatment	% Decrease in Food	Change in Absorbance
0	0	47.7	0.15
50		36.6	0.12
0	50	41.3	0.13
50		34.4	0.11
0	300	21.3	0.07
50		16.3	0.05

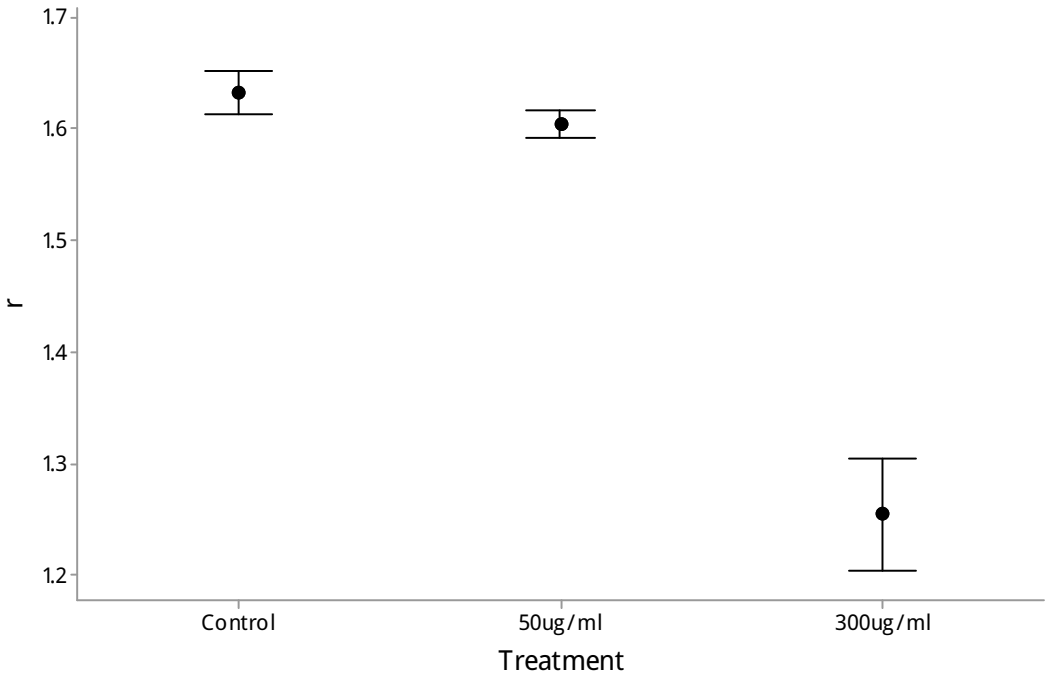
[illegible]

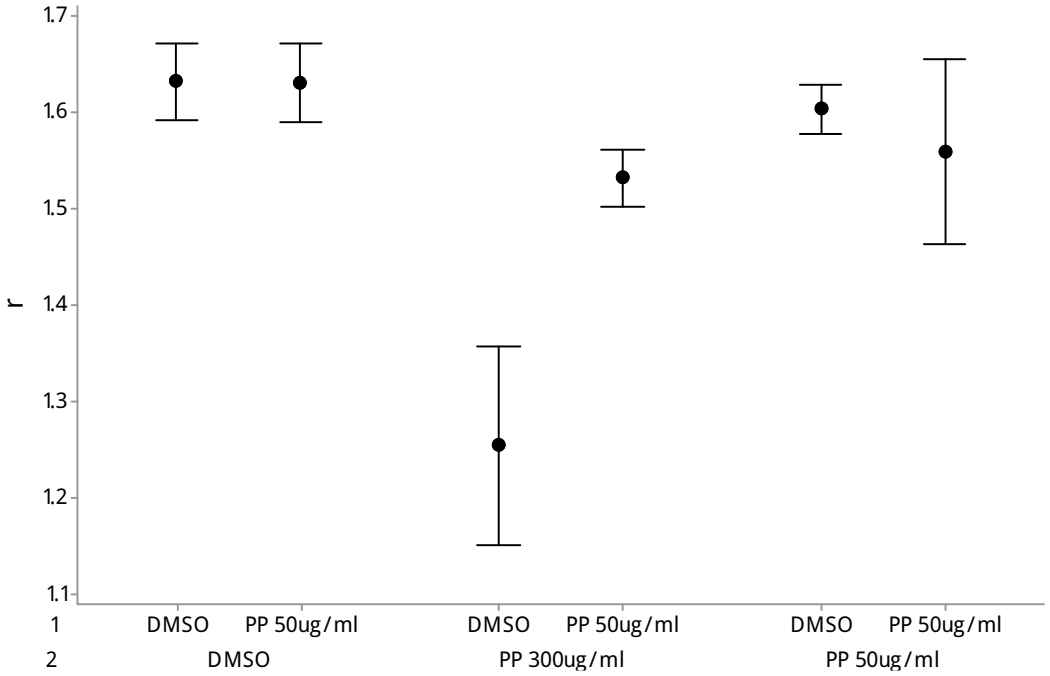
5.3.4 Low dose maternal drug exposure leads to high dose tolerance in progeny.

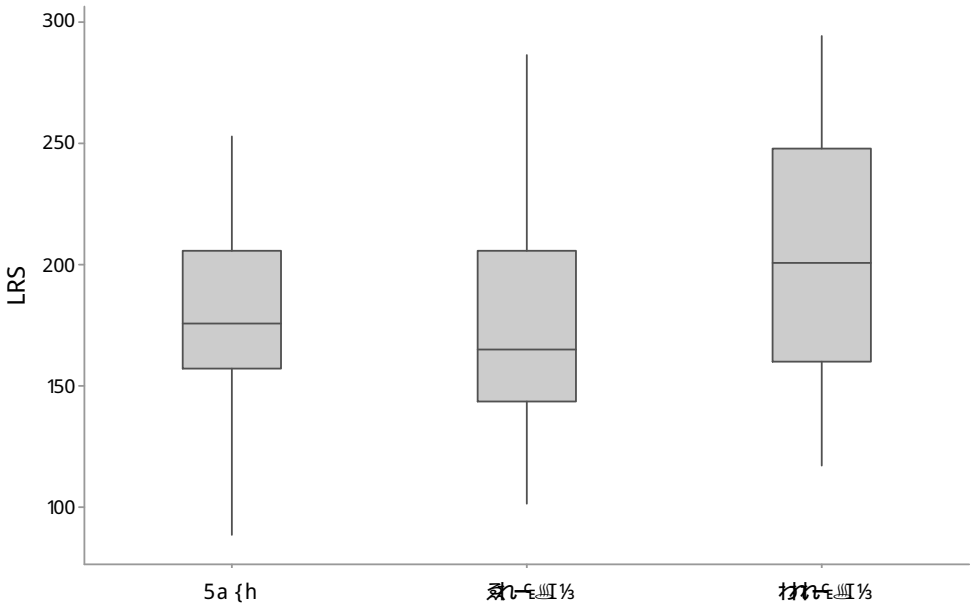
Because of the insoluble nature of albendazole it was necessary to use liquid culture methods to assess drug effects. Here, worms were allowed to grow to gravidity while exposed to various concentrations of each drug and the effects of low and high dose parental exposure on offspring life-history analysed. Albendazole limited reproduction dramatically even at low dosages and as such it was not possible to perform any maternal effect assay using Albendazole. Lower concentrations of Albendazole were not tested as a dose lower than 25 ı g/ml would be lower than any comparable real-world dosage.

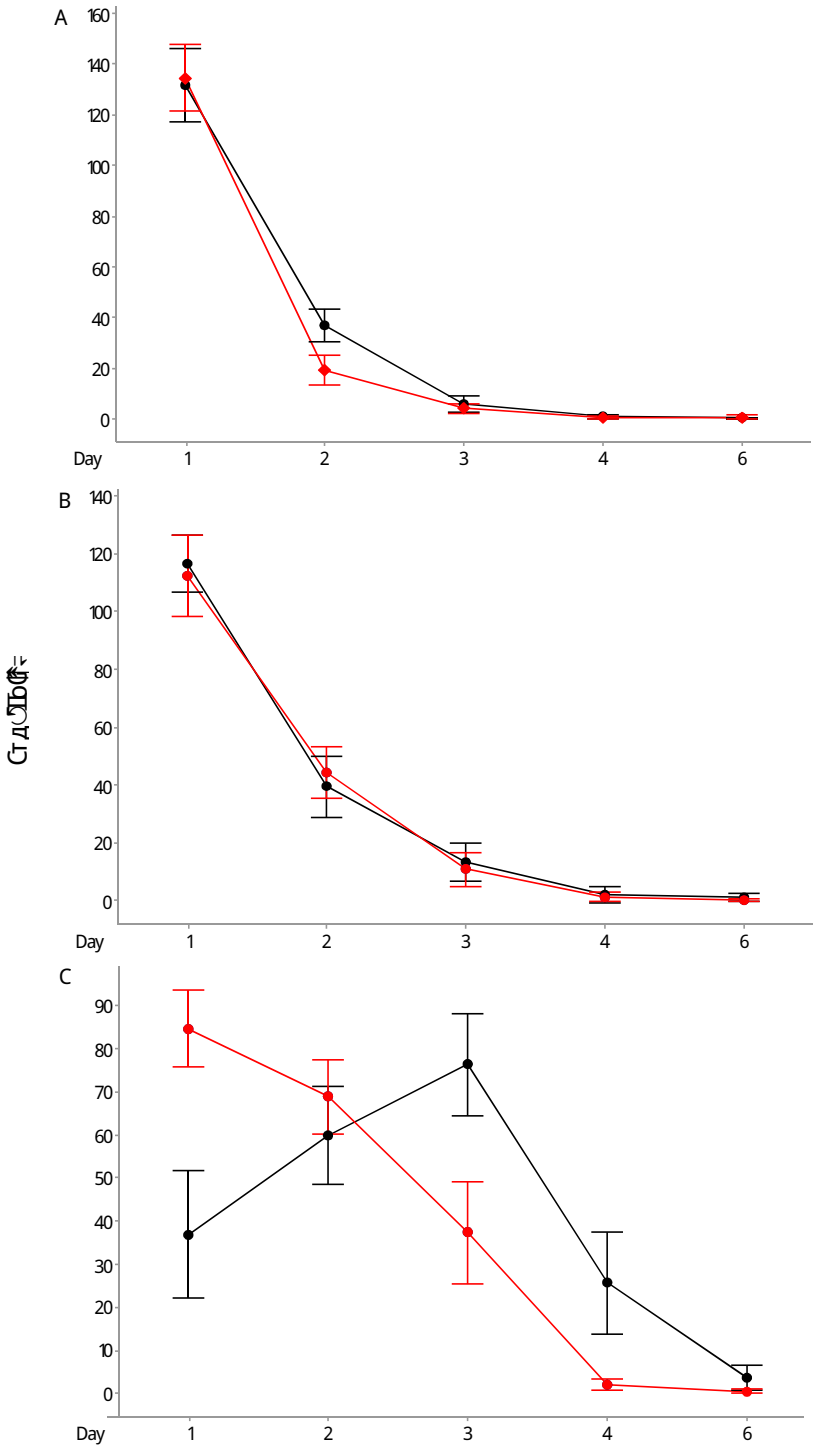
Worms exposed to Pyrantel did produce enough offspring to analyse a maternal effect (i.e. the parents produced enough offspring to expose a comparable number of L1s to the drug treatments). There is an overall developmental delay shown as a reduction in the intrinsic rate of increase (r) in response to Pyrantel that is stronger with a higher dose (i.e. development becomes slower when drug dosage is higher) (figure 5-5). However, when offspring from mothers reared in the presence of a low drug does are exposed to the higher drug does, r is significantly higher than it is for offspring from mothers reared in S-buffer ($p<0.001$, figure 5-6). This change is also reflected in the change of reproductive schedule. When worms are exposed to pyrantel at a dose of 50ı g/ml, reproduction is the same as the control. Fecundity, or lifetime reproductive success (LRS) is not affected by most drug treatments, however at the higher dose of 300ı g/ml, progeny from mothers reared with no drug exposure had a slightly higher LRS ($p=0.017$ by Mann-Whitney test, figure 5-7). When offspring from control mothers (those exposed to no drug) are reared with exposure to 300ı g/ml pyrantel, their reproductive strategy is delayed with a peak in fecundity at day four but offspring from mothers exposed to the drug in a low dose, the reproductive strategy is more similar to the

schedule of the control and low dose offspring with the peak at day one, dropping only slightly at day two (figure 5-9). Whilst LRS does not significantly differ within any treatments, there is a clear developmental change in response to the maternal environment here and a potential indication of a bet-hedging strategy.


1.7
1.6
1.5
1.4
1.3
1.2
r
Control
50ug/ml
300ug/ml
Treatment


1.7
1.6
1.5
1.4
1.3
1.2
1.1
r
1
DMSO
PP 50ug/ml
DMSO
PP 50ug/ml
DMSO
PP 50ug/ml
2
DMSO
PP 300ug/ml
PP 50ug/ml


300
250
200
150
100
LRS
5a {h


A
B
C
Day
1
2
3
4
6

5.4 Discussion

Nematode parasites are responsible for an overwhelming number of diseases and deaths in sub-tropical regions and their control is therefore a major priority for bodies such as the World Health Organisation who are working towards reducing the number of deaths caused by such diseases. It is of paramount importance to slow the rise of resistance throughout human and veterinary medicine, however it is possible that non-genetic mediated responses can contribute to selection as well as genetic mutation and it is important that the potential for such mechanisms is not overlooked. The phenotypic effects of both albendazole and pyrantel are clear. Both drugs hinder development, limit movement, reduce fecundity and ultimately lead to the death of the nematode. In drugs that cause paralysis, locomotion is reduced and, therefore, so is access to food. This may be responsible for direct developmental delays but also indicates that resource limitation is likely occurring in both offspring and parents which is a common cause of maternally mediated phenotypic variation (Bull et al, 2007).

The direct effects of both pyrantel and albendazole have been demonstrated here with a complete lack of reproduction in worms reared on albendazole treated agar plates. The insolubility of the drug likely caused a highly concentrated region on the agar, which meant worms were exposed to albendazole in much higher concentrations than would be needed to negatively affect their development. The plates containing pyrantel absorbed the drug much more efficiently and it was possible to observe the developmental effects of the drug. Delays to development were observed in response to pyrantel treatment but interestingly, lifetime reproductive success was significantly higher following pyrantel treatment. This is also seen in the later maternal effect assay (section 5.3.4).

It was unfortunate that the results from the plate reader were inconclusive. There was an overall trend showing that food was being consumed, but there were extreme differences in reading between individual wells meaning that a truly accurate interpretation could not be made. Given time, it is possible that this method of analysis could be a valuable tool for monitoring resource consumption. Theoretically, if drugs such as those used here are limiting the feeding ability of nematodes, it should be possible to measure the rate of food consumption using similar methods. In this instance, time and resources were limiting factors and other methods were developed to measure nematode development (as in section 5.3.3), however, once perfected, this could be a useful tool to detect drug and dose efficacy. In the pyrantel treatments, readings were clearer and indicated that any potential maternal effect would be a negative one. However, as discussed previously, these results may not be reliable and are contradicted by the final assay which was more robust and therefore more likely to give an accurate example of a maternal effect.

Maternal exposure to low doses of pyrantel results in a clear fitness advantage for offspring. Assuming that the reproductive schedule adopted by worms in the control treatments (no drug) is optimal, with reproduction peaking on the first day, offspring from mothers reared in low dose conditions have a reproductive schedule closer to `normal_ than offspring from mothers that never came into contact with any drug. A change in reproductive strategy may be being utilised in an attempt to ensure offspring were produced before the effects of the drugs delayed reproduction or affected the likelihood of offspring survival which might be indicative of an adaptive response to the predicted presence of the drug in the progeny environment and would be a similar response to classic adaptive maternal effects in response to a predicted environmental stress. Given that in C. elegans early reproduction (high r) is a more successful strategy (Hodgkin & Barnes, 1991), it is likely that the maternal effect

observed here is adaptive and will increase fitness. To investigate this further, it might be interesting to see whether dispersal is affected by the same strategy. If offspring are being produced more quickly when mothers have been exposed to the drug, then other traits are likely to be affected. Direct drug treatment (i.e. no maternal exposure) resulted in delayed reproduction with the majority of offspring being produced on the second day. Very few offspring were produced on the same day as the control of maternal drug treatments, with most worms reproducing at least 24 hours later. This is most likely a direct developmental delay and can be seen as a reduction in the intrinsic rate of increase, r. The fact that this delay is reduced in offspring from drug exposed mothers is important and is likely another adaptive response. There has been little concerns about altered life-history traits affecting parasite evolution or contributing to resistance as, generally speaking, selection will favour worms that mature quickly in response to drug treatment and offspring will be smaller and less fecund (Poulin, 2007). However, analysis of various parasite evolution models has revealed that in some circumstances drug treatment (and sometimes vaccination) may be selecting for increased time to maturity and ultimately bigger, fitter parasites (Lynch et al., 2008).

Of course, any effects demonstrated in C. elegans must be explored in parasitic species in order to fully confirm their existence in parasite as is common practice in drug discovery and pharmacodynamics and kinetic studies first performed using C. elegans. However, maternal effects are seen here in C. elegans in response to low dose treatment, resulting in changes in life-history traits (i.e. development time, age at reproduction etc.) and it is recommended that the potential for such effects be considered when using C. elegans as a model parasite.

Chapter Six

General Discussion and Conclusions

6.1 General Discussion & Conclusions

There is an overwhelming body of evidence that shows how virtually any environmental stress (e.g. temperature, competition, low resource availability, toxicity) can have a direct impact on adaptive ‘plastic’ responses in so many species (see Simons (2011) who reviewed and produced evidence for maternal effects and bet-hedging across 16 phyla in over 100 studies). The study of maternal effects represents an important under-explored area of research in parasitology. For these reasons, further study is needed into maternal effects in both parasitic and non-parasitic species. C. elegans proves to be a useful model species in this area and provides a large quantity of preliminary data to inform further research; however, modelling can only take us so far.

Here I have: undertaken the first analysis of cyclically variable temperature in C. elegans and demonstrated that these varying temperatures can produce maternal effects (Chapter 2); provided evidence that similar responses are seen in entomopathogenic nematodes, the first formal demonstration of trans-generational effects in parasites that may be produced by maternal effects (Chapter 3); and shown that maternal exposure to low concentrations of the anthelmintic compound Pyrantel results in offspring that are better when exposed to high concentrations of the same compound (Chapter 4). To investigate maternal effects in responses to pesticides I also extracted compounds with anthelmintic properties from the African Marigold, Tagetes erecta. These compounds caused direct developmental problems following nematode exposure, however due to the efficacy of these compounds, maternal effects were not observed (Chapter 3). In combination these findings confirm that maternal effects can significantly affect nematode life histories and that they are relevant to parasites.

A greater understanding of maternal effects might lead to an ability to manipulate parasitic development for a specific purpose or environment. For instance, if we are able to show how environmental stress can cause a reproductive shift that increases progeny fitness, how much more important could it be to show how environmental manipulation might decrease progeny fitness. Regular and prolonged anthelmintic chemotherapy is predicted to affect many aspects of parasite population biology. An understanding of the mechanisms behind such responses may greatly impact our understanding of how drug resistance develops in parasites. (Basáñez et al 2012).

Measures of fitness vary between studies with traits such as time to maturity, lifetime fecundity, lifespan and age at reproduction all being used to record the impact of environmental differences such as temperature and resource availability on offspring fitness. Temperature significantly affects many physiological processes and impacts the ecology and fitness of many organisms. Heat stress (or indeed cold stress), i.e. when environmental temperature is outside the expected optimal range), can negatively affect fitness or can induce a hormetic effect when an adaptive response and an increase in fitness is observed following a gradual change in temperature and short exposure stressful conditions (also known as preconditioning [Cypser & Johnson, 2002; Yokoyama et al., 2002; Calabrese et al., 2007; Sørensen et al., 2008]). It is therefore expected that the thermal preference of an organism should be a temperature, or range of temperatures, that maximise fitness. Any deviation from this thermal niche, however temporary, is likely to have an associated fitness cost (Anderson et al., 2011).

Temperature has been well studied but most studies focus on constant temperatures which are unrepresentative of natural conditions. There is growing concern about how informative

these studies are. The effects of slight temperature changes concern climatologists and ecologists alike, yet little is known about the effects of daily temperature cycles and short-term temperature variability (Arrighi et al., 2013). Although C. elegans has been demonstrated to show little, if any, difference in temperature sensitivity between isolates, regardless of their natural climate (Hodgkin & Doniach, 1997), analysis of data presented in chapter two revealed that temperature affects individual fitness and progeny fitness. Specifically, progeny fitness (measured by r and LRS) was significantly affected by the maternal rearing temperature in the N2 strain of C. elegans. Varying temperature seems to have little impact on population growth dynamics, although the proportion of dauer larvae was higher in populations that were grown under variable temperature. C. elegans dauer larvae are much more stress resistant than other larval stages and the greater proportion of these larvae in the variable temperature population may indicate a response to the two temperature extremes (10 and 25éC) experienced. If the sensitive period when the developmental decision between dauer and non-dauer larval development occurs at a time when the temperature is high it may induce dauer development. To investigate this further, a staggered assay could be performed where arrested L1 larvae are placed in a variable temperature incubator and fed (which allows development to continue) at various time points. If there was a difference in dauer proportion between such treatments it may be indicative of a temperature response in dauer production. This would also provide insight into how populations develop in the wild, where temperature is not constant, and may also provide information about how the dauer response interacts with circadian rhythms. To better understand the effects of temperature on Caenorhabditis spp. in general, C. briggsae may also be a viable candidate species as clear genetic and phenotypic differences have been observed between strains isolated in temperate and tropical climates (Cutter et al., 2006), unlike C. elegans.

The effects of temperature on the development of the entomopathogenic nematode, Steinernema carpocapsae have also been demonstrated here (chapter 3). Direct effects follow the same pattern as observed in C. elegans with development being faster at a higher temperature (20éC vs. 25éC). Emerging IJs were smaller at the higher temperature. The effects of temperature on offspring have been well documented and less than optimal conditions usually result in clutches of fewer, larger offspring being produced. Given this, it could be concluded that 25éC is a more optimal temperature for S. carpocapsae development than 20éC due to the smaller IJ size at 25éC. However, fecundity data from these different IJ populations would need to be collected and compared to fully confirm this. Resource limitation also results in the production of fewer, larger offspring and it would be interesting to determine if bacterial proliferation within the host is affected by the outside-host temperature. If the symbiotic bacteria are hindered in their growth at lower temperatures then food availability may be more restricted at lower temperatures which may in turn cause a change in offspring fitness similar to that observed in other species in response to limited food resources (Harvey & Orbidans 2011). Again, fecundity data for the same population would need to be collected to confirm this, as would data on bacterial growth rate, but the size difference in IJs produced at different temperatures reported here indicates that there is a temperature induced effect that may be further confounded by variation in resource availability within the host. The maternal rearing temperature affects progeny fecundity and development (measured by r) in a way that suggests that parent rearing temperature may be more important for progeny fitness than the temperature the progeny experience directly. The worst performing progeny (with the lowest fecundity and slowest development) were those reared at 25éC whose parents were reared at 20éC which indicates that mothers may be producing offspring that are better able to cope with higher temperatures if they too have

experienced the higher temperatures and that offspring whose mothers did not experience the higher temperature produce offspring that do worse at higher temperatures. The 25/25 treatment progeny were also the most variable in terms of their fecundity which may be an indicator of a bet-hedging strategy where some worms are more suited to the higher temperature than others and are therefore, less fecund in a less than optimal environment. When considering rearing techniques for EPN mass production for biological control treatments, if temperature can be manipulated to enhance host-exploitation and parasite development then the efficacy of EPN biocontrol could be much improved (Wonlinska & King, 2009).

Entomopathogenic nematode development was also affected by the host they were reared in. Here, Heterorhabditis downesi infective juveniles were bigger if they developed within a wax moth (Galleria mellonella larva) than if they developed within a mealworm (Tenebrio molitor larva). Again, if size difference can be attributed to food availability then this would indicate that G. mellonella may be a poorer host than T. molitor or that the internal conditions affect bacterial proliferation. Analysis of the composition of the haemolymph of each species would be of interest in this context and would reveal any differences in fatty acid composition and protein content that caused the effects observed. It would also be interesting to see if there was a distinct difference in the host immune response between the two host species that may be affecting both bacterial growth and nematode survival following initial invasion. Miranda et al. (2013) found that progeny production of both H. sonorensis and S. carpocapsae increased under crowded conditions when hosts, Manduca sexta, were reared on a low nutrient diet, but that this effect was reversed in non-crowded conditions. This suggests that both lipid availability and crowding in a host may be crucial for both proliferation of the

bacterial symbiont and for nematode development (Miranda et al. 2013). The differences in progeny quality from different rearing hosts observed here are most clear when the second (progeny) host is T. molitor where parent host significantly affects both progeny fecundity and development (r). Progeny that developed in T. molitor were more fecund when their parents were reared in T. molitor (M/M) than they were if their parents were reared in G. mellonella (W/M) but M/M fecundity was much more variable than W/M fecundity indicating that some progeny were better able to deal with their environment that others within the same clutch. As the same number of nematodes was applied to each insect, any crowding effects would be a result of differences in population growth within the host, and observed effects are therefore most likely a consequence of nutrient limitation. This also may be indicative of a bet-hedging strategy where reproductive strategies are altered via various mechanisms (e.g. increase in fecundity/decrease in size or increase in size/decrease in fecundity) that ensure at least some offspring will survive in a potentially poor environment. To fully elucidate this hypothesis, offspring size data and potentially longevity data would be needed as would the analysis of food availability and host composition for each host species. In terms of mass production of nematodes for biocontrol, parameters that maximise fitness are essential and the traits of importance will be virulence (infectivity), persistence in the soil (or longevity) and behaviour (e.g. dispersal).

Maternal effects were not observed in the experiments described in chapter 4 due to the lack of reproduction in worms exposed to the plant extracts. However, the physiological effects of compounds that are produced by African Marigolds (Tagetes erecta) are clear in C. elegans with fecundity and lifespan reduced by a crude extract from the petals and the complete cessation of reproduction following fractionation of the crude extract. This confirms that marigolds have nematicidal properties and that even if no purified compound is produced

from the plants, their potential for use as a cover crop or green manure is further highlighted here.

In chapter 5, the direct and indirect effects of anthelmintic treatment were investigated and it was found that low level exposure to both Albendazole and Pyrantel reduces fecundity and lifespan. Additionally, an increase in progeny fitness was observed when mothers were exposed to low levels of pyrantel. C. elegans has been used in multiple drug discovery and resistance studies and effects discovered in C. elegans are usually observable in parasites, although C. elegans has a much higher tolerance to anthelmintics than most parasitic species do (Simpkin & Coles, 1981). It is possible that the effects observed here were caused by a decrease in the ability of worms to ingest adequate quantities of food and that the altered reproductive strategies, slowed development and altered fecundities were a result of resource limitation. This would be consistent with many other observed maternal effects in multiple species in response to resource limitation. A difference in food consumption was observed in response to treatment with Pyrantel with food consumption reduced in worms treated with Pyrantel. This effect was not altered in progeny by maternal exposure to the drug, though readings taken with the plate reader were unreliable and of too limited a number to draw any definitive conclusions. However, this method of analysis, given appropriate adjustments, could be used to provide a more extensive data set about the uptake of food by worms exposed to a variety of toxic compounds.

There is evidence of maternal effects driving selection in some species and environmental change has been shown to impact host-parasite coevolution and red queen dynamics (Wolinska & King, 2009). However, this is not always true. Caenorhabditis remanei survivability under heat shock conditions improved after being reared at 30éC when

compared to nematodes reared at 20éC but this effect begins to diminish after ten generations indicating that the importance of pre-exposure is reduced under selection pressure. While parents in optimal environments are capable of producing offspring that can respond to environmental change, parents in stressful environments may be less capable of detecting environmental cues due to selection pressure for increased resistance (Sikkink et al., 2014). Of critical importance to the fitness of parasites is their survival and fecundity within hosts, and the transmission of infection between individuals (Skorping et al. 1991; Anderson & May 1992; Paterson & Viney 2003). A common proxy measure of parasite fitness is the lifetime production of transmission stages and in experimental studies the assumption is routinely made that the fitness of all transmission stages is equal. Such an assumption is also found in the majority of epidemiological models (although see Fenton & Hudson, 2002, where parasite genotypes were allowed to vary the degree to which their progeny varied). A key consequence of maternal effects is that progeny do differ either in the mean or variance of particular traits and that this can affect subsequent development. Thus, maternal effects in parasites clearly have the potential to affect traits of medical or veterinary importance. For example, variation between progeny, particularly for directly-transmitted species, might impact on transmission success via differences in survival of the transmission stage. Alternatively, such variation might change elements of subsequent infection. For instance, in the fish ectoparasite Argulus coregoni experimental manipulation of the time spent searching for a host, and hence the resources available at the start of post-attachment development, revealed a trade-off between accelerated growth and decreased life-expectancy (Hakalahti et al. 2005).

Whilst maternal effects can be attributed to behavioural adjustments which result in developmental change, it is likely that there is a transfer of information across generations that is not genetic, in that there is no change in gene sequence, but that is responsible for

phenotypic change which can be termed ‘transgenerational epigenetic effects’ or ‘soft inheritance’ (Youngson & Whitelaw, 2008). In contrast to Mendelian genetics which can be classed as ‘hard inheritance’ with slow evolution occurring as a result of rare mutations and selection, epigenetics can be classed as ‘soft inheritance’ with changes to gene expression in response to environmental change which provide an advantage to the individual or that individual’s offspring and are adaptive in nature (Bossdorf et al., 2007; Youngson & Whitelaw, 2008). Epigenetics affect gene expression and cause heritable changes without changing DNA sequence. Such changes arise from changes in cytosine residue methylation, chromatin protein modification or through the transfer of small RNAs (Slatkin, 2009). The relationship between maternal effects and epigenetics is complicated and confounded be a lack of an accepted definition of epigenetics. For transgenerational epigenetic effects (i.e. maternal or environmental effects that include phenotypic plasticity) Richards et al. (2010) suggest that the term ‘epigenetic inheritance’ should be used to avoid confusion with wider definitions of epigenetics. However, Bossdorf et al. (2007) express concern about this terminology as it is used to describe both mitotic and meiotic epigenetic modification whereas inheritance is classically used to describe meiotic transgenerational effects. In C. elegans, epigenetic mechanisms have been shown to regulate development and aging with small RNAs transmitted from parent to progeny that persist for two generations before being silenced. This may be a mechanism that contributes to immediate environmentally caused modification whilst still maintaining the germline function (Gonz®lez-Aguilera et al., 2013). Assays completed in chapters two, four and five (using C. elegans) were all carried out using genetically identical inbred lines and the EPNs were highly inbred, therefore any identified changes in gene expression would be likely to be caused be epigenetic variation (Bossdorf et al., 2007). To determine if gene expression had changed in response to environmental changes such as temperature (chapters two and three), host (chapter three) or drug exposure

(chapter five) micro arrays and 2D electrophoresis could be applied and would highlight such changes and determine if environmentally induced phenotypic change is epigenetic in nature. Phenotypic change in response to differentiation of provisioning in offspring is likely to be caused by expression changes in the insulin-like signalling pathway as this pathway appears to be central to most variation in resource allocation within C. elegans (for review, see Murphy & Hu, 2013). To identify this, mutant strains of C. elegans can be used to determine if the maternal environment induces the same phenotypic change in mutants as it does in the wild type, N2. Changes in gene expression could be crudely identified in mutants using 2D electrophoresis as expression would be expected to differ in mutants when compare to N2 under the same conditions. Changes in the insulin-like signalling pathway could be identified using C. elegans mutants. If changes were apparent then further investigations using protein specific antibodies to conduct western blot analyses or chromatin immunosuppression (ChIP) assays could be carried out. These would provide definitive analyses of expression changes in specific genes. Following these, sequencing of phenotypically differing offspring could be performed to rule out mutation therefore indicating epigenetic change.

Here, the parental environment has been shown to affect progeny fitness in C. elegans, S. carpocapsae and H. downesi with multiple environmental variables including temperature, host species, drug exposure and pesticide exposure causing changes in fecundity, development, size, food consumption and lifespan. That these effects have been demonstrated in both a free-living species (C. elegans) and two parasitic species (H. downesi and S. carpocapsae) suggests that maternal or paternal effects are likely to be found in other parasites. The effects demonstrated in entomopathogenic nematodes suggest that the external environment can affect parasite fitness and implies that the host cannot provide complete protection from environmental change. Thus, further study into maternal (or indeed, parental)

effects in parasites is essential. Further investigation of the impact of maternal effects could provide a wealth of information about the ecology of both free-living and parasitic species. Little is known about the natural ecology of C. elegans and studies that use more natural conditions, such as the variable temperature treatment in the present study, contribute to this knowledge. Additionally the knowledge gained here about how parasite fitness varies according to the maternal rearing conditions in entomopathogenic nematodes could ultimately be of great significance for mass production of nematodes for biocontrol, as maximising infective juvenile fitness would contribute to improving the efficacy of biological control using EPN. Finally, the impact of maternal exposure to low doses of anthelmintic drugs on progeny fitness demonstrated here is perhaps the most potentially impactful result. If similar increases in fitness are seen in vertebrate parasites then this could have serious implications in both medicine and agriculture and must be investigated further.

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