STRATEGIES OF REPRODUCTION IN THE ZOOPLANKTON DAPHNIA PULEX: EVOLUTIONARY INSIGHT FROM EXPERIMENTAL DESIGN

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STRATEGIES OF REPRODUCTION IN THE ZOOPLANKTON *DAPHNIA PULEX*: EVOLUTIONARY INSIGHT FROM EXPERIMENTAL DESIGN

by

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ABSTRACT

The research herein concerns reproduction in the zooplankton *Daphnia pulex*. Chapter One introduces the discipline of sexuality research and the model organism *D. pulex*. The first of my two investigations focused on the possibility that the common intracellular parasite *Wolbachia* is responsible for reproductive life-history traits among populations of *D. pulex* from the Great Lakes watershed of North America (Chapter 2). No evidence of any such infection was found among populations exhibiting a variety of *Wolbachia*-like phenotypes. My second investigation explored the effects of crowding and maternal age upon reproduction among both facultatively parthenogenetic (occasionaly sexual) and obligately parthenogenetic (strictly asexual) lines of *D. pulex* (Chapter 3). Crowding was found to increase resting egg production and reduce neonate offspring production among other effects. Inter-genotypic differences were often significant, as were genotypic responses to crowding. Chapter Four suggests improvements to methods of research and effluent testing using *Daphnia*, and proposes a large-scale life-history investigation.

I dedicate this thesis to my dearly departed grandparents: Marjorie McKay, Patrick Fitzsimmons, and Harold and Rachel Rasmussen. They all made sacrifices for their families, and gave me the support to get where I am today.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations used in this thesis are outlined in the Proceedings of the National Academy of Sciences USA **92**(1) vii-x (1995) with the following additions:

♀ ..... female

♂ ..... male

bp ..... base pair

ESD ..... environmental sex determination

FP ..... facultative parthenogenesis

GxE ..... genotype x environment

GLM ..... general linear model

GSD ..... genotypic sex determination

LDH ..... lactate dehydrogenase

OP ..... obligate parthenogenesis

PCR ..... polymerase chain reaction

CO-AUTHORSHIP STATEMENT

I am the principal author for all research herein. I proposed and designed the *Wolbachia* investigation (Chapter 2), and designed the crowding investigation (Chapter 3) with the assistance of my supervisor Dr. David J. Innes. I conducted all research and data analyses. I have written the manuscripts herein, with helpful feedback from Dr. Innes. Authorship for the published *Wolbachia* manuscript (Chapter 2) is Fitzsimmons & Innes, and authorship for the crowding manuscript (Chapter 3) would be the same.

So the question is: if greenflies and elm trees don't do it, why do the rest of us go to such lengths to mix our genes up with somebody else's before we make a baby? It does seem an odd way to proceed. Why did sex, that bizarre perversion of straightforward replication, ever arise in the first place? What is the good of sex?

Richard Dawkins (1976), The Selfish Gene, p. 43

To a miserable organism sitting alone in a singles bar, genetic mixing might not seem worth the bother.

Olivia Judson (2002), Dr. Tatiana's Sex Advice to All Creation, p. 2

Prepare to clone species when all other preservation methods fail.

In multi-point list of potential solutions for species conservation
Edward O. Wilson (2002), The Future of Life, p. 163

CHAPTER 1: An introduction to the field of life-history research and my research on *Daphnia pulex* (Crustacea: Cladocera)

1.1 OVERVIEW

The main thrust of my research is into the broad field of reproduction. Using the model organism *Daphnia pulex*, I undertook two separate but related experiments. Chapter two investigates the possibility of infection among *D. pulex* by intracellular bacteria of the genus *Wolbachia*. Chapter three details my experimental manipulation of daphnid density among both sexual and asexual genotypes, and the resulting reproductive strategies. The present chapter is intended to provide the reader with background information on life-history and sexuality research, *D. pulex* as a model organism, and to introduce my own research against this backdrop. The questions in which I am interested are basic. How should investment in males and females be balanced? How do the life-history strategies of sexual and asexual organisms compare? When is investment in diapause optimal? Most interesting, in my opinion: what evolutionary forces have played a part in shaping reproductive strategies?

1.2 LIFE: A FINE BALANCE

Life is a struggle to survive and reproduce. An awesome variety of strategies have been shaped over evolutionary time in the quest to live in an often adversarial world. Prey evolve predation-avoidance strategies. Competitors evolve antagonistic behaviours. Predators and parasites evolve more efficient strategies to hunt and infect, respectively. Even organisms within one's own species will only cooperate if it will benefit themselves and their genes.

The life-history strategy of each organism is a compilation of trade-offs and compromises. An organism can invest a great amount of resources into armour, burrs, toxins, and other defensive mechanisms. This, however, will leave fewer resources available for growth, reproduction, resource uptake, and so on. Optimal life-history strategies are a fine balance between many competing interests. The success of a given life-history strategy varies over time, and depends upon the surrounding environment. Investment in defensive mechanisms, for instance, is an unnecessary expense in the absence of predators.

1.3 THE PARADOX OF SEX

It has been suggested that if aliens were to come to Earth, it would be sexual reproduction that might cause them to scratch their head-like structures in confusion (Ridley, 1994). Why require two organisms to share investment in offspring as opposed to each organism simply cloning itself? What are the advantages of sex?

Biologists, too, have critically examined sexual *vs* asexual reproduction (Williams, 1975, Kondrashov, 1988, West et al., 1999). Sex seems to be at a numerical disadvantage due to the 'cost of males' (Maynard Smith, 1978). Provided females of both lines produce the same number of offspring, an asexual line that only produces daughters will grow in numbers faster than a sexual line since only females can reproduce. This two-fold advantage to asexuality is a large one to overcome. Since most multicellular organisms reproduce sexually, sex must confer major advantages over asexuality (Bell, 1982).

1.3.1 Costs of sex

In addition to the two-fold 'cost of males,' there are many other costs associated with sexual reproduction. The following list of costs has been modified and expanded from one listed elsewhere (Crow, 1994):

- Process of meiosis: Meiosis is a more complicated, energy-consuming process than mitosis (Solari, 2002).
- Sexual selection: Traits that make one sexually attractive do not necessarily benefit fitness otherwise. In fact, sexually selected traits can be a handicap (Zahavi, 1975, Iwasa et al., 1991, Wiens, 2001).
- Sex limitations: Often sex cannot pass on polyploidy, translocation heterozygotes, or several other traits that can sometimes be beneficial (Crow, 1994).
- Finding mates: Whether it is through nectar production to attract pollinators or producing sounds to attract the opposite sex, there are costs associated with finding mates that can sometimes be substantial (Gerritsen, 1980, Gwynne et al., 1998, Kearney, 2003).
- Sexually transmitted diseases: STDs can spread through a sexual population, but not an asexual one (Knell, 1999).
- Meiotic drive: Selfish gametes can sabotage others, and often result in individual fitness reduction (Crow, 1991) (or increase, Bruggeman et al., 2003). Some have even speculated that segregation distortion has selected for the evolution of two-step meiosis (Hurst & Randerson, 2000).

- No foresight: Only those traits that are beneficial to fitness in the short-term will persist among sexual lines. Asexual genomes may carry maladaptive alleles to a time when they are beneficial again (Dawkins, 1996).
- Sex organs: Although sex organs often serve additional purposes (e.g., urination), those purposes can likely be better served by uncompromising designs.
- Reduced kin selection and cooperation: Sexual organisms may aid their relatives to an extent dictated by their degree of genetic similarity (Petrie et al., 1999, Griffin & West, 2003). With ~100% genetic similarity, asexual individuals should invest as much in the fitness of their kin as they do in their own fitness. This should select for extreme forms of cooperation such as self-sacrificing behaviour (Kurosu et al., 2003).
- Intersexual ontogenetic conflict: Optimum levels of gene expression often differ between sexes (Rice & Chippindale, 2001a). Sex-limited expression patterns can evolve (Rice & Chippindale, 2002), but often gene expression levels reach an evolutionary compromise between optima of each sex (Rice, 1998a, Chippindale et al., 2001).
- Sexually antagonistic coevolution: Mates can manipulate each-other to their own selfish ends. Female fitness may suffer as a result of paternity-ensuring seminal proteins or other yet-unknown mechanisms (Holland & Rice, 1999, Friberg & Arnqvist, 2003, Pizzari & Snook, 2003).

1.3.2 Benefits of sex

The long-term benefit of sex is that it allows greater evolvability, leading to the longer evolutionary existence of most sexual taxa (Williams, 1975, Maynard Smith, 1978), although some asexual taxa seem to be ancient (Arkhipova & Meselson, 2005). Selection, however, takes place in the present without foresight (Dawkins, 1996, Van Valkenburgh et al., 2004), so short-term benefits are required (Partridge & Barton, 2003) unless one is to revert to group selection (Nunney, 1989, Gouyon, 1999). There are many theories proposing short-term benefits of sex, reviewed more extensively elsewhere (Kondrashov, 1993, Hurst & Peck, 1996, Barton & Charlesworth, 1998, Rice, 2002). Below is a summary of the more accepted of these.

- Red Queen: The biotic environment changes rapidly across generations. In order to keep pace with this changing environment, a genome must also change. The most relevant example is that of parasites evolving methods to exploit a population's most common genotype, selecting for change of that genotype via sexual reproduction (Antonovics & Ellstrand, 1984, Hamilton et al., 1990, Lively et al., 1990, Jaenike, 1993, Dybdahl & Lively, 1998).
- Avoiding mutation accumulation: As a result of genetic drift (Andersson & Hughes, 1996, Peck et al., 1999, Otto & Lenormand, 2002, de Visser & Rozen, 2005), selective sweeps (Bachtrog, 2003, Carvalho, 2003, Kim & Stephan, 2003, Hadany & Feldman, 2005), and deleterious transposable element proliferation (Steinemann et al., 1993, Arkhipova & Meselson, 2005), an asexual genome is expected to accumulate mutations of non-lethal deleterious effect over time.

Some of this mutation accumulation is termed 'Muller's ratchet,' illustrating its uni-directionality (Muller, 1964, Felsenstein, 1974, Charlesworth et al., 1993, Rice, 1994, Andersson & Hughes, 1996, Lynch, 1997). If the deleterious effects of mutations act synergistically, then the fitness of asexual genomes should be reduced at an even greater rate than if each effect were independent, according to the deterministic mutation theory (Kondrashov, 1988, Rice, 1998b). Because selection at individual loci is more efficient in sexual than asexual genomes (Rice & Chippindale, 2001b, Betancourt & Presgraves, 2002, Kaltz & Bell, 2002, Hadany & Feldman, 2005), a sexual genome will largely avoid mutation accumulation. A sexual population can further avoid mutation accumulation as a result of sexual selection. The most mutated individuals of the sex attempting to be chosen as mates (usually males) will not be chosen as mates if their reduced fitness can be detected. Both theoretical (Pomiankowski et al., 1991, Agrawal, 2001, Houle & Kondrashov, 2001, Siller, 2001) and experimental (Møller & Mousseau, 2003, Radwan, 2004) evidence suggests sexual selection can thus act as a form of truncation selection to remove deleterious mutations from the population.

- Faster adaptation: Not only do sexual genomes avoid mutation accumulation compared to asexual genomes, but they may also accumulate beneficial alleles more rapidly. Recombination allows separate beneficial alleles on different genomes to combine into a single genome for increased fitness, while asexual genomes must accumulate sequential beneficial mutations over time (Rice & Chippindale, 2001b, Bachtrog & Charlesworth, 2002, de Visser, 2002, Rice, 2002,

de Visser & Rozen, 2005). Selection against a deleterious genome background may also reduce the likelihood of beneficial mutations' fixation among asexuals (Charlesworth, 1994, Peck, 1994, Nachman, 1998, Johnson & Barton, 2002, Hadany & Feldman, 2005).

- Pluralistic advantages of sex: The combined effect of several advantages of sex and recombination may be greater than the sum of their parts. The interactions between the various factors involved are beyond the scope of this paper, but are discussed elsewhere (Charlesworth, 1996, Kövér & Szathmáry, 1999, West et al., 1999, Howard & Lively, 2002).

There is no resolution as to what costs and benefits of sex are the most valid. It is likely that they vary across taxa depending on life-history, population, and habitat characteristics (Haag & Ebert, 2004). Modern bioinformatic and genomic approaches allow unprecedented potential in research of sexuality (Lee & Amon, 2003, Libby et al., 2003, Marais, 2003, Simon et al., 2003, van Dijk & Bakx-Schotman, 2004, Arkhipova & Meselson, 2005, Paland et al., 2005). The evolution and maintenance of sex is not merely a basic academic pursuit. It has direct applications to research in the fields of sex chromosomes (Rice, 1996, Charlesworth & Charlesworth, 2000, Hargreave, 2000), genome-wide patterns of selection (Marín et al., 2000, Nachman, 2002), pathogen evolution (Andersson & Hughes, 1996, Howard & Lively, 2002, Lázaro et al., 2002), agriculture (van Dijk & van Damme, 2000), livestock breeding (Olsen, 1965), and invasive species (Amsellem et al., 2002, Haag & Ebert, 2004, Thompson & Eckert, 2004,

Dybdahl & Kane, 2005), among others. Future research will extend in many directions, including laboratory experiments with appropriate model organisms.

1.4 *DAPHNIA* AS A MODEL ORGANISM

Model organisms allow biologists to explore traits in-depth, and then test findings on other taxa to verify these traits' ubiquity. Choice of model organism influences research methods and results. *Daphnia* spp. are common model organisms among life-history (Weber & Van Noordwijk, 2002, Cáceres & Tessier, 2004, Kerfoot & Weider, 2004, Chadwick & Little, 2005), ecotoxicology (Baird et al., 1989, Dodson & Hanazato, 1995, Hietala et al., 1997, Barata et al., 2002), and ecology (Bittner et al., 2002, Lathrop et al., 2002, Kagami et al., 2004) research. What traits make *Daphnia* such a good model organism? Like *Drosophila melanogaster* and other model organisms, *Daphnia* is easy to raise in the laboratory, is sexually dimorphic, is widely distributed, and has short generation times. Two additional traits make *Daphnia* an exceptional model organism. First, *Daphnia* can produce resting eggs that may survive a variety of stresses over many years. Resting eggs isolated from sediment samples at different depths may be hatched to compare 'time capsules' of *Daphnia* from the past (Kerfoot & Weider, 2004). Second, *Daphnia* have the ability to reproduce clonally. This means animals with the same genome, with the exception of somatic-like mutations, can be raised under a variety of conditions for comparison. Other sexual taxa require either genetic manipulation or extensive inbreeding in order to reproduce pseudo-clonally, with both methods

necessitating large deviations from the organism's natural state. For all of these reasons, *Daphnia* make excellent model organisms for life-history research.

Daphnia also make excellent model organisms for research on sexuality. *D. pulex*, along with several other species of *Daphnia*, consists of lineages that reproduce either via facultative parthenogenesis (FP, occasional sex) or obligate parthenogenesis (OP, strictly asex). FP and OP lines of *D. pulex* differ only in that OP resting eggs are produced mitotically (or in a process that produces the same results as mitosis), while FP resting eggs are produced via sexual recombination (Innes & Hebert, 1988). Both FP and OP lines of *D. pulex* can produce free-swimming neonate offspring clonally. FP *vs* OP mode of reproduction seems to be inherited genetically (Innes & Hebert, 1988). Males produced by OP lines, though paradoxical at first glance, may mate with FP females to produce offspring, some of which are novel OP lines (Innes & Hebert, 1988). I consider the evolutionary factors involved with OP males in the Discussion of Chapter 3. Both FP and OP lines can have the same ploidy level, live in similar nearby habitats, and have indistinguishable life-history traits other than the paternal gamete requirement for FP resting egg formation (Hebert et al., 1989). See the Introduction of Chapter 3 for a more detailed account of the *D. pulex* life cycle.

While *D. pulex* allows comparison of nearly indistinguishable sexual and asexual lines within a species, the use of most other species in sex *vs* asex research requires apples-and-oranges comparisons. Some research makes inter-species comparisons between sexual and asexual species of the same genus (Mantovani et al., 1997). Among aphids, only sexual individuals can produce cold-resistant eggs so comparisons between sexual and asexual animals is complicated by an ecological difference (Simon et al.,

2002). Among many aphids, dandelions, flatworms, and other taxa, comparisons of sexual and asexual lines are complicated by polyploidy among asexuals (Pongratz et al., 2003, Simon et al., 2003, Verduijn et al., 2004). Both diploid and polyploid OP populations of *D. pulex* exist (Hebert, 1987), allowing sexuality comparisons to proceed unhindered by ploidy level confounding factors. It is for the above reasons that I used *D. pulex* as the model organism in my investigation of reproductive strategies among sexual and asexual individuals.

1.5 OVERVIEW OF CHAPTER 2 (*WOLBACHIA* AND *DAPHNIA PULEX*)

D. pulex is a model organism in the fields of life-history and ecology. Research commonly attributes *D. pulex* behaviour to *D. pulex* adaptations. It is known, though, that certain parasites manipulate their hosts' reproductive strategies to their own ends (Majerus, 2003). The most common such parasite is intracellular bacteria of the genus *Wolbachia* (Majerus, 2003). *Wolbachia* is only transmitted through the maternal line of its host, and has adapted ways of increasing its own proliferation, often at the expense of optimal strategies of its host (Stouthamer et al., 1999, Weeks et al., 2002). *Wolbachia*-induced reproductive strategies among hosts include parthenogenesis and female-biased sex ratios (Stouthamer et al., 1999). *D. pulex* genotypes are known to have these very reproductive strategies. The question I endeavoured to answer: is *D. pulex* infected with *Wolbachia*?

Despite their similar reproductive strategies, and despite *Wolbachia* being present in 20 – 76% of the world's arthropod species (Jeyaprakash & Hoy, 2000) including some

crustaceans (Cordaux et al., 2001), no published research had yet investigated the possibility of *Wolbachia* infection among *D. pulex*. I tested for *Wolbachia* infection among 203 *D. pulex* isolates from ponds in the watershed of the North American Great Lakes, exhibiting a range of *Wolbachia*-like reproductive strategies.

I found no evidence of *Wolbachia* infection (for caveats, see Chapter 2 Discussion). It is possible that an alternative parasite is responsible for *D. pulex* reproductive strategies, such as the parasite *Nosema granulosis* which alters reproduction in a different species of crustacean (Kelly et al., 2004). I believe it is prudent to test assumptions whenever possible to prevent invalid conclusions, which is why I tested for the most common arthropod reproductive distorter in a common arthropod model organism. Chapter two has been published in the peer-reviewed Journal of Plankton Research (Fitzsimmons & Innes, 2005).

1.6 OVERVIEW OF CHAPTER 3 (CROWDING, AGING, AND INTERCLONAL VARIATION IN *D. PULEX* REPRODUCTION)

Many populations of *D. pulex* inhabit ephemeral ponds. Because only the resting egg stage can survive over winter, *D. pulex* must utilize cues to predict the onset of conditions that favour diapause more than neonate offspring production. Crowding is known to be one such cue (Ruvinsky et al., 1986, Berg et al., 2001).

I tested the effect of crowding on reproduction among six genotypes of *D. pulex*, four of which were OP. Only limited information is available on the effect of crowding upon reproduction among OP *D. pulex* genotypes (e.g., Innes et al., 2000). My research

also examined the effect of maternal age upon reproductive patterns, revealing some interesting results (Chapter 3). I endeavoured to improve upon previous statistical methods, reported a potential link between crowding and ecdysone levels within daphnids, and proposed several novel evolutionary explanations for daphnid behaviour. With over 30,000 offspring among my data, my analyses were able to detect even slight trends when significant.

A main conclusion of my research is that inter-genotype variation is great, and different genotypes react differently to an environment. An organism's life-history strategy is the product of a genotype x environment interaction. I am not the first to make these conclusions, but still much research is published on a single *Daphnia* genotype in a single environment, assuming species-wide ubiquity. Government regulations often require freshwater effluent to be tested upon a single genotype (originating from anywhere in the world) in environments that vary only in effluent concentration (OECD, 1998, Environment Canada, 2000, USEPA, 2002). I discuss the shortcomings of such environmental regulations and research practices, and propose an improved system, in the concluding chapter of my thesis.

1.7 CHAPTER 1 CONCLUSIONS

Two main threads tie together my two research projects on *D. pulex*. First is the evaluation of the factors influencing reproduction among *D. pulex*. Second is the testing of assumptions. Whether it is testing for a common manipulative parasite when no research had yet done so, or testing for inter-clonal variation which many researchers

ignore, I earnestly believe that assumptions should be tested whenever possible. My research adds to the fields of life-history trade-offs, *Wolbachia* distribution, sex *vs* asex, infochemicals, aging, sexual selection, environmental testing, sex determination systems, genotype x environment interactions, and phenotypic plasticity. Much more research is required, but mountains are ascended one step at a time.

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CHAPTER 2: No evidence of *Wolbachia* among Great Lakes area populations of *Daphnia pulex* (Crustacea: Cladocera)

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2.1 ABSTRACT

Wolbachia endobacteria are commonly associated with a variety of arthropod species as hosts, and induce known changes in their hosts' life-history traits. Despite exhibiting several *Wolbachia*-like life-history traits, and despite being a common model organism, the zooplankton *Daphnia pulex* has not been formally tested for infection with *Wolbachia*. Among 203 isolates exhibiting a range of life-history phenotypes, we found no evidence of *Wolbachia*. This leaves the genes of *D. pulex* as the most likely cause of its own life-history traits.

2.2 INTRODUCTION

Intracellular bacteria of the genus *Wolbachia* are thought to be present in 20 - 76% of the world's arthropod species (Jeyaprakash & Hoy, 2000). It is known to induce several changes in its hosts' life histories, including female-biased sex ratios, parthenogenesis, and male feminization (Stouthamer et al., 1999). Although *Wolbachia* has been found in other crustaceans (Cordaux et al., 2001), we know of no published study to have searched for it in species of *Daphnia*.

The life-history traits of the zooplankton *Daphnia pulex* (Leydig) overlap the life-history traits induced in hosts of *Wolbachia*. Female-biased sex ratios (Innes, 1997) and obligately parthenogenetic lines (Innes & Hebert, 1988, Hebert et al., 1989) are common, and its sex determination system is primarily environmentally-based, making it conceivable for potential male offspring to be turned female by *Wolbachia*-induced hormonal changes. *Wolbachia*-induced host parthenogenesis is typically accomplished

via automictic thelytoky, resulting in 100% homozygosity (Cook & Butcher, 1999). Clonal parthenogenesis via apomictic thelytoky maintains heterozygosity, and has also been found to be induced by *Wolbachia* endosymbionts (Weeks & Breeuwer, 2001), resulting in a non-homozygous genome similar to that of *D. pulex* obligately parthenogenetic clones.

If *D. pulex* is infected with *Wolbachia* then we must resolve the contributions of each to several life-history traits, as has been done recently with a species of spider mite (Vala et al., 2003). If *D. pulex* is not infected with *Wolbachia* then its life-history traits can likely be attributed in large part to its own genes. This would increase the motivation behind finding the genes involved in meiosis suppression and average rate of male production in the soon-to-be sequenced *D. pulex* genome (http://daphnia.cgb.indiana.edu).

2.3 METHOD

D. pulex were sampled from several small ponds in the Great Lakes watershed (North America) in early May 2003, except the clone from pond LP8A which was sampled in Spring 2001 (Table 2.1), using previously described methods (Innes, 1997). These populations represent a geographic cross-section of pond habitats and locations. LP8A, LP8B, and LP9A are near Long Point, Ontario (Innes, 1991). Mar, Morg, Tex, and War are scattered near Ann Arbor, Michigan, being previously described as ponds #74, 69, 72, and 73, respectively (Hebert et al., 1989). Windsor, Ontario contains W3 (Hebert & Crease, 1983), OjibDitch which is near the previously described pond #5

Table 2.1: Populations sampled, their method of reproduction, and successful PCR amplifications for *D. pulex p4m15* and *Wolbachia wsp* loci.

Pond	Method of reproduction:	n clones	*p4m15* amplified	*wsp* amplified
Disp	mixed (cyclical and obligate parthenogenesis)	28	28	0
LP8A	cyclical parthenogenesis	1	1	0
LP8B	cyclical parthenogenesis	21	21	0
LP9A	cyclical parthenogenesis	18	18	0
Mar	cyclical parthenogenesis	25	25	0
Morg	obligate parthenogenesis	13	13	0
OjibDitch	obligate parthenogenesis	16	16	0
Tex	cyclical parthenogenesis	19	19	0
VBA	obligate parthenogenesis	28	28	0
W3	obligate parthenogenesis	15	15	0
War	mixed (cyclical and obligate parthenogenesis)	19	19	0
Total		203	203	0

(Hebert et al., 1989), and Disp which is a newly described pond on Disputed Road. VBA is a newly sampled pond in the Village by the Arboretum, about 1.5 km south of Guelph University in Guelph, Ontario. These populations also represent a cross-section of phenotypes: obligately parthenogenetic and cyclically sexual lines (Innes et al., 2000) (Table 2.1), and lines that vary in their investment in males (Innes & Dunbrack, 1993).

Clones were kept in laboratory conditions as previously described (Innes & Dunbrack, 1993) until their DNA was extracted in early 2004. All *D. pulex* individuals have the ability to reproduce asexually, so clonal individuals were of the same genotype, with the exception of somatic-like mutations from mother to offspring. A total of 203 clones were sampled across 11 ponds, though some were likely clones of others in the same pond at the time of sampling, and therefore redundant.

DNA was extracted from samples of approximately 5 large females per clone using a method modified from http://www.fruitfly.org/p_disrupt/inverse_pcr.html as follows. Samples were ground in an eppendorf tube using a pipet tip before 100 µL of Buffer A (100 mM Tris-HCl pH = 7.5, 100 mM EDTA, 100 mM NaCl, 0.5% SDS) was added. Tubes were incubated at 70 °C for 35 minutes. 200 µL LiCl/KAc solution (1 part 5 M KAc by volume with 2.5 parts 6 M LiCl) was added before tubes were incubated on ice for 15 - 20 minutes. Samples were spun at 13700 g for 15 minutes. Supernatant was transferred into new tubes. 160 µL cold (-20 °C) isopropanol was added, the sample was mixed, and then spun for 15 minutes. We aspirated away the supernatant by vacuum, spun, then aspirated the remaining liquid. Samples were washed twice with cold (4 °C) 70% ethanol, being spun for 2 minutes before supernatant was aspirated away each time. DNA was resuspended in 35 µL double-distilled water and left at 4 °C overnight.

PCR was performed using the primer pair *wsp* 81F and *wsp* 691R, which amplifies an approximately 600 base pair (bp) fragment of the *Wolbachia* surface protein gene *wsp* (Braig et al., 1998). This primer pair has been used to detect a variety of *Wolbachia* strains in a range of crustacean (Cordaux et al., 2001) and other arthropod hosts (Zhou et al., 1998). It is considered the most sensitive of several primer pairs to *Wolbachia* DNA amplification (Hong et al., 2002). A *D. pulex* nuclear microsatellite, *p4m15*, was also amplified for each DNA sample to ensure the extraction procedure produced amplifiable DNA (Primers: *p4m15F*, 5'-TCCACCTCCTTCCTCACCAA, and *p4m15R*, 5'-GCGCGGCAGTGAAATAAATC, courtesy of J. K. Colbourne, Indiana University).

Reaction mixtures for both *wsp* and *p4m15* PCR reactions contained 25 µL total volume: 2.5 µL 10x Buffer (Fisher Scientific, Fairlawn, NJ), 2.5 µL 25 mM $MgCl_2$ (Fisher), 0.5 µL 10 mM dNTPs, 0.25 µL of 10 µM forward and reverse primers, 1 unit of Taq (Fisher), 5 µL DNA solution, and 13.8 µL water. Negative controls consisted of the same PCR reaction mixtures, but without the addition of DNA. *wsp* thermocycling conditions were modified from those previously described for crustaceans (Cordaux et al., 2001) as follows: 94 °C for 3 min, 36 cycles of (94 °C for 30 sec, 53 °C for 45 sec, 72 °C for 1 min), 72 °C for 10 min. The annealing temperature was reduced from the original 55 °C to 53 °C to lower specificity, and hence lower the possibility of false negative results. Approximately 10 µL of PCR product was electrophoresed on 1.5% agarose gels, stained with ethidium bromide and visualized under UV illumination. Images of gels were taken with a digital camera and stored electronically.

DNA was also extracted from a single *Drosophila simulans* individual known to host *Wolbachia*, and this DNA was used as a positive control in each set of *wsp* PCR reactions. All such positive controls amplified the expected ~600 bp band successfully, and all negative controls were free of bands.

2.4 RESULTS

If the *D. pulex* microsatellite PCR amplified the expected band for a sample, but the *wsp* PCR did not, then we concluded the sample did not contain *Wolbachia*. The microsatellite was successfully amplified for all samples, while no samples amplified a band in the expected region for *wsp* PCRs.

A very faint band in the 375 bp region, however, was amplified in the majority of *wsp* PCR samples. We concluded that this amplified DNA was not of *Wolbachia* origin for three reasons. First, the product length of 375 bp is much smaller than the 590-632 bp products amplified from a variety of *Wolbachia* strains (Zhou et al., 1998). Second, its amplification failed at the high annealing temperature of 56 °C while that of the positive control did not. Finally, upon sequencing this product was determined to bear no greater resemblance to *Wolbachia* DNA than would be expected by chance beyond the primer regions.

2.5 DISCUSSION

It is possible that our methods failed to detect *Wolbachia* in samples for several reasons. Our primers, though standard among many *Wolbachia* studies (Schulenburg et al., 2000, Hong et al., 2002), may not amplify the *Wolbachia wsp* locus if mutations have occurred to render them not specific enough. There may also be inhibition of *Wolbachia* DNA amplification by something in the *D. pulex* DNA samples (Jeyaprakash & Hoy, 2000). Or, *Wolbachia* infection of individuals is localized and low-level, leaving insufficient template for amplification.

Though these concerns can never be completely alleviated, we decreased their likelihood of occurrence through several methods. We changed the annealing temperature of our *wsp* PCR reactions to 45, 50, 54, and 56 °C for subsets of samples. While some faint bands were amplified at 45 °C, none were within 200 bp of the region expected of *wsp*. Each of these PCR conditions successfully amplified the ~600 bp band in the positive control. Lower temperatures decrease annealing specificity, which should have allowed amplification of *Wolbachia* DNA if its sequence were fairly similar to that of our primers, or if there were some form of inhibition.

Inhibition was also addressed in a small subsample by successful amplification of small amounts of positive control DNA mixed with sample DNA. Low template level concerns were addressed by using concentrated DNA from many (~15) individuals of a clone, but still finding no *wsp* amplification. Although inhibition has been found in some studies (Jeyaprakash & Hoy, 2000), others have found very diluted DNA to still amplify without difficulty (Wenseleers et al., 2002).

No evidence was found of *Wolbachia* infection among our samples, which represent a cross-section of *D. pulex* phenotypes. We therefore conclude that *Wolbachia* is not responsible for the life-history traits exhibited by the clones involved. Our results are complemented by those of S. West and D. Ebert (unpublished data), who also failed to find PCR-derived evidence of *Wolbachia* among three clones each of *Daphnia magna* Straus and *D. pulex* collected from separate populations in Southern UK.

It is possible that infection with another parasite is responsible for the life-history traits of *D. pulex*, as is the case for several arthropods (Weeks et al., 2002) including crustaceans (Kelly et al., 2004), and more research is needed to test this possibility. The more likely explanation, however, is that the life-history traits of *D. pulex* are the result of its genotype, and genotype x environment effects.

This conclusion supports previous evidence from *D. pulex* cross-breeding that both obligate parthenogenesis (Innes & Hebert, 1988) and the average rate of male production (Innes & Dunbrack, 1993) are traits inherited genetically. This study, therefore, provides increased support for the proposal to find the genes involved in meiosis-suppression and rate of male production in the *D. pulex* genome.

2.6 ACKNOWLEDGEMENTS

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CHAPTER 3: Inter-genotype variation in reproductive response to crowding among *Daphnia pulex*

3.1 ABSTRACT

Crowding is known to have a major influence on reproduction in the freshwater microcrustacean *Daphnia pulex*. I analyzed reproductive output of six different *D. pulex* genotypes under two different density regimes in the laboratory. Four of these genotypes reproduce via obligate parthenogenesis, allowing thorough analysis of the life history strategies of some asexual lines. Among 30109 neonate offspring and 1041 resting egg ephippia collected, several trends were evident. Crowding induced increased resting egg production and reduced neonate offspring production among all genotypes. Offspring sex ratios grew more male-biased with maternal age. The extent, but not direction, of each of these trends varied across genotypes. Offspring sex ratios, and the very direction in which they changed in response to crowding, differed significantly between genotypes with some genotypes producing more and others fewer males in response to crowding. Obligately parthenogenetic genotypes seemed to respond to the crowding stimulus in similar ways as the facultatively parthenogenetic genotypes, as expected from the sexual origins of their genomes. Evolutionary explanations are offered for asexual male production, increased male production with maternal age, coexistence of competing genotypes, and crowding infochemical specificity. The inter-genotype variation in life-history traits observed in this and other investigations calls into question the common practice of extrapolating results from a single *Daphnia* genotype to an entire species.

3.2 INTRODUCTION

Reproduction is fundamental to life. Timing of reproductive investment determines its success, so the utilization of appropriate predictive cues can be just as important as the reproductive investment itself.

Reproductive trade-offs, such as those between short- and long-term reproductive investment, take on an added layer of complexity when an organism can switch between sexual and asexual modes of reproduction. Asexual organisms do not require males, except gynogenetic species for which a sperm stimulus is required to induce asexual reproduction (e.g., Løyning & Kirkendall, 1996). The ability to produce strictly daughters, provided all else is equal including total offspring production, should allow an asexual population to increase in number at twice the rate of a sexual population (Maynard Smith, 1978). This cost of sex is often called the 'cost of males.' Although the field is a large and multi-faceted one, the cost of males is a central component of research on the evolution and maintenance of sex (Barton & Charlesworth, 1998, Rispe & Pierre, 1998).

The freshwater zooplankton *Daphnia pulex* (Leydig) (Crustacea: Cladocera) is well-suited to experimental research on both life-history strategies and the advantages of sexual *vs.* asexual reproduction. Like other model organisms, it is easily reared in the lab, has short generation times, and the basic components of its ecology and life-histories are known from previous research. It may reproduce via clonal free-swimming offspring production or diapausing resting egg production, so trade-offs between short- and long-term reproductive investment can be observed. Clonal offspring production allows

comparison of essentially the same genome across several environmental conditions, which is a major advantage of using *D. pulex* in life-history research.

Daphnia pulex individuals can reproduce either by facultative parthenogenesis (FP) or obligate parthenogenesis (OP). Facultative parthenogenesis is also referred to as cyclical parthenogenesis in much of the literature. Cyclical parthenogenesis, however, inaccurately implies a strictly cyclical pattern to the reproductive cycle governed by an internal clock, and a change in terminology to facultative parthenogenesis has been suggested (Tessier & Cáceres, 2004). Both FP and OP *D. pulex* readily produce clonal offspring, but FP lines require sex and recombination to produce resting eggs, while OP line females produce resting eggs asexually with resulting genotypes matching those that would be expected from mitosis (Hebert, 1987). Method of reproduction is believed to be inherited genetically (Innes & Hebert, 1988).

Many OP lines produce males, despite biologists' insistence that asexual organisms should not do so. These males are functional, and can mate with FP females to produce a mixture of FP and OP offspring (Innes & Hebert, 1988). *D. pulex* obligate parthenogenesis has multiple origins, many of which are likely the result of such FP-OP matings (Paland et al., 2005). Novel asexual lineages are believed to have also resulted from matings between asexual-lineage males and sexual-lineage females in other species (Weinzierl et al., 1999, Simon et al., 2002).

It is important to note that although a clonal line may be obligately parthenogenetic, it will retain much of the genetic history of sexual *D. pulex* (Lynch et al., 1989). Cues to induce male production, for instance, may no longer be relevant to OP

lines, but with genomes that are essentially frozen OP lines may remain responsive to these cues.

The goal of the present study was to observe the effects of crowding and age on reproduction among six different genotypes of *D. pulex*. Four of these genotypes were OP, while two were FP. The number of genotypes prevent broad conclusions of FP *vs* OP life-history strategies since inter-clonal variation can be substantial in *D. pulex*. Instead, I thoroughly explored both the mode (resting eggs *vs* neonates) and quantity of reproduction among these six genotypes and the factors influencing their variation. This study also entailed a detailed exploration of reproductive life-histories to explicitly test the effect of age on *D. pulex* reproduction.

3.2.1 Life cycle of *Daphnia pulex*

The life cycle of *D. pulex* must be considered in the context of its habitat; ephemeral freshwater ponds. It is only the resting egg stage of the *D. pulex* life cycle that can survive over winter or when the pond has dried up. *Daphnia* resting eggs may hatch the following year, or may remain buried and hatch many years later (Kerfoot & Weider, 2004). Normally two resting eggs are encased within an ephippium (plural: ephippia), which is a hard dark case that protects the eggs from stresses. As mentioned previously, these resting eggs are produced either sexually or asexually for FP and OP lines, respectively. Although many northern OP lines are polyploid (Hebert, 1987), allozyme electrophoresis results (not shown) led me to believe the genotypes used here were diploid.

The production of free-swimming offspring (called neonates) is clonal for both FP and OP lines of *D. pulex*. Sex is determined environmentally with genetic contributions (Innes & Singleton, 2000). Thus both male and female offspring are identical genetically. Neonates are produced in broods that range in size from several to dozens. All neonates within a brood are usually the same sex, although mixed-sex broods are not uncommon (Innes, 1997). Male and female neonates resemble small versions of their adult equivalents, and can be distinguished morphologically based on the presence of a distinct second antennule among males.

A key component of *D. pulex* fitness is a genotype's ability to produce an optimal number of offspring via neonate production to maximize the number of resting eggs produced before pond conditions deteriorate. The cues used by *D. pulex* to induce male- and resting-egg production vary across genotypes within and between habitats (reviewed in Discussion). Crowding is one of these inductive cues (Berg et al., 2001).

3.3 METHOD

3.3.1 Clonal isolates

Specimens were sampled from four ephemeral ponds in the Great Lakes watershed of North America in May, 2003 using previously described methods (Innes, 1997). Disp and VBA ponds are in Windsor and Guelph, Ontario, respectively (Fitzsimmons & Innes, 2005). Morg and War are near Ann Arbor, Michigan, being previously described as ponds 69 and 73 respectively (Hebert & Crease, 1983). At least a

dozen clonal isolates were individually maintained from each of these ponds in synthetic zooplankton media (Lynch et al., 1986) in conditions described elsewhere (Innes & Dunbrack, 1993). Cups of clonal strains were fed 3.5 mL of algal slurry from an aquarium system daily. The goldfish in the aquarium were fed fish food flakes, and their excretions provided nutrients for a small variety of phytoplankton and bacteria to thrive. Though the small random fluctuations in this algal community prevent precise quantifications, its multi-taxa composition is a more natural food than single algal strains, and may increase *Daphnia* reproductive output (Sanders et al., 1996).

The clones chosen for the experiment had been observed with males on multiple occasions in the lab. This non-random sampling of genotypes was purposeful, as only genotypes capable of producing males were desired. One clone from each of Disp and VBA was chosen, as were two clones from each of Morg and War. The two clones from Morg and War were genetically unique, as inferred from allozyme electrophoresis. It was originally believed that all six of these clones were OP, based on previous research with these populations (Hebert et al., 1989) and phenotypic heterozygosity at the lactate dehydrogenase (LDH) allozyme locus, which is usually correlated with obligate parthenogenesis (D. J. Innes, pers. comm.). Verification of mode of reproduction, however, strongly suggested the clones Disp and War2 were FP. Verification entailed allowing females to produce ephippia in the absence of males. OP daphnids can produce diapausing resting eggs within ephippia in the absence of males, whereas FP daphnids can only produce an empty ephippium. Since OP daphnids may also occasionally produce empty ephippia, however, a single empty ephippium is insufficient evidence for facultative parthenogenesis. More than 20 ephippia were opened for each of Disp and

War2 without the presence of resting eggs, while ephippia containing resting eggs were found for each of the other clones within the first 3 being opened. We therefore assume Disp and War2 to be FP.

Additional FP and OP genotypes were included in the experiment initially, and would have allowed more general tests of FP *vs.* OP reproductive investment. However a sports-related injury (from which I have now fully recovered) limited use of my hands and necessitated a reduction in the number of genotypes tested. We do not feel it prudent to test for general differences between FP and OP *D. pulex* reproductive investment from such a small sample.

Three successive parthenogenetic generations of each clone, originating from a single female of each clonal culture, were raised in identical conditions in an incubator prior to the experiment. This was done to control for any potential maternal or grandmaternal effects and to prevent mortality of clones unaccustomed to lab conditions (Antunes et al., 2003). These conditions were 15° C, 16L:8D photoperiod, 3.5 mL of algae daily, and 5 females per 80 mL cup of media. Female neonates were obtained from the final generation within 24 hours of being released from a brood and used in the experiment.

3.3.2 Experimental set-up

Translucent 140 mL plastic cups were filled with 40 mL of synthetic zooplankton media. Either one or ten female neonates of a clone were included in a cup, for Alone and Crowded conditions, respectively. It should be noted that even the Alone condition may be considered somewhat crowded to *Daphnia* (Banta & Brown, 1929, Hobæk &

Larsson, 1990). Eight replicate cups were used for each clone in each crowd condition (2 density conditions x 6 clones x 8 replicates = 96 cups). Cups were maintained in incubators at 15° C, with a short-day photoperiod of 8L:16D. Alone and Crowded cups were fed 1.9 and 3.8 mL of algae daily respectively, which was an ample amount for each condition.

Every other day the daphnids were transferred by pipet to a cup of new media, and the contents of the old media poured through a 250 μm plankton mesh to collect all neonates and ephippia. Neonates were temporarily immobilized in a shallow, weak ethanol-water solution to allow easier visualization and counting under a dissecting microscope. Neonate sex was determined based on visualization under the microscope. The numbers of male and female neonates were recorded along with number of ephippia. Ephippia were not opened to count the number of resting eggs within. After data collection, neonates and ephippia were discarded.

Data collection continued until the specimens reached the age of 38 days, at which point the experiment was terminated. Although *D. pulex* individuals can live longer than one month in a lab environment, their lifespan is likely shorter than one month in their natural habitat (Dudycha, 2004), so it made little sense to extend the experiment beyond 38 days of age. Spare replicates were kept for most clones, under identical conditions as others, to replace dead experimental daphnids. No daphnids in the Alone condition died in the experiment, but a small number died in the Crowded condition. Most of these were immediately replaced with individuals of the same genotype, age, condition, and stage of reproduction (i.e., carrying ephippia, carrying early-stage brood, etc.). This resulted in balanced statistical analyses.

3.3.3 Data analyses

All data were analyzed using MINITAB 14.1, and all graphs were produced using Prism 4.0. The General Linear Model (GLM) was the primary method used to evaluate each of the four major aspects of the data: Ephippia production, experiment-long fecundity, brood size, and sex ratio.

To test the various factors influencing variation in ephippia production, the factors of Density and Clone were included along with their interaction. The response variable was the total ephippia production over the length of the experiment per adult female in experimental cup. If ephippia production were no different between Alone and Crowded conditions, then this per-female ephippia production would not differ between density conditions. Ephippia data were consolidated over the length of the experiment as opposed to analyzed at individual data points for two reasons: non-consolidated data produced residuals that violated all assumptions required for GLM, and Age was determined to not be a significant factor influencing ephippia production in these analyses (results not shown).

Total fecundity over the length of the experiment was calculated using neonate data only, since any incorporation of ephippia in fecundity would necessitate a weighting factor to be applied to it, which may or may not be accurate. With total neonate production per female over the length of the experiment as the response variable, terms in the model were Density, Clone, and Density x Clone interaction.

The assumption is made in many *Daphnia* studies that male and female neonates cost the same amount of resources to the mother (Korpelainen, 1992, Innes, 1997). This

assumption is based largely on analyses by Barker & Hebert (1986) in which separate mixed model ANOVAs were performed on each of four clones of *Daphnia magna* Straus, testing for the influences of brood sex and date of brood release (i.e., age of mother) on brood size within each clone. In each of these analyses the date of brood release, but not brood sex, had a significant effect on brood size (Barker & Hebert, 1986). Since the sex of a brood had no significant effect on the number of neonates in the brood, males and females were assumed to cost the same resources for the mother. Similar within-clone comparisons of male and female brood sizes were conducted on a single clone of *D. magna* (Hobæk & Larsson, 1990) and three clones of *D. pulex* (Innes & Singleton, 2000).

I tested my data in two ways, using a separate mixed model ANOVA for each relevant clone with the terms Age and BrdSex as was done by Barker & Hebert (1986), as well as using the GLM outlined below. Brood size could only be evaluated from daphnids in the Alone density condition, since separate broods could not be distinguished in the Crowded condition. The few mixed-sex broods observed in the Alone condition were excluded from analysis, so as not to confound the BrdSex factor. Because Morg2 and VBA did not produce both all-male and all-female broods in the Alone condition, all data for these clones were excluded from brood size analyses evaluating the effect of brood sex. I believe the GLM analysis evaluates the assumption of equal resource costs for male and female neonates in a more robust manner than clone-specific ANOVAs. The GLM incorporates all relevant clones' data into one model, and adds the Clone factor to determine the level of variation in brood size that can be explained by variation between clones. If my analyses were to find brood sex to significantly influence brood

size, then assumptions of equal resource cost for male and female neonates would require re-evaluation.

The GLM to evaluate factors influencing brood size had the number of neonates in a brood (BrdSize) as its response variable. The terms in the model were: Age of mother at brood release (Age, regression term), Clone, brood sex (BrdSex), and the two-way interactions Age x Clone, Age x BrdSex, and Clone x BrdSex. This GLM was evaluated using the same four clones mentioned above. If all factors involving BrdSex were found to be insignificant, then a new GLM would be created that would eliminate such terms and only include the factors Age, Clone, and their interaction. This GLM would include data from all six clones, since there would be no reason to exclude Morg2 and VBA for producing broods of one sex only.

Although sex ratio is a common subject of study, its analysis is difficult with *Daphnia*. Because all neonates within a *D. pulex* brood tend to be the same sex, assumptions of sex independence are violated for within-brood siblings. For this reason, a common way to analyze sex ratios or sex allocation in *Daphnia* research has been to classify each brood as all-female, or containing any males (e.g., Hobæk & Larsson, 1990, Olmstead & LeBlanc, 2002). Because broods could not be distinguished from each other in the Crowded condition of my data, this method would not suffice for my purposes. Thus I totalled all neonate data across the length of the experiment for each replicate, producing lifetime sex ratios (total males over total neonates produced). This Sex Ratio was the response variable for my analysis, and the terms in the GLM were Density, Clone, and Density x Clone interaction.

Using consolidated data for sex ratios prevented the evaluation of Age as a factor influencing sex ratio variation. To evaluate its influence, another GLM was analyzed using only Crowded condition data, not consolidated over the length of the experiment. Data from the clone VBA was excluded since no males were produced in the Crowded condition. With Sex Ratio as the response variable, the terms of the model were Age (regression), Clone, and Age x Clone interaction.

To evaluate whether any effect of Age on sex ratios is due to an extraordinary bias of brood sex among first broods of *D. pulex*, I also compared sex ratio data between sequential broods. Since separate broods could not be distinguished in the Crowded condition, only data from the Alone condition were used. Data from Morg2 and VBA were excluded since neither produced both male and female broods in the Alone condition. Each replicate's broods were distinguished by the order in which they occurred. T-tests of the brood data were carried out comparing the sex ratios of first broods with those of second broods, and between second and third broods.

3.4 RESULTS

A total of 30109 neonates and 1041 ephippia were collected during the experiment (Table 3.1). The assumptions of residuals' independence, homogeneity, and sums of zero are made in any analyses based on General Linear Models (GLMs), and are met for all analyses here.

Table 3.1. Numbers of neonates and ephippia collected in the experiment. Clonal method of resting egg production is designated as facultative parthenogenetic (FP) or obligately parthenogenetic (OP). Daphnids were kept at densities of one (Alone) or ten (Crowded) in 40 mL of liquid.

Clone	Density	Male Neonates	Female Neonates	Total Neonates	Sex Ratio (% Males)	Ephippia
Disp (FP)	Alone	453	114	567	79.89	0
	Crowded	2925	585	3510	83.33	42
	Total	3378	699	4077	82.86	42
Morg1 (OP)	Alone	71	1154	1225	5.80	0
	Crowded	730	2163	2893	25.23	179
	Total	801	3317	4118	19.45	179
Morg2 (OP)	Alone	10	463	473	2.11	20
	Crowded	42	332	374	11.23	345
	Total	52	795	847	6.14	365
VBA (OP)	Alone	2	1558	1560	0.13	0
	Crowded	0	6599	6599	0.00	95
	Total	2	8157	8159	0.02	95
War1 (OP)	Alone	553	1194	1747	31.65	0
	Crowded	3060	4897	7957	38.46	70
	Total	3613	6091	9704	37.23	70
War2 (FP)	Alone	1589	101	1690	94.02	0
	Crowded	919	595	1514	60.70	290
	Total	2508	696	3204	78.28	290
Overall	**Alone**	**2678**	**4584**	**7262**	**36.88**	**20**
	Crowded	**7676**	**15171**	**22847**	**33.60**	**1021**
	Total	**10354**	**19755**	**30109**	**34.39**	**1041**

3.4.1 Ephippia production

Of the 1041 ephippia collected in this experiment, only 20 (1.92%) came from daphnids in the Alone condition, all of which were from the Morg2 clone (Table 3.1). This is reflected in the statistical results. The Density x Clone interaction explained a significant amount of the variation in experiment-long ephippia production per adult female ($p < 0.001$, Table 3.2). The individual terms Density and Clone also explained significant amounts of variation ($p < 0.001$ for each, Table 3.2), but this must be interpreted with caution since the interaction term is significant and takes precedence. The extent of the increase in number of ephippia produced from Alone to Crowded conditions depended upon the clone (Fig 3.1).

3.4.2 Fecundity

The total number of neonates produced by each daphnid varied according to both clone and density condition, with crowded individuals of each clone producing fewer neonates per-daphnid than those in the Alone condition (Table 3.1, Fig 3.2). The interaction Density x Clone explained a significant amount of the variation in neonate production ($p < 0.001$, Table 3.2). The individual terms Density and Clone also explained significant amounts of this variation ($p < 0.001$ for each, Table 3.2). All GLM-based assumptions of residuals were met. As would be expected, there was a significant negative correlation among Crowded condition daphnids between the numbers of neonates and ephippia produced over the length of the experiment (Pearson correlation $r = -0.799$, $p < 0.001$, Fig 3.3). A daphnid can carry either a brood of neonates or an ephippium at one time, resulting in a trade-off.

Table 3.2. General Linear Model results for several analyses. See Methods (Data Analyses) for details on each analysis and factor.

Factor	d.f.	Seq SS	Adj MS	F	p
Ephippia					
Density	1	74.016	74.016	248.13	< 0.001
Clone	5	119.306	23.861	79.99	< 0.001
Density x Clone	5	27.302	5.460	18.31	< 0.001
Error	84	25.056	0.298		
Total	95	245.681			
Fecundity					
Density	1	256739	256739	294.87	< 0.001
Clone	5	187254	37451	43.01	< 0.001
Density x Clone	5	65517	13103	15.05	< 0.001
Error	84	73136	871		
Total	95	582647			
Brood Size					
Age	1	23718.4	19895.4	366.16	< 0.001
Clone	5	14356.4	543.2	10.00	< 0.001
Age x Clone	5	8354.6	1670.9	30.75	< 0.001
Error	248	13475.3	54.3		
Total	259	59904.7			
Sex Ratio					
Density	1	0.03109	0.01089	0.64	0.427
Clone	5	9.33902	1.87192	109.60	< 0.001
Density x Clone	5	0.99187	0.19837	11.61	< 0.001
Error	82	1.40054	0.01708		
Total	93	11.76251			

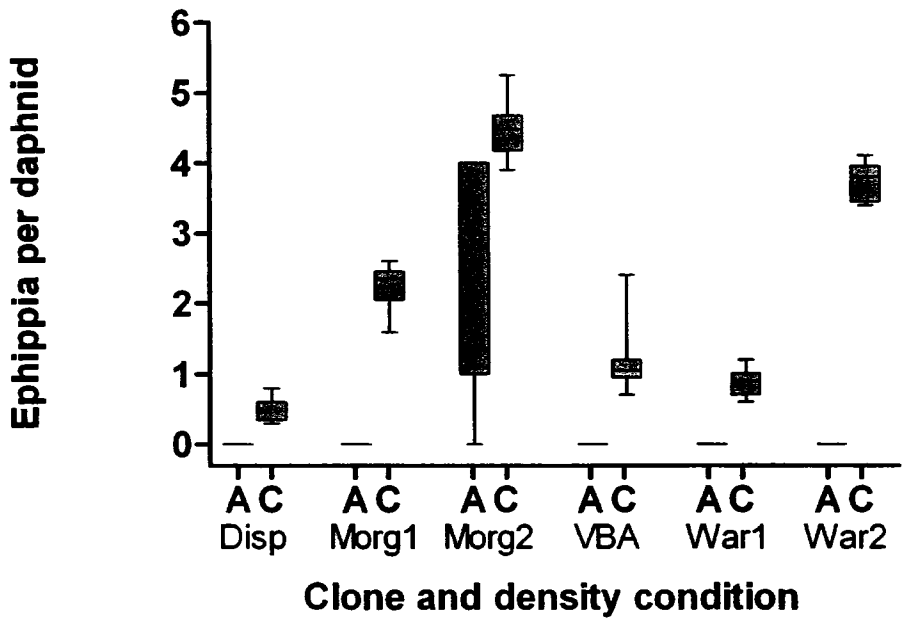

Figure 3.1: Per-daphnid ephippia production among clones in the Alone (A) and Crowded (C) conditions. Boxes include the median and span the 25th – 75th percentiles. Whiskers span the range of the data.

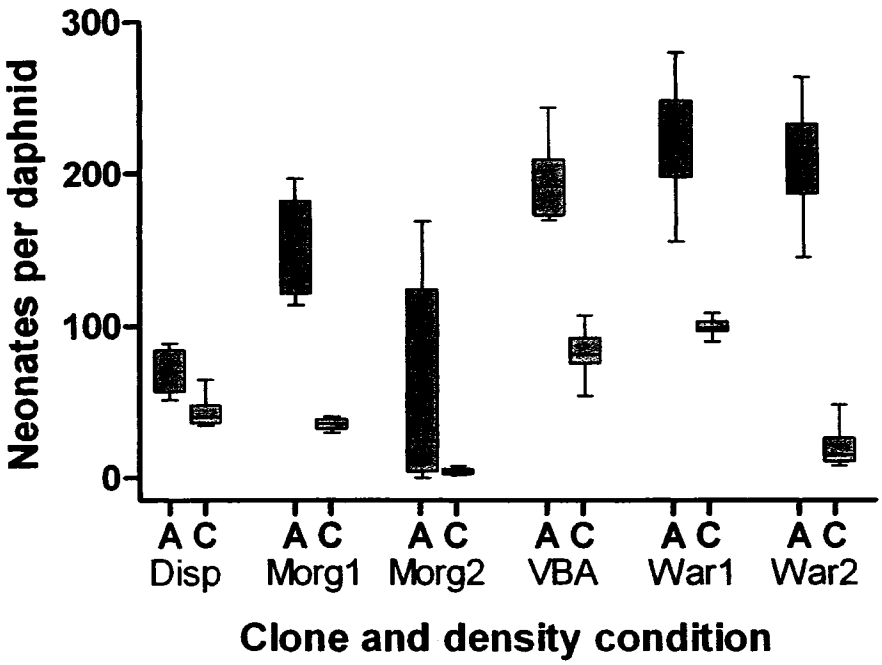

Figure 3.2: Experiment-long fecundity per daphnid among clones in the Alone (A) and Crowded (C) conditions. Boxes include the median and span the 25^{th} – 75^{th} percentiles. Whiskers span the range of the data.

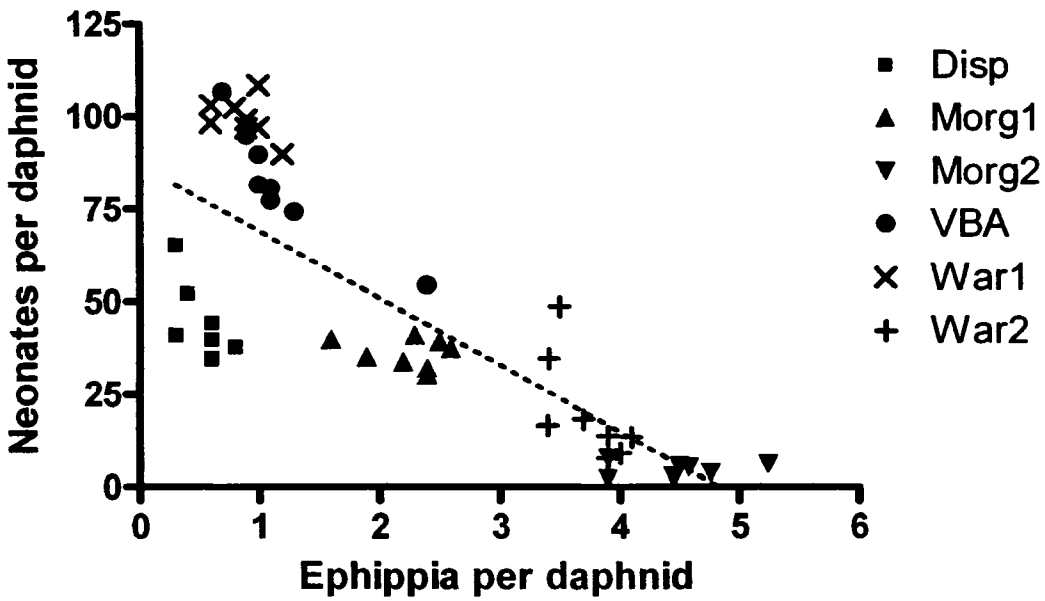

Figure 3.3: Experiment-long production of neonates and ephippia per daphnid among clones in the Crowded condition. The six different clones are designated by points of different shapes, with eight replicates each. The black dotted line represents the linear regression among all data points.

3.4.3 Brood size

Like Barker & Hebert (1986), I tested for the influence of brood sex and age on brood size in separate ANOVAs for each of the four appropriate alone-condition clones in my data set. Age did ($p < 0.001$ for each clone, Table 3.3), and brood sex did not ($p > 0.05$ for each clone, Table 3.3), explain a significant amount of brood size variation in each of the four clones analyzed. This same pattern held true when Age was substituted with the reproductive event order (i.e., each brood or ephippium is a reproductive event and ranked sequentially, data not shown). These results suggest the assumption of broods of each sex being the same size, and that there is no significant difference in resource costs for the mother between male and female neonate offspring, is valid.

The GLM evaluating effects on brood size among Alone condition data for the four appropriate clones failed to reveal significant effects of any term involving BrdSex. Thus, all terms involving BrdSex were discarded and a new GLM was evaluated incorporating all six clones. In this GLM, the interaction Age x Clone explained a significant amount of variation in the number of neonates in each brood ($p < 0.001$, Table 3.2). The terms Age and Clone alone also explained a significant amount of this variation ($p < 0.001$ for each, Table 3.2), but should be interpreted only after their interaction is considered. All GLM-based assumptions of residuals were met. The size of most clones' broods increased with age (Pearson correlation $r = 0.484$, $p < 0.001$), albeit to different degrees (Fig 3.4). Individuals of the Disp clone, however, produced slightly smaller broods with age (Fig 3.4a). Age x Clone remained significant even when Disp was

Table 3.3. Results of ANOVA analyses of brood size variation for each of four individual clones with respect to the factors of time and brood sex. Time is represented either by daphnid age (a) or reproductive event order (b).

Clone	Factor	d.f.	Seq SS	Adj MS	F	p
a)						
Disp	Age	1	191.53	246.13	16.26	< 0.001
	BrdSex	1	54.74	54.74	3.62	0.064
	Error	42	635.73	15.14		
	Total	44	882.00			
Morg1	Age	1	3268.1	3168.5	47.20	< 0.001
	BrdSex	1	2.6	2.6	0.04	0.846
	Error	39	2618.3	67.1		
	Total	41	5889.0			
War1	Age	1	7663.8	7396.8	127.59	< 0.001
	BrdSex	1	79.7	79.7	1.37	0.248
	Error	39	2261.0	58.0		
	Total	41	10004.4			
War2	Age	1	4266.6	3566.2	80.27	< 0.001
	BrdSex	1	0.5	0.5	0.01	0.913
	Error	47	2088.2	44.4		
	Total	49	6355.4			
b)						
Disp	Event	1	150.48	191.68	11.66	0.001
	BrdSex	1	41.34	41.34	2.52	0.120
	Error	42	690.18	16.43		
	Total	44	882.00			
Morg1	Event	1	2750.0	2660.2	33.18	< 0.001
	BrdSex	1	12.5	12.5	0.16	0.696
	Error	39	3126.5	80.2		
	Total	41	5889.0			
War1	Event	1	7198.1	6908.1	97.98	< 0.001
	BrdSex	1	56.7	56.7	0.80	0.375
	Error	39	2749.6	70.5		
	Total	41	10004.4			
War2	Event	1	4160.7	3467.3	74.51	< 0.001
	BrdSex	1	7.6	7.6	0.16	0.688
	Error	47	2187.1	46.5		
	Total	49	6355.4			

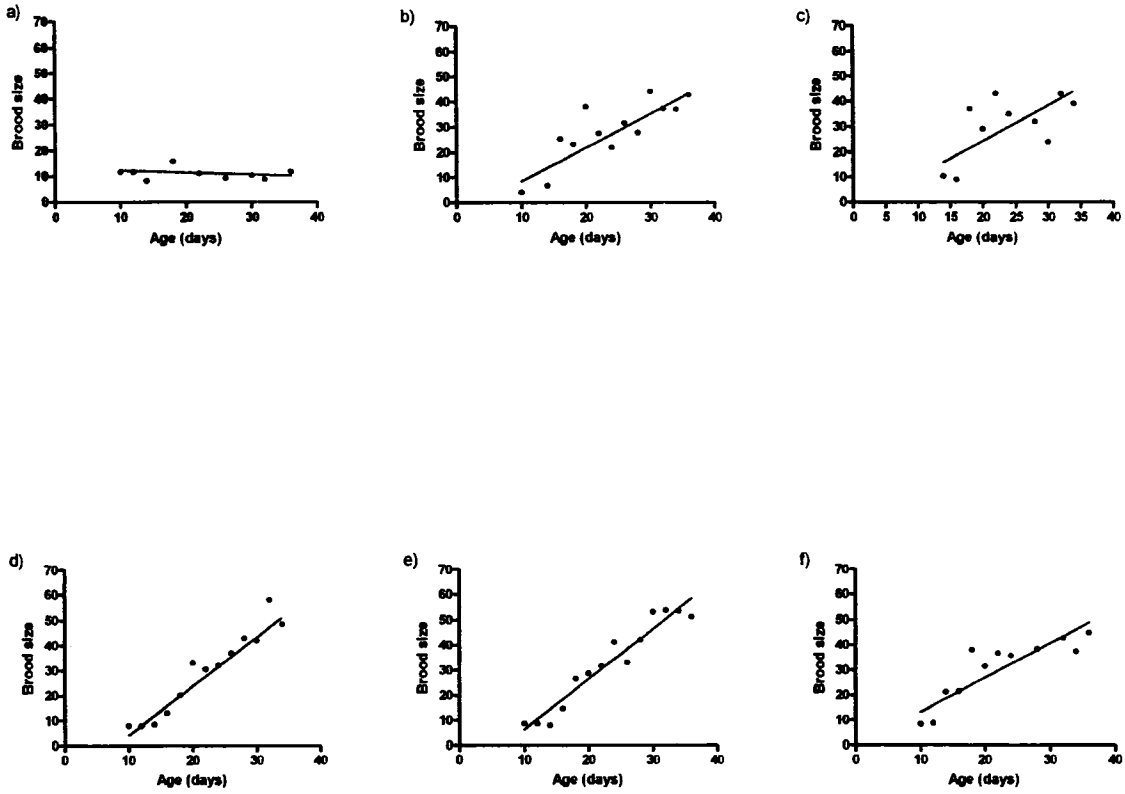

Figure 3.4: Number of neonates in broods of individuals in the Alone condition, plotted against age of the mother in days. Symbols plot the mean brood size among replicates at a given maternal age. Regression lines across maternal lifetime are shown. Graphs are separated by genotype: a) Disp, b) Morg1, c) Morg2, d) VBA, e) War1, and f) War2.

removed from the analysis ($F_{4, 202} = 6.98$, $p < 0.001$), as did the individual terms Age ($F_{1, 202} = 357.78$, $p < 0.001$) and Clone ($F_{4, 202} = 4.54$, $p = 0.002$).

3.4.4 Sex ratio

The sex ratio of all neonates produced over the length of the experiment varied among clones, and each clone responded differently to crowding. Some clones responded in opposite ways. Morg1 produced a greater proportion of males, while War2 reduced its proportion of males in the Crowded condition (Fig 3.5). The interaction of Density x Clone was responsible for a significant amount of the variation in experiment-long sex ratios ($p < 0.001$, Table 3.2). The term Clone alone was also a significant influence on sex ratios, but as with other analyses the interaction term must take precedence over solitary terms if significant. All GLM-based assumptions of residuals were met.

To evaluate the potential influence of Age on sex ratios, non-consolidated Crowded condition data was analyzed. Unlike neonate data from the Alone condition, which tended to be all-male or all-female, Crowded condition data was often intermediate due to several daphnids producing broods in the same time frame, often of different sexes. The Age x Clone interaction explained a significant amount of the variation in sex ratios according to our analyses ($F_{4, 286} = 2.52$, $p = 0.041$, Fig 3.6). Both Age ($F_{1, 286} = 108.92$, $p < 0.001$) and Clone ($F_{4, 286} = 7.99$, $p < 0.001$) alone also explained significant variation in sex ratio. All GLM-based assumptions of residuals were met. Each of the five appropriate clones analyzed under Crowded conditions increased their sex ratio in favour of males with age (overall Pearson correlation $r = 0.391$, $p < 0.001$).

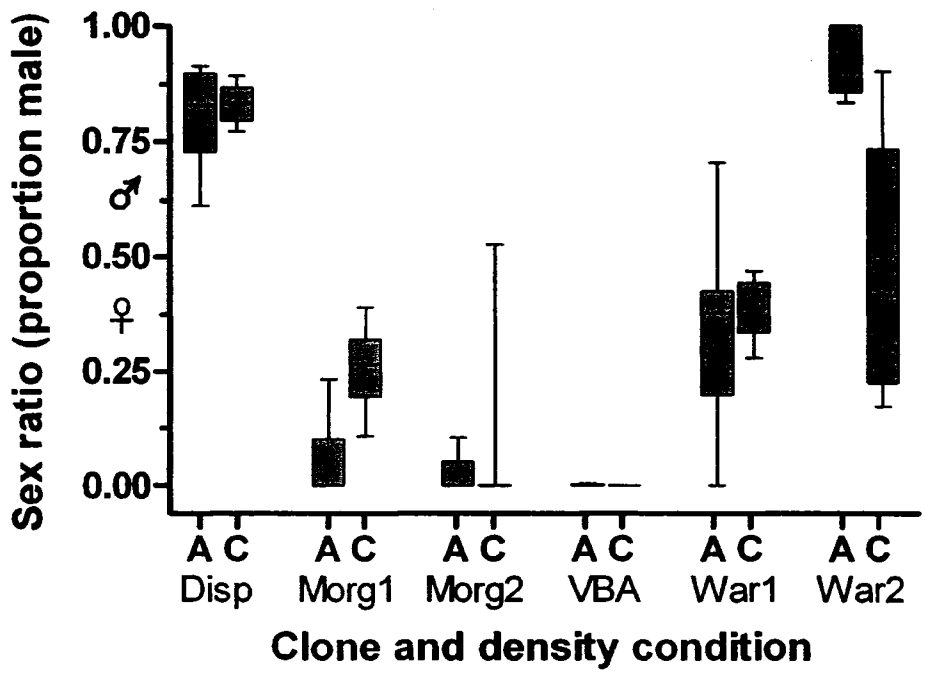

Figure 3.5: Overall sex ratios (proportion male) of all neonates among clones in the Alone (A) and Crowded (C) conditions. Boxes include the median and span the 25^{th} – 75^{th} percentiles. Whiskers span the range of the data.

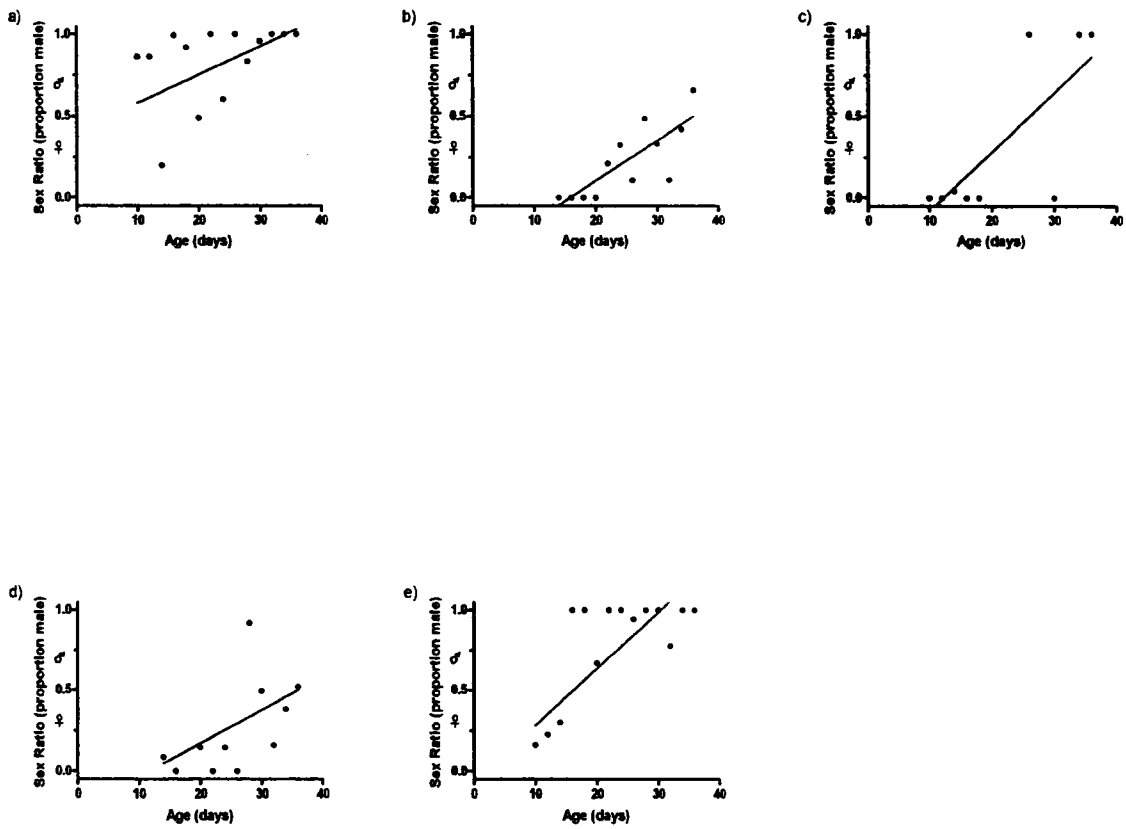

Figure 3.6: Sex ratio (proportion male) of neonate offspring of five clones in the Crowded condition, in relation to age of mother in days. Symbols plot the mean sex ratios among replicates at a given maternal age. Regression lines across maternal lifetime are shown. Graphs are separated by genotype: a) Disp, b) Morg1, c) Morg2, d) War1, and e) War2.

To further evaluate the influence of age on sex ratios, I considered whether the age effect was simply due to heavily female-biased first broods. At least one previous study had found no first broods to be male among the three *Daphnia* clones analyzed (Stross, 1969). Using brood data from the Alone condition I compared sequential broods' sex ratios using t-tests. I excluded from my analysis all mixed-sex broods and all data from the clones Morg2 and VBA for reasons explained above. Brood data from the remaining four clones was consolidated according to reproductive event, with a replicate's first brood or ephippium given a rank of one, its second reproductive event a two, etc. The mean and standard error sex ratios of broods as the first three reproductive events were 0.202 ± 0.070, 0.470 ± 0.089, and 0.589 ± 0.088, respectively. Broods that were daphnids' first reproductive event were all-male on several occasions, but were significantly more female-biased than broods that were daphnids' second reproductive event ($t_{58} = -2.36$, $p = 0.022$). Each subsequent brood increased its propensity toward male production, but with insignificant differences between broods (sex ratio differences between second and third broods as reproductive events, $t_{61} = -0.95$, $p = 0.347$).

3.4.5 Abnormal neonate and mixed-sex brood analyses

A total of 114 of the 19755 female neonates collected during this experiment (0.58%) were deformed morphologically as described below. Only three of these abnormal neonates (2.63%) were observed in the Alone condition. Abnormal neonates were produced by each of the experiment's six clones. The assumptions based on residuals necessary for GLMs were violated, preventing its use for analysis of the factors influencing the proportion of female neonates that were deformed. Instead, correlations

were analyzed with respect to the fraction of female neonates that were abnormal and the density of adult daphnids. There was a significant positive correlation between the density of adult daphnids and the fraction of female neonates that were abnormal (Pearson correlation $r = 0.108$, $p = 0.019$). Thus as adult density increased, so did the proportion of daughters with developmental abnormalities. Age was significantly and negatively correlated with the fraction of female neonates that were abnormal ($r = -0.115$, $p = 0.012$), suggesting older daphnids produced relatively fewer abnormal daughters.

Among daphnids in the Alone condition in this experiment, 13 out of 278 broods (4.68%) contained both males and females. Other studies have found mixed-sex broods to occur at frequencies of ~11% for *D. pulex* (Innes, 1997), and 7.6% (Barker & Hebert, 1986) and 5.6% for *D. magna* (Kleiven et al., 1992) under various conditions.

There has been speculation as to whether or not there is a genetic component to the propensity to produce mixed-sex broods (Innes, 1997). With only 13 mixed-sex broods, the present data set is too small for formal analysis of influential factors. I simply point out that each of the six clones produced at least one mixed-sex brood, with War1 producing the most such broods with six (data not shown).

3.5 DISCUSSION

Crowding provided an important stimulus in reproductive decisions of *D. pulex*. Both the type and amount of reproduction varied across density conditions, but remained consistent within each condition within each genotype. This is yet another example of intraclonal phenotypic plasticity in response to environmental stimuli (Lee, 1984, Lushai

et al., 2003, Printes & Callaghan, 2003). Different genotypes responded to the crowding stimulus in ways that were often similar but sometimes contradictory.

3.5.1 Reproductive responses to crowding

The per-daphnid rate of ephippia production was significantly higher for crowded daphnids than for those that were alone. In fact, only one of the experiment's six genotypes produced any ephippia in the Alone condition. Genotypes differed in the extent of their ephippia production increase with crowding, but all increased. Crowding can likely be used by *D. pulex* as a predictive cue of upcoming events that would benefit diapause more than neonate production. Crowding may either result from the growth of a population that is often followed by a steep decline (Dudycha, 2004, Nelson et al., 2005), or decreasing water levels as a result of the pond drying up. Either of these situations would favour investment in diapause as opposed to neonate production.

Other research has also found ephippia production among *D. pulex* to vary across clones (Innes et al., 2000, Tessier & Cáceres, 2004) and to increase as a result of crowding (Lürling et al., 2003), sometimes in a clone-specific manner (Berg et al., 2001). Research using other species of *Daphnia* has yielded similar results for inter-clonal variation in ephippia production among *Daphnia pulicaria* Forbes (Cáceres & Tessier, 2004, Tessier & Cáceres, 2004), and an increase in ephippia production with crowding among clones of *D. magna* (Ferrari & Hebert, 1982, Carvalho & Hughes, 1983), also in a clone-specific manner (Yampolsky, 1992). Some studies have found crowding to only increase ephippia production when combined with additional stimuli, such as reduced food levels (Olmstead & LeBlanc, 2001) or reduced food levels and low-light

photoperiods (Kleiven et al., 1992), but these studies considered only a single clone so their broad applicability may not be valid (see below). Stimuli other than crowding have also been suggested to induce *Daphnia* ephippia production including: photoperiod (Deng, 1996, Deng, 1997b), food level (Deng, 1996), fish kairomones (Ślusarczyk, 1995), density of males (Innes, 1997, Innes & Singleton, 2000), clonal competitive ability (Loaring & Hebert, 1981), parasite resistance (Mitchell et al., 2004), and the contrast between mother and offspring food levels (LaMontagne & McCauley, 2001). Clearly more research is required to evaluate the effects of each stimulus on ephippia production in various species of *Daphnia.*

Just as the effects of crowding on ephippia production were uni-directional but differed in magnitude across clones, crowding reduced per-daphnid experiment-long fecundity in all clones, but to various degrees. This result is supported by previous research on *D. pulex* in which the total number of neonates produced per daphnid was reduced in response to crowded conditions, but in a clone-specific manner (i.e., significant clone x density interaction) (Innes & Singleton, 1994, Innes & Singleton, 2000, Berg et al., 2001). Other research has found that offspring produced by crowded *D. magna* mothers were more tolerant of toxins than those produced by non-crowded mothers (Baird et al., 1991). This suggests that under crowded conditions mothers may allocate resources to fewer, high-quality offspring that are competitive, and many weaker offspring when there is no sign of impending competition with other daphnids. Other explanations are possible, so this hypothesis should be tested by comparing reproductive output of daphnids originating from uncrowded and crowded mothers, both alone and in competition with other daphnids. If the hypothesis were true, offspring from crowded

mothers would have the higher fitness of the two in a competitive environment. In the absence of competition, total offspring production among all daughters of an uncrowded mother should be greater than the equivalent grand-offspring production of a crowded mother. Microparasites (Lass & Bittner, 2002), fish kairomones (Weber & Declerck, 1997, Lass & Bittner, 2002), and temperature (Loaring & Hebert, 1981) are among the other factors that have been suggested to influence *Daphnia* fecundity with effects that often vary across clones.

As with other studies, I found a significant negative correlation between the total numbers of ephippia and neonates produced among daphnids in the crowded condition because a daphnid may only carry an ephippium or a brood at one time (Loaring & Hebert, 1981, Ruvinsky et al., 1986, Yampolsky, 1992, Berg et al., 2001). This represents an intrinsic trade-off between neonate and resting egg production.

A fraction (0.58%) of the female neonates produced in this study were abnormal morphologically. They were characterized by their small size and deformed carapaces and antennae. These developmental abnormalities closely resemble those observed among neonates of *D. magna* individuals exposed to fenarimol (Mu & LeBlanc, 2002). Mu & LeBlanc (2002) further investigated the basis for the developmental abnormalities they observed, and found fenarimol exposure during embryo development to result in reduced ecdysone levels among neonates. We undertook no such biochemical tests of ecdysone levels among daphnids in our experiment. Age was negatively and adult density was positively correlated with the fraction of daughters that were deformed. Further research is required to thoroughly evaluate the embryological reasons for this

developmental abnormality and the metabolic and ecological influences on its underlying factors.

Brood size, like fecundity, varied significantly across clones in the Alone condition. As most daphnids grew older, they produced broods with greater numbers of neonates. The magnitude of this age (and size) effect varied across clones (i.e., clone x age effect), with Disp daphnids notably not increasing their brood sizes with age. Previous research has found that the linear increase of brood size with age levels off in old age among two clones of *D. magna* (Glazier, 1992). Other research has also found that the effect of age upon brood size is one that can be modified by other cues. Upon exposure to a microsporidian parasite, *D. magna* have been found to increase reproduction at young ages and decrease reproduction when old (Chadwick & Little, 2005).

3.5.2 Offspring sex ratios

Fisher's rationale behind selection for a 1:1 operational sex ratio holds true only if sons and daughters cost equal resources to their mothers (Fisher, 1930, Carvalho et al., 1998). Therefore, the relative costs of male and female neonates to mothers must be considered before *D. pulex* sex ratios can be evaluated in full. Three previous studies have found no difference in sizes of male and female broods among clones of *D. magna* (Barker & Hebert, 1986, Hobæk & Larsson, 1990) and *D. pulex* (Innes & Singleton, 2000). One of these studies used only a single clone, so its results should be treated with caution (Hobæk & Larsson, 1990). The statistical methods used in the other two studies lacked the power of the one used here because they evaluated each clone separately.

Whether I used within-clone tests or all-clone models, I found no significant difference in the sizes of male and female broods among male-producing clones in the Alone condition. I therefore conclude that if there are differential costs in male and female neonate production, they are either minute or resources are redistributed from other functions to accommodate the more expensive sex. It is likely that male and female neonates cost the same to a mother, thus sex allocation and sex ratio of neonates can be considered equal.

One clone (VBA) was reluctant to produce males under either density condition, producing only 2 males among 8159 neonates. This was surprising, as numerous males had been observed in stock cultures of this clone prior to this experiment. This provides further evidence that the cues to male production vary across clones.

Among all five male-producing clones in crowded conditions, there was a significant increase in the sex ratio in favour of males as daphnids grew older. This was true to such an extent that the two FP clones (Disp and War2) were producing male broods almost exclusively by the end of the experiment. Although the effects of seasonal progression on the ratio of males in ponds has been studied (Barker & Hebert, 1986, Innes, 1997), little research has explicitly examined changes in offspring sex ratio within the lifetime of individual daphnids. Increased male production with age has been observed with a single clone of *D. magna* (Hobæk & Larsson, 1990), and another study found no first-broods of three *Daphnia* clones to be male (Stross, 1969). A *D. magna* clone in a study by Olmstead & LeBlanc (2001) only increased its male production with age when under certain environmental conditions and the juvenile hormone analog methoprene was added, otherwise this clone reduced its male production with age.

More research is required into the effects of age upon offspring sex ratios, but I will speculate on potential selective pressures for increased male production with age. These pressures assume sexuality, and focus on sexual selection. Sexual selection is of little current relevance to OP lines, but may still be entrenched among OP genomes due to their sexual origins.

I assume males to be more variable in reproductive output than females. Little is currently known about sexual selection among *Daphnia* (Brewer, 1998) other than males can fertilize >20 females in one day, and mate with females regardless of genetic similarity (Winsor & Innes, 2002). Future research on pre- and post-copulatory sexual selection under a variety of conditions would be beneficial for our understanding of *Daphnia*. Below are three potential evolutionary reasons for increased male production with maternal age.

First, increased male-production with age may be a bet-hedging strategy, whereby males are only produced after it has been ensured that some females have been produced, since males are a riskier investment. It has been demonstrated mathematically that generations of low fitness affect the overall geometric mean of fitness more than those of high fitness (Vrijenhoek, 1998). Thus a 'safe' female-first strategy may be beneficial even if the various low- and high-reproductive generations of an early male production strategy would otherwise seem to cancel each-other out.

Second, assuming males are better able than females to translate high fitness into increased offspring production, if offspring quality increases with maternal age then selection should favour an increased male-bias with maternal age (Trivers & Willard, 1973). This could be a simple result of older, larger females being able to invest more

resources into offspring. Indeed, a study using two genotypes of *D. magna* found females to produce eggs of greater mass with age (Glazier, 1992). In support of this resource-investment-in-males theory, birds in low-food conditions have been found to reduce their investment in male offspring by preferentially starving them, presumably because daughters' fitness is compromised less by low resources than that of sons (Nager et al., 1999).

Finally, in addition to being able to allocate increased resources to offspring to increase their quality, an older mother may also indirectly ensure genetic quality for her offspring. A long-lived daphnid is generally of higher quality than those that died at a younger age. Therefore, the increased male production among older daphnids may have been selected as a way for high-quality genotypes to produce the offspring sex that can best convert high genetic quality into increased reproductive output.

Offspring sex ratios of *Daphnia* are often reported to become male-biased when the mother is exposed to either crowded conditions, or the cues within *Daphnia*-crowded water (Hobæk & Larsson, 1990, Kleiven et al., 1992, Innes et al., 2000, Innes & Singleton, 2000). It is often suggested that increased male production is induced by similar stimuli as ephippia production, just at a more relaxed level (Ferrari & Hebert, 1982, Kleiven et al., 1992). This is supported by *Daphnia* field sample studies finding high levels of males in a pond or lake two weeks before high levels of ephippial females (De Meester & Vanoverbeke, 1999, Cáceres & Tessier, 2004, Spaak et al., 2004). In addition to crowding, stimuli reported to alter *Daphnia* offspring sex ratios include photoperiod (Ferrari & Hebert, 1982, Korpelainen, 1986, Innes et al., 2000, Zhang &

Baer, 2000), temperature (Korpelainen, 1986), food level (Ferrari & Hebert, 1982, Zhang & Baer, 2000), and exposure to fish kairomones (Boersma et al., 1998).

In contrast to some of the literature cited above, my results do not support a simple relationship between increased crowding and increased male-bias among *D. pulex* sex ratios. I found significant differences not only between clones' sex ratios, but also between clones' sex ratios in response to increased density. The clones Morg1 and War1 increased their sex ratio in favour of males under dense conditions, while War2 behaved in the opposite way by producing fewer males under dense conditions. Disp, meanwhile, did not alter its sex ratio much either way with crowding.

How can my findings of inter-clonal variation in sex ratio responses to density be reconciled with the above-cited literature describing a simple positive relationship between crowding and sex ratios? The experimental design of some studies prevented detailed quantification of offspring sex ratios in response to crowding (Innes & Singleton, 2000), or only studied the effects of crowding when combined with another stimulus and thus could not tease apart the effects of the two cues (Innes et al., 2000). Two studies used the same single genotype of *D. magna* and thus could not detect inter-clonal variation (Hobæk & Larsson, 1990, Kleiven et al., 1992).

Studies that have tested for inter-clonal variation in sex ratios have found it, among clones of *D. pulex* (Innes & Dunbrack, 1993, Innes, 1997, Innes et al., 2000, Innes & Singleton, 2000), clones of *D. pulicaria* (Tessier & Cáceres, 2004), and both clones (Korpelainen, 1986) and populations (Ferrari & Hebert, 1982) of *D. magna*. In fact, the significant clone x density interaction affecting sex ratios in the present study was also found to significantly influence sex ratios in other studies on *D. pulex* (Ruvinsky et al.,

1986, Innes & Singleton, 1994) and *D. magna* (Yampolsky, 1992). A population x density interaction similarly influenced sex ratios in a component of a study on *D. pulex* that did not distinguish between clones (Berg et al., 2001). Other research has reported different clones responding with sex ratios biased in opposite directions in response to crowding (Innes & Singleton, 1994).

Applicability of results from the laboratory to nature are difficult to assess. However, other researchers have found their lab results to match well with field results for *D. pulex* (Innes, 1997, Tessier & Cáceres, 2004), providing reason to believe the same may be true for the present experiment.

3.5.3 Male production among asexual *D. pulex*

Previous research suggests male production among OP genotypes is much-reduced compared to male production among FP genotypes (Innes et al., 2000). Any male production at all among OP clones seems paradoxical, but may in fact be an adaptive trait. Males from OP clones can mate with FP females to create novel OP lines. There is evidence that obligate parthenogenesis has been spreading among North American *D. pulex* populations in a contagious fashion through such FP-OP matings (Paland et al., 2005). These new OP lines are expected to benefit from the half-genome contribution of the FP mother, as it is expected to be both locally-adapted and less mutated than that of the OP father (Simon et al., 2003).

Although selection may favour male production among OP clones in areas near FP populations of *D. pulex* (Innes et al., 2000), why would male production continue among OP clones far away from FP populations? Asexual genomes by their very

definition do not recombine with others, and thus change very little over generations. Selection at each locus is reduced in an asexual genome (Rice & Chippindale, 2001, Betancourt & Presgraves, 2002, Kaltz & Bell, 2002, Hadany & Feldman, 2005), so traits may be retained after they have ceased to be adaptive. Put simply, an asexual genome has trouble separating itself from its maker. This applies to sexual selection strategies (see above), male production, and other traits adaptive to sexuality.

It would be interesting to contrast levels of male production from OP populations at the FP-OP overlap range of middle North America with those from the Northeast of the continent whence obligate parthenogenesis is believed to have originated, and where obligate parthenogenesis is now ubiquitous among *D. pulex* populations (Paland et al., 2005). Is selection against male production in the Northeast strong enough to have eliminated those genotypes with heavy male production and hence reduce genotypic diversity? Or, is it weak enough that its effects have been largely overridden by the overall fitness of the rest of the genome? Has sufficient time passed for selection against male production to have had an effect? It is also possible that males serve a yet-unknown selected function, and their continued production is in fact adaptive.

3.5.4 Sex determination among *Daphnia*

Because offspring sex is determined by non-genetic factors post-fertilization, *Daphnia* are classified as having environmental sex determination (ESD). Genotypic sex determination (GSD), in contrast, occurs at conception based on genes that are often carried on sex chromosomes (Valenzuela et al., 2003). Although some argue for continued and clear distinctions to be made between ESD and GSD systems (Valenzuela

et al., 2003), there is growing support for viewing ESD and GSD as ends of a spectrum between which a grayscale range of systems may lie (Sarre et al., 2004). Sex expression of *Daphnia* has been described as a result of genotype-by-environment interactions (Yampolsky, 1992, Innes, 1997, Innes & Singleton, 2000), a description my results support.

The majority of ESD research has involved reptiles and fish with temperature-dependent sex determination. There is a growing body of evidence among such species for both hereditary and environmental factors to play influential roles in sex determination (Bull et al., 1982, Conover & Heins, 1987, Conover et al., 1992, Lagomarsino & Conover, 1993, Rhen & Lang, 1998, Janzen & Morjan, 2001, Shine et al., 2002, Valenzuela et al., 2003, Sarre et al., 2004). The hereditary factors involved may also include maternal effects, maternal choice of nesting spot, and other non-genetic effects, but are likely to at least involve genetic factors (Rhen & Lang, 1998).

What are the genetic factors involved in *Daphnia* sex determination? Given the multiple opportunities to influence sex determination from the environment to the embryo, there are many genes that may influence *Daphnia* sex. In the present experiment, there is the potential for inter-clonal differences in the rates of production of 'crowding infochemicals', detection of crowding infochemicals by mothers, production of sex determining hormones (likely methyl farnesoate, Olmstead & LeBlanc, 2002), detection of these hormones by embryos, and embryo response to hormone levels. Each of these traits should have a genetic basis which may vary across genomes. These are also likely to be threshold traits, which are favoured in temporally varying environments,

with critical ranges controlled by multiple loci (Roff, 1996). Thus the number of genes involved in the *Daphnia* 'environmental' sex determination system could be quite large.

3.5.5 *D. pulex* crowding infochemicals

Having discussed the effects of crowding on *D. pulex*, let us now consider the very nature of this crowding stimulus. What are the infochemicals used by *Daphnia* to detect crowding, and how specific are they? Although physical interaction with other daphnids may increase the stimulus, the mere water in which daphnids have lived is sufficient to induce a crowding response among *Daphnia* (Larsson, 1991). Six kairomones have recently been isolated from *D. pulex* that induce morphological changes in algal prey (Yasumoto et al., 2005). Whether these kairomones are the crowding infochemicals detected by fellow daphnids remains to be seen, but their synthesis allows future research to explore the possibility.

The specificity of *Daphnia* infochemicals is a matter of evolutionary importance. If daphnids are able to distinguish between infochemicals emitted by males and females then they could conceivably adjust their offspring sex ratios or reproductive behaviour accordingly, as some monoecious plants do depending on the amount of pollen they detect (López & Domínguez, 2003). The population demographics experienced by a mother, however, may not reliably predict those her offspring will experience at sexual maturity, which may be a reason why some bird species, for instance, do not employ this strategy (Bensch et al., 1999). A study using a single genotype of *D. magna* failed to reveal any distinction between male and female cues in swarming behaviour (Crease & Hebert, 1983).

If genotypes of *Daphnia* are able to distinguish between their own infochemicals and those of others in the same species, then they would be better able to determine outbreeding opportunities. Within-clone matings are known to produce offspring with fitness that is variable but reduced on average (Innes, 1989, Innes & Dunbrack, 1993, Deng, 1997a, Salathé & Ebert, 2003). Perhaps *D. pulex* adjusts its reproduction in response to outbreeding opportunities, just as female ambrosia beetles adjust their offspring sex ratio depending on outbreeding opportunities (Peer & Taborsky, 2004).

Are infochemicals detected across species within the genus *Daphnia*, or even within the family Cladocera? Inter-species detection of infochemicals decreases their reliability of prediction, and may select for the utilization of alternative predictors of environmental conditions (Serra et al., 2005). As with *Daphnia*, crowding induces a sexual response in many rotifers (Carmona et al., 1993). However, both experimental and theoretical evidence suggests some rotifers use the progression of generations as a sex-inducing cue instead of crowding, likely because crowding cues are not species-specific and hence lack accuracy (Serra et al., 2005). What little research conducted on inter-species detection of *Daphnia* infochemicals has used single genotypes, so inter-species response levels could not be compared to intraspecific inter-genotype response levels (e.g., Hobæk & Larsson, 1990, Lürling et al., 2003).

Many aspects of *Daphnia* infochemicals have implications that extend to experimental design. In a study on *D. pulex* response to water inhabited by stickleback fish, the fish were fed *Daphnia* (Weetman & Atkinson, 2002). Is it possible that some of the observed effects were not the result of stickleback kairomones, but in fact *Daphnia* alarm cues in the water? Aphid alarm cues are known to indirectly induce the production

of dispersing offspring through a 'pseudo crowding' effect (Kunert et al., 2005). And in a study on the effects of crowding upon *D. pulex* reproduction, water used for all clones came from one pond (Berg et al., 2001): a not-uncommon method among *Daphnia* laboratories (e.g., Deng, 1997b). The authors point out that this may have differentially affected the clone originating from this pond compared to the other clones in the experiment, but rate this an unlikely possibility (Berg et al., 2001).

3.5.6 Coexistence of competing genotypes

Both in this experiment and in others like it, a variety of reproductive strategies are found among *Daphnia* clones. Some variation in life-history strategies may be attributed to local adaptation between different habitats (Korpelainen, 1992, De Meester, 1996, Declerck et al., 2001). Coexistence of multiple clones within a habitat, however, is an especially intriguing issue. Whether it is at the level of genotypes within a species or species within a community, coexistence is a fundamental aspect of Biology. There are many factors involved in coexistence, reviewed elsewhere (Kassen, 2002, Ackerman & Doebeli, 2004, Bonsall et al., 2004, Vellend & Geber, 2005). I will focus discussion of *D. pulex* clonal coexistence on three areas: Differential resource use between clones, genotype x environment (GxE) interactions, and the persistence of maladaptive genotypes.

Intra-clonal competition for resources has been found to be greater than inter-clonal competition in *D. pulex* (Tagg et al., 2005a). A recent experiment found genetically diverse *Daphnia obtusa* (Kurz) to increase in numbers at the expense of genetically uniform competitors in laboratory mesocosms, likely as a result of food niche

diversification (Tagg et al., 2005b). If different clones utilize slightly different, albeit largely overlapping, resources then clonal diversity may be more easily maintained than if they were competing for the exact same resource.

Not only do *D. pulex* clones utilize slightly different resources, but they also interact differently with the environment. The present study illustrated clearly that different clones responded in different ways to crowding. Such GxE effects have been found in almost every study to have tested for them, with genotypes responding differently to stimuli that include photoperiod (Korpelainen, 1986, Deng, 1996, Tessier & Cáceres, 2004), food levels (Epp, 1996), toxins (Baird et al., 1991, Hietala et al., 1997), predator kairomones (Spitze, 1992, Weber & Declerck, 1997, Weber & Van Noordwijk, 2002, Michels & De Meester, 2004, Pauwels et al., 2005), and temperature (Loaring & Hebert, 1981, Mitchell & Lampert, 2000).

The permanent lake environment of some *Daphnia* species has extended periods of a consistent environment, during which selection may act as a sieve against clones maladapted to the particular environment (De Meester, 1996). The ephemeral pond environment of *D. pulex*, on the other hand, is one that is not only spatially but also temporally heterogeneous. Both during a season and across seasons, a pond's environment will change with respect to food levels, temperature, crowding, parasite levels, photoperiod, and so on. Each genotype's fitness will vary with its environment (Kojima, 1971), with interactions between multiple environmental stimuli affecting genotypes differently (Deng, 1996, Hietala et al., 1997). There are therefore opportunities for multiple clonal strategies to be selected for, each being competitively superior in a different environment.

There is also the possibility that many of the clonal strategies found in nature and in this experiment are not adaptive at all. Each season resting eggs hatch that may not be adaptive in the current environment (Berg et al., 2001, Mitchell et al., 2004). Selection against a genotype's life-history strategy, whether originating from egg bank hatchings or mutations, may take many generations before the genotype is eliminated from the population (Lynch et al., 1998). The natural boom-and-bust cycles of ephemeral pond populations (Dudycha, 2004) are also likely to weaken selection, with most individuals thriving during the boom and dying during the bust periods regardless of genotype (Nelson et al., 2005). Negative frequency dependent selection may also favour the rare phenotype even if the common phenotype is normally most effective, such as predator-mediated selection on multiple coexisting predator-avoidance strategies (Weber & Van Noordwijk, 2002). Thus *D. pulex* clonal coexistence can be explained in large part by differential resource use, differential environment optima, and continued persistence of maladaptive genotypes.

3.5.7 Research implications and directions

This experiment and others like it have provided ample evidence for different genotypes of *Daphnia* responding differently to stimuli. A great deal of published research ignores inter-genotype variation, drawing species-wide conclusions from a single genotype of *Daphnia* (e.g., Kleiven et al., 1992, Alekseev & Lampert, 2001, LaMontagne & McCauley, 2001, Weetman & Atkinson, 2002, Kessler & Lampert, 2004, Mikulski et al., 2005). Drawing species-wide conclusions from multiple replicates of the same genotype can be considered a form of pseudoreplication, and should be avoided (Hurlbert,

1984). Testing regulations of freshwater effluent established by governments worldwide require no more than one genotype of *Daphnia* to be used (OECD, 1998, Environment Canada, 2000, USEPA, 2002). In fact, some researchers and regulations have advocated the widespread use of a specific genotype to aid repeatability between laboratories (Baird et al., 1991, OECD, 1998).

By allowing tests to be conducted on a single genotype, government regulations are making an improper assumption of low inter-genotype variation. If repeatability is the justification for these regulations, then universal usage of a specific *battery* of genotypes would improve upon current regulations' repeatability. If labour costs to company laboratories for using several instead of single genotypes is the rationale behind current regulations, then we must re-examine the value we place on the well-being of our aquatic environment (De Meester & Declerck, 2005).

The findings of this investigation may apply to several fields of research including: life-history tradeoffs, diapause investment, sex *vs* asex, intra-clonal variation, effects of stresses on sex ratios, and the impact of stresses on a biological community. Future research on the ecology, ethology, and evolution of *Daphnia* should thoroughly explore both sides of genotype x environment interactions. The present study observed neonate and ephippia production among six genotypes in an environment that varied only in density. To study more effects on more genotypes in multiple environments would vastly add to our understanding of biological systems.

3.6 CONCLUSIONS

The results of this study contribute to our knowledge of life-history strategies of *Daphnia*. Six genotypes of *D. pulex* were raised under two different density regimes, and reproductive output observed. My conclusions are as follows:

1. Crowded individuals invested more in resting eggs, and less in neonates.
2. Obligately parthenogenetic genotypes, like facultatively parthenogenetic genotypes, can modify male production depending upon their density condition.
3. Inter-genotype variation was significant for both mode and amount of reproduction within density conditions.
4. Responses to density conditions differed significantly among genotypes, especially with respect to offspring sex ratios.
5. Significant genotype x environment effects demonstrate the necessity of research using several genotypes and several environments to make broad conclusions with confidence.

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CHAPTER 4: Implications and proposals arising from my research

4.1 SUMMARY

In Chapter 3, I briefly discussed the shortcomings of *Daphnia* research that assume species-wide ubiquity from single genotypes and environments. Both sides of the genotype x environment interaction should be more fully explored. This chapter can be divided into two sections. The first thoroughly outlines my criticism of single-genotype research, especially among government regulations for freshwater effluent testing. I propose an alternative procedure that should improve validity and repeatability. In the second section of this chapter, I propose a large life-history experiment on *D. pulex*. This experiment would be a grand undertaking, the results of which may be valued by many researchers.

4.2 CONSTRUCTIVE CRITICISM OF SINGLE-GENOTYPE RESEARCH ON *D. PULEX*

My research and that of others provides ample evidence that different genotypes of *D. pulex* respond differently to stimuli. However intuitive inter-clonal variation should be, it is neglected by too many laboratories and research protocols. The ability of *Daphnia* to clone themselves is an attribute that makes them a useful model organism (Colbourne et al., 2005), but also one that allows shortcuts to be taken. Too many articles have been published that infer species-wide conclusions from a single genotype of *Daphnia* (e.g., Kleiven et al., 1992, Alekseev & Lampert, 2001, LaMontagne & McCauley, 2001, Weetman & Atkinson, 2002, Kessler & Lampert, 2004, Mikulski et al., 2005, Rinke & Vijverberg, 2005). These extrapolations can sometimes be true; mortality

from toxin exposure is likely to hold true across most genotypes within a species, albeit likely at varying concentrations. Specific environmental interactions, life-history strategies, and concentration level thresholds, however, have been inferred from experiments using a single genotype, which is a practice of questionable prudence. Research conducted on a single genotype of other species, even with multiple independent replication, is only considered acceptable under extraordinary circumstances such as rare mutations. A lower standard should not apply to *Daphnia* research.

4.2.1 Shortcomings of environmental testing procedures

Daphnia is a favourite model organism for aquatic toxicology research, and the use of a single genotype is commonplace (e.g., Zhang & Baer, 2000, Hanazato & Hirokawa, 2004, Kashian & Dodson, 2004, Smolders et al., 2005). Testing regulations of freshwater effluent established by governments worldwide require no more than one genotype to be used (OECD, 1998, Environment Canada, 2000, USEPA, 2002). In fact, some researchers and regulations have advocated the widespread use of a specific clone to aid repeatability between laboratories (Baird et al., 1991, OECD, 1998).

The underlying sentiment behind advocacy of single-genotype testing must be to reduce variation caused by differences between genotypes. This reasoning is logical, but fails the purpose of effluent testing. If the purpose of testing effluent is to evaluate its impact on local fauna, then tests using a variety of local genotypes would be optimal. This would, however, limit repeatability across laboratories, among several concerns relating to the use of local organisms (USEPA, 2002 section 6.2.3.1). Thus there is a tradeoff between applicability of tests to local fauna and repeatability across laboratories.

4.2.2 A proposal to improve government testing protocols

A solution to this tradeoff can be easily reached. A small number (~10) of genotypes could be established for each country or continent (e.g., Canada, European Union, etc.), stock supplies of which would be available from sources within or approved by the government. Government regulations would require tests to use this battery of genotypes, unless use of others is justified. Although these genotypes would likely not have originated from the locale into which the effluent will be dumped, they would have at least originated from the appropriate country / continent, which is not necessarily the case at present. Test results could be easily verified by other laboratories, provided the current stringent guidelines for temperature, feeding, etc. are kept to aid repeatability (OECD, 1998, Environment Canada, 2000, USEPA, 2002). Results may differ in alternative environmental regimes, but to test for every scenario might be overly labourious.

4.2.3 Justification for proposing policy improvement

Currently it is not commonplace for academic researchers to advocate policy changes as a result of their research, as I am doing. Several prominent freshwater biologists have recently called on their colleagues to take a more active role in linking research with policy (De Meester & Declerck, 2005, Franklin, 2005). My research, as well as that of many others (Spitze, 1992, Innes & Singleton, 1994, Weber & Van Noordwijk, 2002), suggests that inter-genotype variation prevents prudent extrapolation from behavioural and life-history research using a single genotype of *Daphnia*. Others have found similar inter-genotype variation in toxicology research (Baird et al., 1989,

Barata et al., 2002). By allowing tests to be conducted on a single genotype, government regulations are thus making an improper assumption / shortcut. If repeatability is the defence of these regulations, then universal usage of a specific *battery* of genotypes would improve upon current regulations' repeatability. If labour costs to company laboratories for using several instead of single genotypes is the rationale behind current regulations, then we must re-examine the value we give to the well-being of our aquatic environment (De Meester & Declerck, 2005).

4.3 LIFE-HISTORY EXPERIMENT PROPOSAL

Future research on the ecology, ethology, and evolution of *Daphnia* should thoroughly explore both sides of genotype x environment interactions. The present study observed neonate and ephippia production among six genotypes in an environment that varied only in density. My observations amount to a mere peek into the complete life-histories of these animals. To study more effects on more genotypes in multiple environments would reveal a more complete and accurate picture of life-history strategies.

Each environmental variable affecting *D. pulex* (temperature, predator kairomone level, etc.) can be visualized as an axis in multi-dimensional space. Genotypes will vary in their life-history response and fitness at each point in this space. I propose that a grand investigation be conducted on the effects of multiple environmental conditions on several genotypes of *D. pulex*. The environmental variables available to manipulate include: photoperiod, temperature, pH, salinity, concentrations of various natural toxins, calcium

concentration, density of daphnids, food composition and abundance, kairomones of various predators, and exposure to various parasites. The responses of daphnids to be observed include: egg mass and size, neonate mass and size, size and mass at maturity, size at death, age at maturity, instar duration, lifespan, brood sizes, sex ratio, carapace thickness, ephippia pigmentation, phototactic behaviour, small-scale swimming behaviour, and heat-shock protein expression levels.

To conduct a grand experiment with all of the above parameters would be a huge undertaking requiring much labour and time (but little technology). To undertake such an investigation solely in the interest of *Daphnia* research would be of questionable merit. I would argue that *D. pulex* be used as a model organism to model life-history strategies and tradeoffs, and that *D. pulex* would make a more suitable organism than almost any other for such data-gathering. Its clonal capabilities allow essentially the same genotypes to be tested in multiple conditions. Inbreeding or genetic manipulation is required for the same feat among strictly sexual species. Among asexual animal species, few are as commonly and easily reared in the laboratory, and the literature on *D. pulex* ecology and evolution is unmatched. The *D. pulex* genome will soon be sequenced (http://daphnia.cgb.indiana.edu), which will allow unprecedented investigation of the genetics behind life-history traits (Colbourne et al., 2005).

There is reason to believe that the results of such an investigation would be applicable to nature from the laboratory. Other research has found lab results for *D. pulex* to agree with field results (Innes, 1997, Tessier & Cáceres, 2004). This would be aided by realistic stimulus levels that may be encountered in an organism's natural habitat (Korpelainen, 1986).

Many basic biological questions could be addressed with such an investigation. What is the tradeoff between reproduction and growth (Wheelwright & Logan, 2004)? Do genotypes from ponds with fish respond differently to fish kairomones than naïve genotypes (Boersma et al., 1998)? What is the tradeoff between responses to stresses and reproductive investment (Weber & Declerck, 1997)? Under what conditions do mothers adjust their offspring sex ratio in favour of males (Trivers & Willard, 1973, Kobayashi et al., 2003, Suorsa et al., 2003)? More questions can be imagined, and even more are possible if genetic analyses are integrated with the investigation.

Although the hypotheses surrounding this investigation are exciting, they are not essential to the success of the project. Like genome projects, surveys of the stars, and mapping the oceanic floor, this mega-project would not be strictly hypothesis-driven. The purpose of this project would be to map life's responses to environmental variables, using a system that allows many copies of the same genotype to be used. Predator-prey researchers would use the data set for their purposes, and sex ratio researchers for their own. Hitherto unknown trends may be detected that open novel avenues of investigation, as happened in 2003 when the human Y chromosome sequence revealed palindromes of unforseen size and yet-unknown function (Hawley, 2003, Rozen et al., 2003, Skaletsky et al., 2003).

In addition to collecting massive amounts of data, mega-projects also confer the benefits of increased exposure, prestige, and often funding to a field of research (Godfray, 2002). The project I have proposed would collect a large amount of life-history data at a fraction of the cost of other large projects. I believe the ultimate goal of life-history research to be a noble one: to tell the complete story of life. The project I have proposed

would certainly fall short of that goal, but I sincerely believe the field of life-history research would benefit from the data and exposure such a project would bring.

4.4 THESIS CONCLUSIONS

The results of my research contribute to our knowledge of life-history strategies of *Daphnia*, and their influences. My main conclusions are as follows:

1. There is no evidence of *Wolbachia* infection among Great Lakes area populations of *D. pulex*.
2. Among the genotypes used in Chapter 3, crowded individuals invested more in resting eggs, and less in neonates.
3. I found increased evidence for *D. pulex* sex determination to involve a combination of environmental and genetic influences.
4. Genotypes differ significantly in both their mode and amount of reproduction within density conditions.
5. Responses to density conditions differ significantly between genotypes, especially with respect to offspring sex ratios among the genotypes I tested in Chapter 3.
6. Significant genotype x environment effects demonstrate the necessity of research using several genotypes and several environments to make broad conclusions with confidence.

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