EVOLUTION OF REPRODUCTION AND STRESS TOLERANCE IN BRACHIONID ROTIFERS

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EVOLUTION OF REPRODUCTION AND STRESS TOLERANCE IN BRACHIONID ROTIFERS

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To Ginny and Dale Smith.

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SUMMARY

Stress can be a driving force for new evolutionary changes leading to local adaptation, or may be responded to with pre-existing, ancestral tolerance mechanisms. Using brachionid rotifers (microzooplankton) as a study system, I demonstrate roles of both conserved physiological mechanisms (heat shock protein induction) and rapid evolution of traits in response to ecologically relevant stressors such as temperature and hydroperiod. Rapid evolution of higher levels of sex and dormancy in cultures mimicking temporary waters represents an eco-evolutionary dynamic, with trait evolution feeding back into effects on ecology (i.e., reduced population growth). I also reveal that prolonged culture in a benign laboratory environment leads to evolution of increased lifespan and fecundity, perhaps due to reduction of extrinsic mortality factors. Potential mechanisms (e.g., hormonal signals) are suggested that may control evolvability of facets of the stress response. Due to prior studies suggesting a role of progesterone signaling in rotifer sex and dormancy, the *membrane associated progesterone receptor* is assayed as a candidate gene that could show positive selection indicating rapid divergence. Despite some sequence variation that may contribute to functional differences among species, results indicate this hormone receptor is under purifying selection.

Detailed analyses of multiple stress responses and their evolution as performed here will be imperative to understanding current patterns of local adaptation and trait-environment correlations. Such research also is key to predicting persistence of species upon introduction to novel habitats and exposure to new stressors (e.g., warming due to climate change). Perhaps one of the most intriguing results of this dissertation is the

rapid, adaptive change in levels of sex and dormancy in a metazoan through new mutations or re-arrangements of the genetic material. This suggests species may be able to rapidly evolve tolerance of new stressors, even if standing genetic variation does not currently encompass the suite of alleles necessary for survival.

CHAPTER 1

INTRODUCTION

Environmental Stress and the Biota, with an Emphasis on Thermal Stress and Hydroperiod

Stress is recognized as an important force structuring ecosystems and driving evolution, imposing selection pressure for adaptations to increase fitness and affecting persistence of species and populations (Parsons 1991, Chippindale et al. 1998, Bijlsma and Loeschcke 2005, Sabo and Post 2008). Stress responses determine limits of species' tolerance ranges for environmental conditions and extremes (Roelofs et al. 2008). Due to different physiological tolerances among taxa, what constitutes stress varies among species and can be difficult to define (Bijlsma and Loeschcke 2005, Liancourt et al. 2005). Typical expectations are for organisms to be most stressed under conditions outside those normally experienced in their environment (Bijlsma and Loeschcke 2005). However, frequent exposure to environmental conditions does not guarantee that those conditions are not perceived as stressful. For example, organisms in the highly variable intertidal region are exposed to greater thermal fluctuations than subtidal taxa, but still induce a heat shock response at temperatures experienced in their natural habitat (Tomanek 2010). Here I define stress as anything causing a reduction in performance, growth and reproduction, or viability—something endangering the function or survival of organisms or causing a harmful change (Bradshaw and Hardwick 1989, Parsons 1991, Schimel et al. 2007, Sabo and Post 2008). Specifically I focus on externally imposed environmental stressors.

Studies of stress tolerance are particularly timely considering increasing anthropogenic changes to the environment. Stressful conditions may include fluctuating but predictable environmental changes such as salinity shifts in estuaries (Elliott and Quintino 2007), or atypical conditions like those encountered upon migration to a new environment (Ghalambor et al. 2007). Stress also includes pulse events resulting in mortality and thereby potentially clearing space for new colonizers; such disturbances may alter diversity, food chain length, and ecosystem processes (Connell 1978, Schimel et al. 2007, Sabo and Post 2008). Anthropogenic influences can add new sources of stress or exacerbate natural ones by altering or destroying habitats. In toads, elevated levels of the stress hormone corticosterone are associated with low habitat availability or high levels of fragmentation (Janin et al. 2011). Anthropogenic effects can indirectly impose stressful conditions as well, exemplified by human-mediated increases in greenhouse gases that are contributing to climate change (Crowley 2000). Elevated carbon dioxide levels and climate change are predicted to impact an array of environmental factors including temperature, precipitation and drought events, and ocean acidification; these in turn can affect species' survival and distribution (Pearson and Dawson 2003, Brooks 2004, Reusch and Wood 2007, Hofmann and Todgham 2010).

Temperature constitutes one of the most obvious environmental conditions altered by climate change (Reusch and Wood 2007, Hofmann and Todgham 2010). Research has already documented effects of global warming on ecosystems and the biota, including altered phenology—the timing of seasonal events such as flowering in plants, and egg-laying in birds (Dunn and Winkler 1999, Hughes 2000, McCarty 2001, Miller-Rushing and Primack 2008). In Lake Baikal, warming over the past 60 years is associated with

shifts in the planktonic community, including over a 300% increase in cladoceran density (Hampton et al. 2008). Thermal regimes and climate change influence a variety of properties from shifts in species distributions to individual physiology (Harley et al. 2006, Hofmann and Todgham 2010). At the cellular level, elevated temperatures can alter enzymatic reaction rates, or denature proteins and disrupt membrane fluidity (Hofmann and Todgham 2010). Furthermore, thermal shifts exert indirect effects by modifying other environmental conditions. In aquatic habitats altered temperature and precipitation patterns as anticipated under climate change can influence hydroperiod, the length of inundation in temporary waters such as vernal pools (Brooks 2004, 2009).

In addition to temperature, hydroperiod presents a key source of stress. For both terrestrial and aquatic taxa, dehydration is listed as one of the most critical forms of stress (Watanabe 2006), and for aquatic species habitat desiccation can lead to mortality. Hydroperiod is one of the most important forces structuring communities in temporary pools (De Meester et al. 2005, Jocque et al. 2010). Hydroperiod impacts community composition and distribution of a diverse range of taxa, including aquatic macrophytes, macro- and microinvertebrates, and amphibians (Schneider and Frost 1996, Snodgrass et al. 2000, Brock et al. 2003, Céréghino et al. 2008). Typically, species richness is higher in more permanent systems, but ephemeral waters may house rare species (Collinson et al. 1995, Wellborn et al. 1996, Serrano and Fahd 2005, Frisch et al. 2006, Fahd et al. 2007). Hydroperiod also affects the relative importance of biotic interactions; higher abundance and diversity of predators can increase the role of predation in more permanent water bodies (Schneider and Frost 1996, Wissinger et al. 2003, De Meester et al. 2005, Marion and Hay 2011).

Stress Tolerance and Evolution of Adaptations

Stressful conditions may exceed a species' tolerance limits resulting in extirpation or emigration, but some individuals may persist through phenotypic plasticity (Piersma and Drent 2003), physiological stress response mechanisms (Sørensen 2010), or life history adaptations such as dormancy (Cáceres 1997, Brock et al. 2003). Tolerance mechanisms of organisms are wide-ranging. Responses to stress include changes in gene expression, homeostasis and folding of proteins, organization of the cytoskeleton, metabolic activity, and progression through the cell cycle (Kültz 2005, de Nadal et al. 2011). Gene transcription can be up- and/or down-regulated within minutes of stress exposure (de Nadal et al. 2011). Associations of traits with environmental gradients may suggest evolved adaptations; indeed, most research on evolved responses to climate change consists of correlative studies (Reusch and Wood 2007).

Commonalities in the stress response have been studied at the level of evolutionary conservation across taxa, as well as responses to different types of stressors. It has been suggested that signal transcription factors differ more between soil invertebrates and plants than effector genes in the stress response, with effector (endpoint) genes including heat shock proteins that act as molecular chaperones (Roelofs et al. 2008). In contrast, in a review focusing on yeast, *Drosophila melanogaster*, and mammals, de Nadal and colleagues (2011) propose stress sensors and effectors are less conserved than signal transduction pathways such as stress-activated protein kinase pathways. In comparing responses to different types of stressors experienced by soil invertebrates and plants, Roelofs et al. (2008) report that greater similarities exist in genomic and transcriptomic regulation in response to drought, salinity, and cold, with

heavy metals or heat affecting more distinct molecular pathways. Genes involved in oxidative stress responses are affected by several different types of stressors (e.g., hot or cold temperatures) (Roelofs et al. 2008). Research spanning archaea, eubacteria, yeast, and humans pinpoints several molecular chaperones including heat shock proteins (e.g., DNAJ/HSP40, HSP60 chaperonin, and HSP70) as part of the minimal stress proteome, a suite of universally conserved proteins implicated in the cellular stress response (Kültz 2005). Heat shock proteins facilitate survival of several stressors besides heat (Feder and Hofmann 1999, Sørensen 2010). Despite these attempts to elucidate conservation or divergence of responses across different stressors, and by different organisms, this remains a very broad field of research requiring more information to facilitate comparisons.

In addition to cellular physiological responses, adaptations to stress include life history modifications and dormancy. In temporary waters, organisms from aquatic insects in the larval stage to zooplankton and fishes tend to show accelerated development and shortened life cycles compared to their permanent water counterparts (Valdesalici and Cellerino 2003, Wissinger et al. 2003, Suhling et al. 2005, Schröder et al. 2007). More rapid development is thought to be adaptive, allowing completion of the life cycle before habitats dry. When spatial migration is not possible and adults cannot survive in the habitat, dormancy provides a means for persistence (Cáceres 1997). Dormancy—a resting stage comprised of arrested development and/or metabolism—can be subdivided into quiescence (initiated and maintained by external conditions) and diapause (regulated by internal physiology) (Hand 1991, Cáceres 1997). In ephemeral habitats diapause is employed by plants and many invertebrate phyla, including sponges, zooplankton,

insects, and even higher taxa such as some fishes (Cáceres 1997, Hand and Podrabsky 2000, Simon et al. 2002, Brock et al. 2003, Valdesalici and Cellerino 2003, Watanabe 2006). Diapause is often associated with sex, particularly among facultative or cyclical parthenogens that alternate between asexual and sexual reproduction (Simon et al. 2002, Brock et al. 2003, Serra and Snell 2009). Sex itself may foster survival in changing or heterogeneous environments, for example, by providing new genetic variation through meiotic recombination and segregation (Hurst and Peck 1996, Burt 2000, Becks and Agrawal 2010). The association between sex and dormancy also may lead to eco-evolutionary feedbacks, whereby ecology influences evolution, which in turn impacts ecological dynamics (Schoener 2011). For instance, ecological factors such as stress levels may influence the evolution of sex, and ultimately incur a cost to population growth (Stelzer 2012).

Associations of species or traits with stressful habitat conditions may reflect species selection or sorting where environmental conditions filter species based on pre-existing adaptations or traits (Jablonski 2008), or local adaptation and evolution *in situ* (Kawecki and Ebert 2004). It is generally accepted that stress has a role in evolution; environmental stress has been defined by some as a driving force for evolved adaptations to environmental change (Parsons 1991, Bijlsma and Loeschcke 2005). Yet evolutionary mechanisms and causes of trait-environment correlations can be challenging to identify, and often are poorly understood (Badyaev 2005). Manipulative experiments can be used to test whether fitness differences across environmental gradients are due to local adaptation to a habitat feature of interest (Kawecki and Ebert 2004).

Experimental evolution research is beginning to shed light on the process of adaptation to stressful conditions and rapid evolution. In these studies, environmental conditions are manipulated but the researcher does not directly select for a specific adaptive trait; evolution results from differential fitness among individuals via natural selection (Buckling et al. 2009). Experimental evolution approaches have uncovered several instances of rapid evolution. Rapid evolution can be considered as contemporary evolutionary changes that occur swiftly enough to affect ecological dynamics (Hairston et al. 2005, Ellner et al. 2011). For instance, Fussmann et al. (2007b) suggest rapid evolution occurs within ~1000 generations. Rapid evolution in response to stress, and stress-induced hypermutation (elevated genomic mutation rates), are features of diverse bacteria and eukaryotes (Goldman and Travisano 2011, Ram and Hadany 2012). Using experimental evolution techniques, researchers can track the roles of either standing genetic variation or new mutations in providing the raw material for rapid evolution and stress tolerance. Several studies including both experimental evolution and theoretical approaches document an important role of initial levels of variation, and attribute rapid evolution of resistance to predators, parasites, or competitors to shifts in initial clone or genotype frequency (Duffy and Sivars-Becker 2007, Fussmann et al. 2007b, Becks et al. 2010, Turcotte et al. 2011). Other studies have tracked microbial evolution to abiotic stressors, including responses to thermal stress (Duncan et al. 2011), and *de novo* mutations facilitating rapid evolution of ultraviolet radiation tolerance (Goldman and Travisano 2011).

The ability for such evolutionary change can be described as evolvability (Pigliucci 2008), and research on the nature of evolvability may facilitate understanding

of the evolution of cellular and/or life history adaptations to stress. Here the definition of Colegrave and Collins (2008) is followed; these authors describe evolvability as the capacity to respond to the force of natural selection through creation and use of genetic variation. Typically, evolvability is thought to be augmented by modularity, robustness, and genetic exchange or recombination (Pigliucci 2008, Masel and Trotter 2010). Modularity may be viewed as independence of phenotypic characters or their underlying genetic networks, and robustness as the average effect of perturbations on phenotypes, with high robustness associated with greater resistance to changes (Pigliucci 2008, Masel and Trotter 2010). Nonetheless support for the role of these factors in evolvability is mixed. Some studies indicate stress response networks may be grouped into evolutionary modules, and others suggest that modularity may increase evolvability in some cases and be inversely related in others (Hansen 2003, Griswold 2006, Singh et al. 2008).

Invertebrate Study Systems and Evolution of Stress Tolerance

Invertebrates represent a particularly intriguing group for investigations of the stress response, both due to studies suggesting their capacity for rapid evolution, and the need for research on hitherto under-studied groups to advance understanding of general paradigms. Invertebrates outnumber vertebrates in sheer numbers of species and play important roles in ecosystem functioning (Wilson 1987). Their diverse life history strategies and typical ease of culture compared to vertebrates make them useful for manipulative assays of stress tolerance adaptations. Within the Bilateria, the Ecydsozoa (e.g., nematodes, arthropods), Deuterostomia (including echinoderms, tunicates, and chordates), and Lophotrochozoa or Spiralia (e.g., rotifers, mollusks) represent three superclades (Tessmar-Raible and Arendt 2003, Paps et al. 2009, Edgecombe et al. 2011).

Lophotrochozoans contain some genes once thought to be vertebrate-specific (Tessmar-Raible and Arendt 2003), and are said to be the most morphologically diverse bilaterian branch (Halanych and Borda 2009). Thus while the Lophotrochozoa have been under-studied compared to the other two superclades, research on this group may help address gaps in knowledge and understanding of evolutionary trajectories from more basal metazoans through such derived taxa as vertebrates (Tessmar-Raible and Arendt 2003).

The focal subjects of my dissertation are members of the *Brachionus plicatilis* species complex (Phylum Rotifera). Rotifers are zooplankton comprising over 2000 species of microscopic metazoans that live in freshwater and marine environments; though less common, their habitats also include thin water films as found on mosses and liverworts (Wallace and Smith 2009, Wallace and Snell 2010). Rotifers' breadth of habitats, from desert rock pools to pockets of melted water in ice (Wallace and Smith 2009), can present a variety of stressors such as temperature extremes and desiccation. Short lifespans around 10–20 d and ease of culture (Snell et al. 2012) facilitate use of brachionid rotifers in life history assays. Reports of rapid evolution in the group and eco-evolutionary dynamics recommend their utility for experimental evolution studies of the stress response (Fussmann 2011, Stelzer 2012). Brachionid rotifers are in subclass Monogononta, a group of cyclical parthenogens that primarily reproduce asexually, but through sex produce diapausing embryos capable of withstanding conditions (e.g., desiccation) lethal to active animals (Wallace and Smith 2009, Robles-Vargas and Snell 2010, Wallace and Snell 2010).

The overall aim of my research is to advance understanding of evolutionary trajectories including rates and drivers of evolution, with an emphasis on evolution of the

stress response and reproduction in brachionid rotifers. I hypothesize that the stress response in *Brachionus* spp. derives both from conserved stress response systems, as well as ability for rapid contemporary evolution facilitating local adaptation to new levels or types of stress.

In the second chapter, I posit that functional conservation of HSPs as part of a universal stress response manifests in a role of *hsp* genes in rotifer thermotolerance. Specifically, I test genes encoding members of the DNAJ/HSP40, HSP60 chaperonin, HSP70, and HSP90 protein families for their roles in brachionid heat shock survival. Detailed phylogenetic analysis of relatedness of the specific *hsp* genes assayed with thermoregulatory HSPs across eukaryotes is beyond the scope of this study, and will require future work to obtain the complete rotifer gene sequences. In general, members of these four HSP families are known to contribute to organisms' thermal stress response, but individual genes and families involved in thermotolerance vary among species (Guimarães et al. 2011, Hahn et al. 2011, Leggat et al. 2011, Mikulski et al. 2011, Miot et al. 2011). Differences even exist in *hsp* gene expression between coral-algal symbionts (Leggat et al. 2011). In rotifers heat shock proteins have been associated with various stressors ranging from temperature to toxic metals (Cochrane et al. 1991, Wheelock et al. 1999, Rios-Arana et al. 2005), but prior studies relied on correlations of stress exposure and protein expression. I test the role of multiple *hsp* genes, using interference RNA (Fire et al. 1998, Snell et al. 2011) to individually suppress *hsp* genes and assay their necessity for surviving heat shock.

In the third chapter, I test the ability for rapid evolution of adaptations to hydroperiod using laboratory cultures mimicking ephemeral or permanent ponds. This

experimental evolution study assesses life history traits, including the frequency of sex and diapausing embryo production, for adaptive changes. In the fourth chapter, I analyze rapid evolution of lifespan and fecundity in these experimental cultures in greater detail. I discuss controls on the evolvability of rotifer lifespan and reproduction, including the potential for hormonal regulation. Hormones are bioregulatory compounds that coordinate physiological processes (local, autocrine; adjacent, paracrine; or distant, endocrine) and alter cell function or gene transcription (Ketterson and Nolan 1999, Denver et al. 2009). These signaling molecules may possess key roles in evolvability.

In the fifth chapter I report on molecular evolution of a sex steroid receptor—the *membrane associated progesterone receptor* (*MAPR*). Exogenous additions of progesterone can increase production of brachionid diapausing embryos, and *MAPR* is the only known progesterone receptor gene in rotifers to date (Snell and DesRosiers 2008, Stout et al. 2010). Reproductive proteins often exhibit signs of rapid evolution (Civetta and Singh 1998, Swanson and Vacquier 2002); thus this hormone receptor was anticipated to show positive selection in the *Brachionus plicatilis* species complex. Finally, the sixth chapter presents conclusions and future directions. In all, this dissertation reports on ecology and evolution of the stress response in brachionid rotifers, including tests of both extant adaptations such as heat shock proteins, and the ability for rapid evolution upon exposure to or removal from environmental stressors.

CHAPTER 2

THREE HEAT SHOCK PROTEINS ARE ESSENTIAL FOR ROTIFER THERMOTOLERANCE

(This chapter has been published in the Journal of Experimental Marine Biology and Ecology at http://dx.doi.org/10.1016/j.jembe.2011.11.027.)

Abstract

Heat shock proteins (HSPs) are important molecules in the stress response of organisms from prokaryotes to mammals, and thus may be useful biomarkers for environmental stress. Here we characterize the functional roles of genes belonging to four distinct families of HSPs (*hsp40*, *hsp60*, *hsp70*, and *hsp90*) in the monogonont rotifer *Brachionus manjavacas*. Because *B. manjavacas* inhabits ponds of varying thermal regimes, including ephemeral ponds that may experience temperature fluctuations, HSP-mediated thermotolerance likely is important to its survival and adaptation. Using interference RNA (RNAi), we provide the first conclusive evidence that HSPs are required for rotifer survival following heat stress. Effective RNAi-mediated suppression of all *hsp* genes except *hsp90* was verified via quantitative PCR. *Hsp40, hsp60,* and *hsp70* are required for rotifer thermotolerance ($P<0.05$); however, our data do not indicate *hsp90* is essential. Quantitative PCR further revealed immediate up-regulation of *hsp40* mRNA following heat stress. Additionally, we demonstrated expression of *hsp40* mRNA in multiple tissues using fluorescent *in situ* hybridization. Our characterization of mRNA expression and functional roles for four distinct *hsp* genes provides a baseline for molecular-level comparisons of the stress response of rotifers with other taxonomic groups, and the technique for in-depth studies of the role of specific genes in rotifer stress

responses. Considering the potential for ambient temperatures to impact species survival, competitive interactions, and body size of individuals, thermotolerance may be an important influence on zooplankton community structure.

Introduction

Heat shock proteins (HSPs) comprise several families of constitutive and inducible proteins with conserved roles in both housekeeping and stress tolerance (Parsell and Lindquist 1993). Heat shock proteins promote stress survival by targeting damaged proteins for degradation or proteolysis, or by serving as molecular chaperones that aid refolding of proteins denatured by stresses such as heat shock (Parsell and Lindquist 1993, Feder and Hofmann 1999, Buckley et al. 2001). Numerous studies have demonstrated that the general role of HSPs in the stress response is conserved from prokaryotes to higher eukaryotes, but the specific genes involved and conditions for induction may vary among taxa (Parsell and Lindquist 1993, Feder and Hofmann 1999). Much of the foundational literature relies on protein electrophoresis or autoradiography studies, in which the proteins detected may constitute one or several HSPs of similar size (Feder and Hofmann 1999). More recently, studies have attributed specific HSPs and their encoding genes to particular stressors, and investigated the role of HSPs in adaptation of natural populations (Clegg et al. 2001, Tanguay et al. 2004, Sørensen 2010).

Rotifers are a group of micrometazoans for which in-depth studies are only beginning to reveal genes involved in the heat shock response. As basal consumers rotifers play an important role in aquatic food webs, forming a link between the microbial food loop and classical food web, and are found in diverse aquatic and limnoterrestrial

habitats (Wallace and Smith 2009). Thus, research on the ability of rotifers to withstand environmental perturbations may be important to forecasting the health of aquatic food webs. Moreover, in rotifers HSPs may serve as bioindicators for stressors ranging from heavy metal and crude oil pollution to thermal stress and ultraviolet radiation (Cochrane et al. 1991, Wheelock et al. 1999, Rios-Arana et al. 2005, Kim et al. 2011). In nature, stressful conditions such as heat exposure are associated with both active populations and dormant structures, e.g., the resting eggs (diapausing embryos) produced during sexual reproduction of cyclically parthenogenetic monogonont rotifers (Denekamp et al. 2009).

Thermal stress is particularly relevant to rotifer populations given they may experience thermal fluctuations, especially in shallow and temporary water bodies (Stemberger 1995, Denekamp et al. 2009, Dupuis and Hann 2009). Rotifers in the *Brachionus plicatilis* species complex inhabit brackish environments that may vary in hydroperiod, with habitats being wet permanently or only episodically (García-Roger et al. 2006). While this species can persist desiccation by entering a metabolically quiescent state as diapausing embryos, the embryos in dry sediment banks may be exposed to high temperatures (García-Roger et al. 2005). In the brine shrimp *Artemia*, higher HSP expression in encysted embryos has been linked to natural adaptation to warmer habitats (Clegg et al. 2001). In rotifers less is known of the role of HSPs in natural adaptation, but recent research shows *hsp* genes are expressed by diapausing embryos (Denekamp et al. 2009). In active populations, temperature preferences affect rotifers' ability to survive at different food concentrations and may impact competitive ability (Stelzer 1998, Ortells et al. 2003). Moreover, in aquatic systems higher temperatures are associated with shifts to smaller zooplankton individuals and species, which tend to be an inferior food source for

fish (Moore and Folt 1993). Considering the ramifications of thermal regimes for aquatic community structure, mechanisms such as the HSP pathways that regulate heat tolerance and potentially species survival are worthy of further study.

A major limitation of all HSP research to date in rotifers, and many studies in other taxa, is the restriction of functional studies to primarily correlation-based assays of expression. Recent development of methods to suppress gene expression in rotifers via interference RNA (RNAi) (Snell et al. 2011) provides a method we employ here to directly test the role of HSPs in thermotolerance. We individually suppress *hsps* belonging to four distinct families to compare their roles in survival following thermal stress. Because expression may vary across tissue types (Sørensen 2010), we performed fluorescent *in situ* hybridization (FISH) to investigate mRNA localization, and used quantitative PCR (qPCR) to assess thermally-induced up-regulation of one *hsp* gene. We hypothesized that all four *hsps* would be essential to survivorship following thermal stress, with mRNA induction following heat shock, and anticipated that expression would be localized to certain organs (e.g., reproductive organs critical to fitness).

Methods

Thermotolerance Bioassays

Interference RNA (RNAi) via transfection of rotifers with double-stranded RNA (dsRNA) synthesized *in vitro* was used to individually suppress one of four *hsps* (*hsp40*, *hsp60*, *hsp70*, and *hsp90*), or a non-*hsp* control (*actin*), following (Snell et al. 2011). All bioassays were conducted with *Brachionus manjavacas* (Russian strain), formerly *B. plicatilis* (Fontaneto et al. 2007). Culture conditions followed (Snell et al. 2011).

Our work extends the collection of *hsp* sequences for *Brachionus* in GenBank; most published sequences to date are from *Brachionus ibericus*. Primers for synthesis of *actin* and *hsp90* are as in (Snell et al. 2011). Primers for the three other *hsp* genes were designed in this study (Table 2.1) based on *hsp40* and *hsp60* sequences in *Brachionus* spp. expressed sequence tag (EST) libraries developed by D. Mark Welch and colleagues, e.g., (Suga et al. 2007), and the *hsp70-3* EST sequence in (Denekamp et al. 2009). PCR amplicons of all five genes were cloned and sequenced as in (Smith et al. 2011) to confirm amplification of the target genes from *B. manjavacas* genomic DNA (GenBank accessions HQ901982-HQ901986). Blastn searches of GenBank with our *hsp40, hsp60,* and *hsp90* sequences revealed >80% identity to *Brachionus ibericus* sequences for *hsp40, hsp60,* and *hsp90α1* (Kim et al. 2011), but did not yield a DNA sequence for a *Brachionus* homolog of *hsp70-3*. Lack of brachionid *hsp* sequences in GenBank limits comparisons within the genus, but comparison with other organisms confirms the identity of genes studied here. Blastn matches of *hsp40* with ≥80% identity and E-values ≤6e-06 include *hsp40/dnaJ* homologs in the silk moth *Bombyx mori* and pea aphid *Acrythosiphon pisum*. For *hsp60*, blastn matches with >80% identity and E-values ≤3e-20 include homologs to *hsp60* in the chironomid *Polypedium vanderplanki* and snail *Biomphalaria glabrata*. For *hsp90*, the sequence in *B. manjavacas* already has been published (Snell et al. 2011); a blastn search reveals a match to *hsp90* from the planarian *Dugesia japonica* with 76% identity and an E-value of 8e-61. Our *hsp70-3* sequence matches the *B. plicatilis hsp70-3* EST of (Denekamp et al. 2009), GenBank accession FM930314, with 89% identity, and an E-value of 5e-160.

Table 2.1. Primers and GenBank accession references for genes in this study. Sizes are internal to PCR primers. For T7 PCR, the PCR primers were preceded at the 5' end with the bacterial T7 promoter sequence TAATACGACTCACTATAGG.

Gene	PCR primers (5'-3')	GenBank accession	Size (bp)	qPCR primers (5'-3')
hsp40	for: GGCTTTGACTGGTTTCAAAC rev: TGGCTAGCACATTGAACTCG	HQ901983	347	for: TGCTCTTCAGCATCGACAGG rev: GCAAAATGGCGAAATTGAAC
hsp60	for: GAAAGATTGGCCAAATTGAG rev: TCTTTGCTGGCATCATAACC	HQ901985	313	for: GGCATTTTGAGAGCTCGTCG rev: GAAGGTATCGTCCCAGGTGG
hsp70-3	for: CATCACCCTTAGCTGGAAAGG rev: AAGAAGGTCGATGCTTTTGG	HQ901984	431	for: TGCCCAGGGAGAAAATAGTG rev: GGCAACTTCAACCTCAAAGG
hsp90	for: ACCGACCCCATTGACAAGTA rev: CAATCGGATGGTCAGGATTT	HQ901986	326	for: GGAGTTGCCAGAAGACGAAG rev: GCAGCCCATCCATACTGACT
actin	for: CCGCGACCTGACTGACTATT rev: GCTTCGAAATCCACATGCTT	HQ901982	493	for: GCATCCACGAGACCACCTAT rev: TAGGATCGAACCACCAATCC

For each gene, approximately 60 neonate (24 h post-hatch, previously maintained at 25°C) females transfected with dsRNA for a single gene (Snell et al. 2011) were transferred into 0.6 mL thin-walled PCR tubes in ca. 100 µL of 15 ppt artificial seawater (ASW; Instant Ocean salts). Tubes were incubated at 40°C for 1 h for heat shock exposure; heat shock at this temperature has been shown to induce significant mortality in the closely related congener *B. plicatilis* (Wheelock et al. 1999). After exposure, rotifers were incubated in 15 ppt ASW at 25°C for 24 h, and percent mortality was recorded. Initially, four separate heat shock exposures were conducted on rotifers that had been transfected with dsRNA for the *hsp60* or *actin* gene. Mortality between animals transfected with *hsp60* versus *actin* was compared by a paired t-test, with pairs constituting percent mortality for *hsp60* and *actin* treatments in each of the bioassays (N=4). Verification of impacts on mortality (see Results) motivated investigation of other *hsps*. In these subsequent bioassays rotifers were transfected with *hsp40*, *hsp70-3*, *hsp90*, or *actin*; results were analyzed with a one-way ANOVA, and Dunnett t-tests for *post-hoc* comparisons. Because assays of *hsp40*, *hsp70-3*, and *hsp90* were conducted later we

analyzed the results independently from that of the original *hsp60* bioassays to account for any unknown, uncontrollable variables, and compared their effects on survivorship to that of *actin* control treatments performed simultaneously. All statistical analyses were conducted in IBM SPSS v. 18 on the arcsine square root transformed percentage mortality.

Fluorescent *In Situ* Hybridization

To identify localized regions of *hsp40* expression within rotifers, fluorescein-labeled dsRNA probes were prepared for each gene using T7 PCR product as the DNA template; T7 product was made as in (Snell et al. 2011). Probe was synthesized in a final volume of 19.5 µL with 10 µL of T7 template, 40 U T7 RNA Polymerase (Promega), 1X transcription buffer (Promega), 1X DIG RNA labeling mix (Roche), and 7.69 mM DDT (final concentrations). After a 2 h incubation at 37°C, dsRNA was ethanol precipitated, and re-suspended in 5 µL RNase-free water. Probes were prepared separately for both *actin* and *hsp40* genes.

The protocol for hybridizing the probe to animals and solution preparation was adopted from (Tautz 2000), with minor modifications as follows. Briefly, for each gene probe (*hsp40* or *actin*) *B. manjavacas* neonate females 24 h post-hatch were exposed to 1 h heat shock at 40°C, or as a non-heat shock control left at room temperature (~22°C) in 15 ppt ASW. Subsequent steps were performed separately on heat-treated and control females. Immediately after the 1 h exposure, animals in ASW were prepared for hybridization by anesthetizing ~40 females with club soda, then fixing them with formalin (~1%). For prehybridization, ASW was replaced with 200 µL phosphate buffered Tween 20 (PBT); subsequently another 200 µL PBT containing 30 µg/mL

Proteinase K was added. After an incubation of 2–5 min. at room temperature, the reaction was terminated by addition of 200 µL PBT containing 2 mg/mL glycine. For hybridization, animals were washed for 10 min. in hybridization solution. Rotifers were heated in the solution for 20 min. at 45°C. Meanwhile, 2 µL of the dsRNA fluorescein probe was added to a 5 µL solution of hybridization buffer containing 2 mg/mL sonicated salmon sperm DNA. Probe and sperm DNA were denatured 3 min. at 100°C, cooled on ice, then added to the rotifers for a 2 h hybridization at 65°C. To wash, supernatant was replaced with 400 µL pre-warmed hybridization solution, and incubated 10 min. at 65°C. The process was repeated for a second wash with 400 µL PBT.

For analysis, washed females were transferred to a microscope slide for visualization on a BH-2 Olympus compound microscope (100X magnification) with a fluorescein filter. Fluorescent pixel intensity was quantified in the corona (apical ciliated organ for motion and sensory perception), ovary/vitellarium, and developing oocytes within females using ImageJ (http://rsbweb.nih.gov/ij/). To account for background fluorescence, a control probe was prepared and hybridized to another ~40 females as above, but using 10 µL sterile water in lieu of T7 template DNA during probe synthesis. Hence, any fluorescent activity from this reaction would be attributed to carry-over and incorporation of individual DIG-labeled ribonucleotides. Mean pixel intensity for the water control in each tissue region, averaged for 8–10 females, was subtracted from pixel intensity in the corresponding tissue for each female hybridized to *actin* or *hsp40* dsRNA probe as a normalization step. Experiments were repeated three times for each gene probe and temperature exposure (~22°C room temperature control, or 40°C heat shock).

Images were assessed for fluorescent pixel intensity of *hsp40* or *actin* in each of the three tissue regions with a MANOVA, performed in SPSS.

Quantitative PCR

Quantitative PCR of individual rotifers was used to confirm that *hsp* expression was indeed suppressed by RNAi, and to determine whether differential expression of rotifer *hsp* genes was elicited in response to thermal stress. To validate RNAi suppression of *hsp* expression, *B. manjavacas* females were transfected as described above with dsRNA to initiate RNAi, or with phosphate buffered saline (PBS) as a control. Transfected neonates (ca. 24 h old) were maintained at 25°C with no heat shock. In a separate experiment to evaluate changes in *hsp* expression in response to thermal stress, neonate females were either exposed to a 1 h 40°C heat shock or kept for 1 h at room temperature (~22°C, no heat shock control). These rotifers were not transfected, so *hsp* activity was not artificially suppressed.

Individual rotifers were preserved in 5 µl RNAlater (Qiagen) and total RNA was extracted from each rotifer using the RNeasy Micro Kit (Qiagen) following modifications in (Snell et al. 2011). The qPCR primers for *actin* and *hsp90* as well as qPCR conditions using EXPRESS One-Step SYBR GreenER Kit (Invitrogen) followed (Snell et al. 2011), and primers for *hsp40*, *hsp60* and *hsp70-3* were designed based on EST sequences as above (Table 2.1). Amplification and detection of target (*hsp*) and reference housekeeping (*actin*) genes were conducted using a Mastercycler Realplex 2 (Eppendorf) for 12 replicate rotifers for each treatment and corresponding control. Expression levels of target genes were normalized to *actin* levels. Relative expression of target genes in target dsRNA-transfected rotifers versus PBS control rotifers (validation of RNAi

knockdown), or heat-shocked rotifers versus room temperature controls (stress response induction), was determined using the ΔΔCT method (Livak and Schmittgen 2001). Statistics also were performed on ΔC_T values (Yuan et al. 2006), using t-tests to reveal significant differences in expression of treated rotifers relative to controls. Relative expression was used to calculate percent knockdown (% KD) calculated as: % KD = $(1 - 2^{-\Delta\Delta C_T}) \times 100$ (Cheng et al. 2008).

Results

Thermotolerance Bioassays

Significantly higher mortality after heat shock occurred in rotifers transfected with dsRNA for *hsp60* compared to *actin* (one tail t-test, P=0.030; Figure 2.1). Likewise, one-way ANOVA for bioassays on the remaining three *hsps* demonstrated a significant effect of the gene used in transfections on survivorship (P=0.004). *Post-hoc* Dunnett t-tests (one-tail) comparing *hsp* to *actin* transfections revealed the effect was attributed to knockdown of *hsp40* (P=0.004) and *hsp70-3* (P=0.001), with no significant effect of *hsp90* (P=0.075).

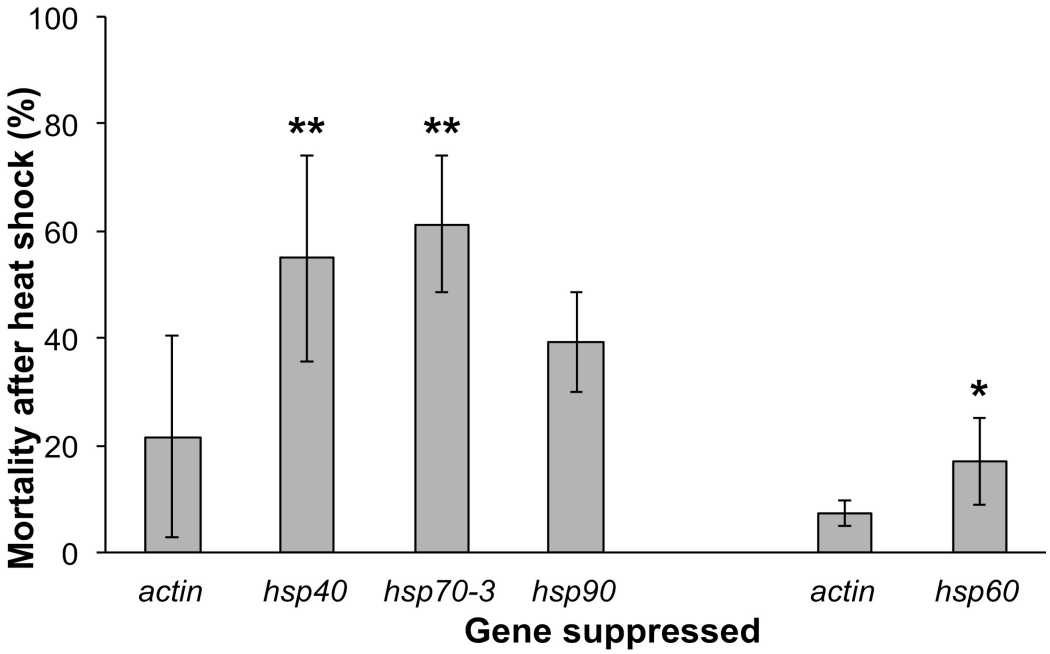

Figure 2.1. Mean mortality and standard deviation 24 h following heat shock in rotifers transfected with *actin* (control) or *hsp* genes. Significantly higher mortality from *hsp* relative to *actin* transfectants was compared by a t-test (four bioassays of *actin* versus *hsp60*) (right side) or ANOVA with Dunnett t-tests (five bioassays of *actin* versus *hsp40*, *hsp70-3*, and *hsp90*) (left side). Significant results are indicated as * (P<0.05) or ** (P<0.01).

Fluorescent *In Situ* Hybridization

Both *hsp40* and *actin* mRNA were expressed in the corona, ovary/vitellarium, and internal developing oocytes of the rotifers. MANOVA comparing the treatment of heat shock and no heat shock on expression of *hsp40* or *actin* did not show a significant effect of gene, nor an interaction effect of heat treatment and gene, on mRNA levels (fluorescent pixel intensity) in the three aforementioned tissue regions. However, heat treatment did significantly affect mRNA levels (P=0.01). *Post-hoc* univariate F tests to examine the effect of treatment in each tissue type revealed significant increases in mRNA levels (pixel intensity) due to heat shock (P<0.05, Figure 2.2).

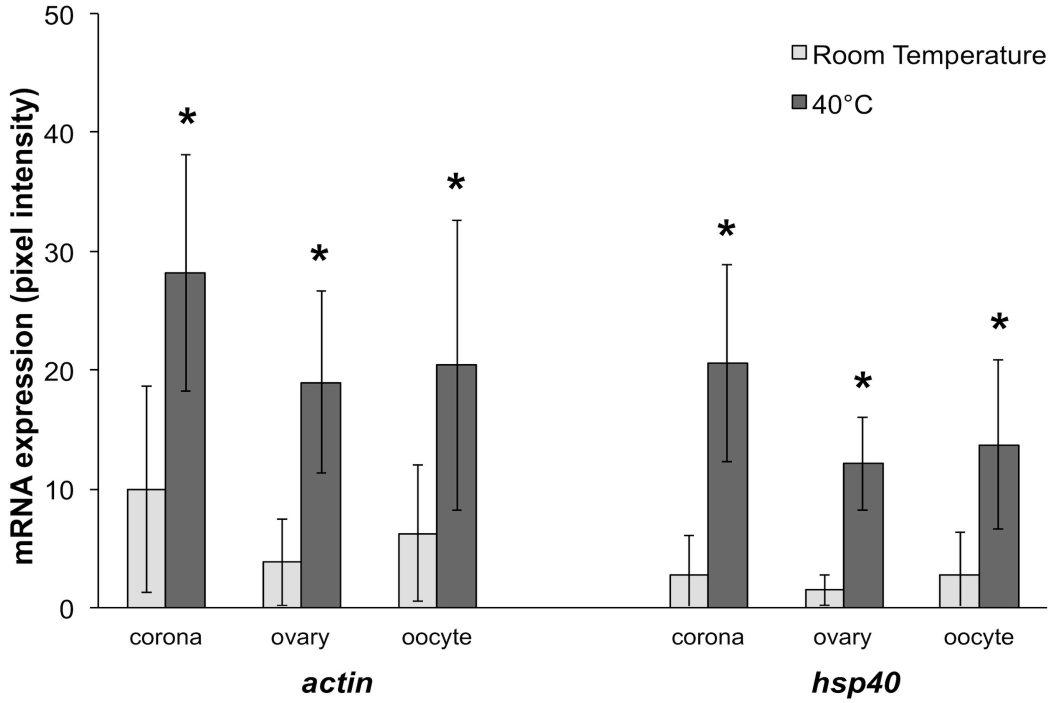

Figure 2.2. Mean pixel intensity and standard deviation for fluorescent *in situ* hybridization. Data for three tissue types showing mRNA levels of *hsp40* and *actin* following 1 h heat shock (40°C) or 1 h at room temperature (~22°C, control). Significantly greater expression following heat shock (P<0.05) is indicated by *.

Quantitative PCR

Transfection of *B. manjavacas* with *hsp* dsRNA resulted in a 0.8–6.9-fold reduction in expression of each respective gene relative to the PBS control treatment (Figure 2.3A). While knockdown of *hsp40*, *hsp60,* and *hsp70-3* genes was efficient, that is, all biological replicates (N=12) exhibited 100% knockout of gene expression, *hsp90* demonstrated variability in knockdown success with a mean of 46.3±7.6% knockdown of gene expression (Figure 2.3B). T-tests determined rotifers transfected with *hsp40*, *hsp60,* and *hsp70-3* dsRNA demonstrated significantly lower expression of the target gene relative to the PBS control based on ΔC_T values (P<0.0001); however, expression of *hsp90*-treated rotifers was not different from PBS controls (P=0.44). In tests to determine

whether *hsp40* was induced in response to heat stress, a 3.5-fold increase in *hsp40* expression was observed. This represented significant up-regulation of the *hsp40* gene in rotifers exposed to 1 h of heat shock at 40°C relative to incubation at room temperature, based on ΔC_T values (-7.86±0.90 and -6.06±1.24, respectively; t-test, P=0.0006) (Figure 2.4).

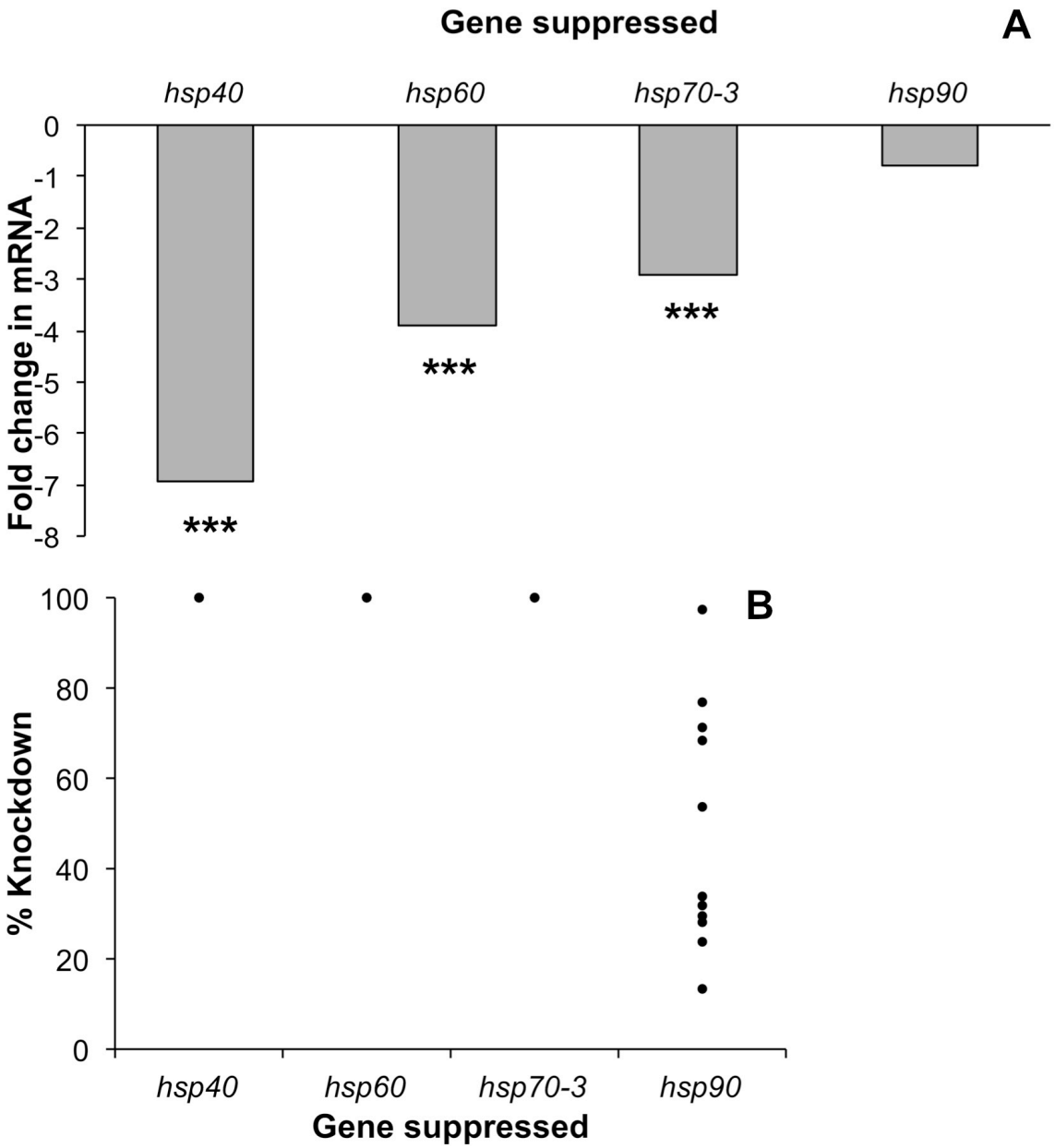

Figure 2.3. Quantitative PCR verification of gene suppression. (**A)** Fold change in *Brachionus manjavacas* gene expression due to RNAi treatments with heat shock genes relative to PBS controls. Negative values indicate reductions in gene expression due to treatment. Gene expression was normalized to the *actin* housekeeping gene. *** indicates a significant difference (P<0.0001) in expression of target gene, based on ΔC_T, between rotifers transfected with *hsp* dsRNA and PBS controls. (**B**) Percent knockdown of gene expression in individual rotifers (N=12 per treatment) due to RNAi treatments with *hsp* genes relative to PBS controls. Circles indicate the percent knockdown within an individual rotifer after transfection with dsRNA for the target gene.

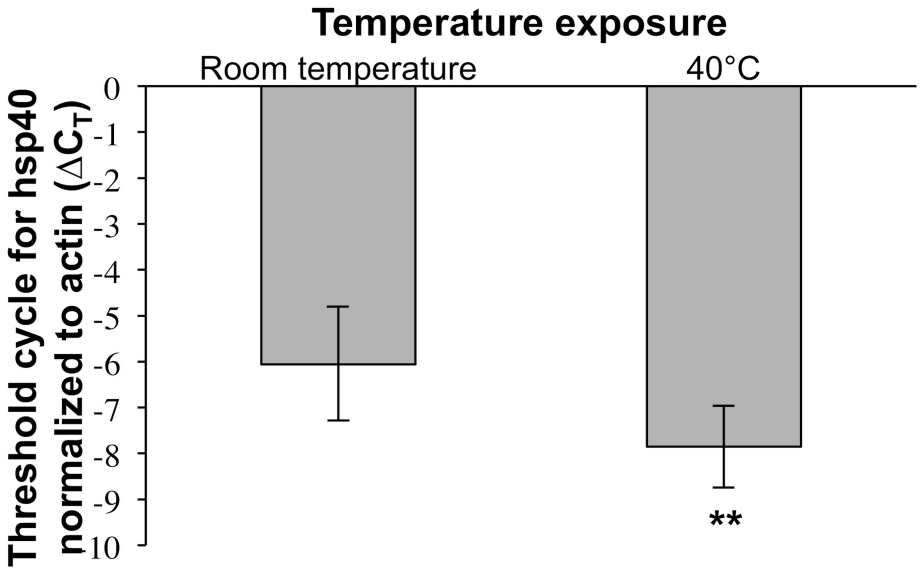

Figure 2.4. Up-regulation of *hsp40* gene expression in heat-shocked rotifers (N=12 individuals per treatment), represented by cycles of qPCR amplification required to reach a target threshold. Values of ΔC_T result from normalizing threshold cycle number for *hsp40* by subtracting threshold cycles for amplification of the reference gene (*actin*); negative cycle values result from higher cycle numbers for reference amplification. The lower ΔC_T for 40°C heat shock versus room temperature (~22°C) indicates a higher quantity of *hsp40* mRNA in the rotifer and hence reduced need for amplification, with ** indicating a significant difference (P<0.01).

Discussion

Here we report the first test in rotifers to definitively demonstrate the necessity of specific *hsp* genes for thermotolerance. Using RNAi to suppress expression of *hsp* genes, this study provides empirical evidence that *hsp40*, *hsp60,* and *hsp70-3* are essential for thermotolerance in rotifers. By assessing multiple genes we shed light on the potential for the role of a multi-protein complex or multiple chaperone pathways in rotifer thermotolerance, as *hsp40*, *hsp60*, and *hsp70-3* were each, individually required for survival following heat shock (Figure 2.1).

Quantitative PCR revealed 100% knockdown efficiency of *hsp40, hsp60,* and *hsp70-3* genes, evidence that the increase in rotifer mortality following RNAi and heat

shock can be attributed to suppression of *hsp* mRNA (Figure 2.2). While suppression of *hsp90* did not decrease thermotolerance, qPCR demonstrated only 46.3% mRNA knockdown, or a 0.79-fold reduction attributed to RNAi. Thus additional study is needed to improve knockdown efficiency in order to conclusively determine the influence of *hsp90* on rotifer thermotolerance. In the congener *B. ibericus*, basal levels of the *hsp90* ortholog exceed those of 12 other *hsp* genes (Kim et al. 2011); hence high constitutive levels of *hsp90* may explain our difficulty suppressing its activity.

For *hsp40*, the only *hsp* gene in which we evaluated induction of expression in response to heat stress, qPCR did show induction of expression following 1 h heat shock. Heat shock responses are rapid and can be detected shortly after the onset of increased temperatures (Morimoto 1993, Parsell and Lindquist 1993, Feder and Hofmann 1999). In a study of *B. ibericus* induction of four *hsp* genes, maximum up-regulation in response to ultraviolet radiation occurred within 3 h of exposure (Kim et al. 2011). Here, the apparent up-regulation of both *hsp40* and *actin* after heat stress, as demonstrated with FISH bioassays, indicated that the magnitude of the *hsp40* up-regulation found during qPCR might be masked due to the use of *actin* as a housekeeping gene to calculate relative expression. Thus induced *hsp40* expression is likely to be higher than determined in this analysis. Future studies are warranted to better characterize these genes as constitutive or induced by heat shock in rotifers.

Contrary to expectations for localization, expression of *hsp40* and *actin* was demonstrated in multiple regions, with fluorescence indicating mRNA presence in the corona as well as reproductive tissues. Because the organs of rotifers largely are syncytial (Wallace and Smith 2009), lack of cellular compartmentalization may lead to a more

global distribution of *hsp* mRNA. The *hsp40* gene was selected for further analysis (qPCR and FISH) due to its having a significant but intermediate effect on rotifer mortality, with mean mortality after *hsp40* suppression being slightly below that for *hsp70-3* (Figure 2.1). Hence it was chosen as a representative *hsp* gene with intermediate, but significant, effects on thermotolerance. Further studies are warranted on additional *hsp* genes.

Although the 40°C heat stress used in our study is unlikely to be commonly experienced by *B. manjavacas* in nature, with the thermal tolerance of the closely related *B. plicatilis* as ca. 5–29°C (Walker 1981), rotifers have been found at temperatures as high as 40°C in waterways receiving thermal effluents (Oden 1979). Rotifers in such extreme environments, as well as active populations in shallow waters or the diapausing embryos in dried sediment banks of ephemeral ponds, may be particularly dependent for survival on stress response systems such as HSPs. Of interest is the fact that the gene for *hsp70-3*, shown here to be required for thermotolerance, also is expressed in *B. plicatilis* diapausing embryos (Denekamp et al. 2009). Moreover, while some rotifer species are polythermal, others have narrow temperature ranges, and egg production may cease at high temperatures (May 1983, Bērzinš and Pejler 1989). Investigating the potential for differential *hsp* expression across taxa may shed insight into their thermal sensitivities. With the potential for temperature to impact zooplankton species richness, competitive interactions, and body size, as well as for cascading effects on higher trophic levels such as planktivorous fish, thermotolerance may be an important driver for aquatic community structure (Moore and Folt 1993, Stemberger et al. 1996, Stelzer 1998).

Conclusions

By demonstrating the role of specific genes in the rotifer stress response and a way for controlling expression of at least three *hsps* genes, we extend the utility of rotifer HSPs as biomarkers for stressful conditions and natural adaptation. Previous studies have suggested a role for a ca. 60 kDa protein in rotifer stress tolerance (Cochrane et al. 1991, Rios-Arana et al. 2005). Members of the HSP70 family are implicated in natural adaptation to thermal stress in a range of taxa, including *Drosophila*, lizards, and brine shrimp (Clegg et al. 2001, Sørensen 2010). Our finding that *hsp40* suppression also impacts heat shock survival in *B. manjavacas* may reflect coordination among heat shock proteins; indeed (Fan et al. 2003) has argued that a major role of HSP40 is the regulation of HSP70 activity. With the potential for climate change to cause elevated temperatures (Moore and Folt 1993), better knowledge of the ability of organisms to respond to heat stress will be critical to forecasting the effects on aquatic communities.

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CHAPTER 3

RAPID EVOLUTION OF SEX FREQUENCY AND DORMANCY AS HYDROPERIOD ADAPTATIONS

(This chapter has been submitted to the Journal of Evolutionary Biology.)

Abstract

Dormancy can serve as an adaptation to persist in variable habitats, and often is coupled with sex. In cyclically parthenogenetic rotifers an asexual phase enables rapid population growth, whereas sex produces diapausing embryos capable of tolerating desiccation. Few studies have experimentally tested whether sex-dormancy associations in temporary waters reflect evolution in response to the short hydroperiod selecting for diapause ability. Here we demonstrate evolution of higher propensity for sex and dormancy in ephemeral rotifer cultures mimicking temporary ponds, and lower propensity in permanent cultures. Results are consistent with rapid evolution, with evolutionary changes occurring in a short timeframe (385 d, ~84 generations), and affecting ecological dynamics (population density). We also provide insight into mechanisms for rapid evolution in basal metazoans. The evolutionary change observed likely involved new mutations and/or recombination, rather than being simply a shift in clone or genotype frequency.

Introduction

In ephemeral aquatic systems, taxa from macrophytes to zooplankton produce dormant stages, e.g., resting eggs (diapausing embryos) or seeds that remain quiescent during dry spells and hatch when water returns (Brock et al. 2003). For many facultative

asexuals and cyclical parthenogens that engage in asexual and sexual reproduction, dormant stages are the product of sex (Simon et al. 2002, Brock et al. 2003, Cáceres and Tessier 2004, Serra and Snell 2009). Sex-dormancy associations are found in organisms ranging from aphids to zooplankton (Simon et al. 2002, Serra and Snell 2009); sex and dormant seed banks also play a role in persistence of aquatic plant communities (Lokker et al. 1997). Yet little is known of the mechanism and pace of evolution of dormancy and sex as adaptations to stressors such as desiccation.

Research on the loss of sex and dormancy suggests the potential for these traits to undergo rapid evolution, whereby contemporary evolutionary changes occur swiftly enough to affect ecological dynamics (Hairston et al. 2005, Ellner et al. 2011). Several studies have documented sex loss in the cyclically parthenogenetic rotifer (zooplankton) *Brachionus calyciflorus* in periods of months to years (Bennett and Boraas 1989, Fussmann et al. 2003, Becks and Agrawal 2010, Stelzer et al. 2010). Typically, asexual brachionid females produce subitaneous female eggs; the females also excrete a protein signal into the water that accumulates with increasing rotifer density (Snell et al. 2006). At high densities, the signal reaches a threshold concentration that induces sex in a process analogous to microbial quorum sensing (Kubanek and Snell 2008). A fraction of the asexual brachionid females start producing sexual daughters; the rest continue with asexual reproduction. The sexual daughters produce males (which are smaller, haploid, and do not feed), or if fertilized by a male, produce diapausing embryos that hatch after a period of obligate dormancy (Wallace and Smith 2009, Wallace and Snell 2010). Yet some rotifers lose ability for sex. It has been proposed that brachionid sex loss constitutes an eco-evolutionary feedback, with the ecological dynamic of density-dependent sex

induction undergoing evolution, which in turn impacts population densities (Stelzer 2012).

Sex-dormancy associations and their evolution provide an opportunity for investigating both the role and mechanism of rapid evolution. The significance of eco-evolutionary changes and rapid evolution has been questioned, with preliminary evidence suggesting these forces do play important roles in community and ecosystem structure and function (Ellner et al. 2011, Becks et al. 2012). Rapid evolution of sex and dormancy may be critical for species' survival, particularly given the potential for climate change to alter hydroperiod and habitat permanence (Brooks 2004). Field-based and theoretical studies show correlations of rotifer traits associated with sexual reproduction and dormancy to hydroperiod (Serra and King 1999, Schröder et al. 2007, Serra and Snell 2009, Gilbert and Diéguez 2010, Campillo et al. 2011), with the tendency for higher levels of sex and dormancy in ephemeral habitats. Several studies have attributed rapid evolution and eco-evolutionary dynamics to clonal selection—including assays of rotifer sex loss (Fussmann et al. 2003), aphid growth rate (Turcotte et al. 2011), and cladoceran parasite resistance (Duffy and Sivars-Becker 2007, Fussmann et al. 2007b). However, the necessity of pre-existing variation and generality of clonal selection as the mechanism for rapid evolution of traits such as dormancy remain unknown.

Here we describe an experimental evolution study designed to test the hypothesis that the requirement for dormancy in environments mimicking ephemeral or permanently filled aquatic habitats results in rapid evolution of diapause and sex, and report on the role of clonal selection. Laboratory *Brachionus plicatilis* s.s. cultures were maintained for 385 d divided into six, 9-week growth seasons; ephemeral systems were reset (restarted

from diapausing embryo hatchlings) at the end of each season but permanent cultures were not reset. Hence ephemeral cultures underwent five rounds of sex between seasons, whereas in permanent cultures sexual embryos were removed and only asexual propagation contributed to population growth. We compared the cultures for evolutionary changes in sex induction propensity, sex frequency, and the production and hatching frequency of diapausing embryos. We also assayed lifespan and fecundity for both asexual and sexual females to test for evolution of traits less directly associated with sex and dormancy, and monitored population density to assess the cost of sex to population growth. Results are consistent with rapid evolution of higher propensity for sex and dormancy in ephemeral as compared to permanent cultures.

Methods

Cultures and Hydroperiod Treatments

Subcultures of 15 separate lineages of *Brachionus plicatilis* s.s. from the pond Poza Sur, Spain were provided by our colleague Dimas-Flores. Seven lineages corresponded to the "high density sex" group (high density for sex induction; range, 7.8–149.7 females mL^{-1}), and eight represented the "low density sex" group (2.2–30 females mL^{-1}) described in (Carmona et al. 2009). We confirmed identity of all lines as *B. plicatilis* s.s. with restriction fragment length polymorphism of *cytochrome c oxidase subunit 1* and gel electrophoresis, following (Berrieman et al. 2005). Ten asexually produced clonemates from each of the 15 lineages were used to inoculate each of six chemostat containers; chemostats were maintained for 385 d.

Cylindrical Cellift^{TM} bioreactors (Ventrex; Portland, ME, USA), each holding ca. 570 mL of medium, were used as chemostat (continuous flow) culture containers, with a

flow rate of ca. 150 mL d^{-1} controlled by a Manostat cassette pump. Inflow medium consisted of the green alga *Tetraselmis suecica* grown on F medium (Guillard and Ryther 1962), and diluted with 15 ppt artificial seawater (ASW, Instant Ocean® sea salts) to 1 million cells mL^{-1}. Chemostats were maintained in an Environmental Growth Chamber at 22°C below a 40 Watt fluorescent light; location of the chemostats was randomized and altered every 9 weeks.

Three chemostats were randomly chosen to represent an ephemeral environment, and three a permanent environment. Resets of ephemeral chambers were conducted to mimic the desiccation and refilling of an ephemeral pond. At the end of 9 weeks, all chemostats were cleaned to remove algal buildup on the walls. Debris settled at the base of the chemostat—including diapausing embryos, which sink—was collected and set aside. Removal of diapausing embryos was conducted to reflect conditions of a deep permanent lake, where embryos may sink to layers too deep for exposure to hatching stimuli (e.g., light) (García-Roger et al. 2005). At the reset each ephemeral chemostat was re-inoculated with fresh algae and hatchlings of diapausing embryos that had been harvested from its chamber 6 weeks prior and kept in diapause (dark, 5°C) to promote hatching. Immediately before a reset approximately 800 diapausing embryos per ephemeral chemostat were incubated for ~74 h (22°C, constant light from the 40 Watt lamp) and all hatchlings were used to refill (restart) the chemostat (Figure 3.1). Permanent chemostats were re-inoculated with their adult animals and old medium after chamber cleaning and removal of debris and diapausing embryos. Periods between these 9-week resets are referred to as growth seasons. Conditions in permanent chemostats allowed for continuous asexual growth. A total of 5 resets between seasons in ephemeral

chambers; however, interrupted asexual growth and required re-colonization by hatchlings of sexually produced diapausing embryos.

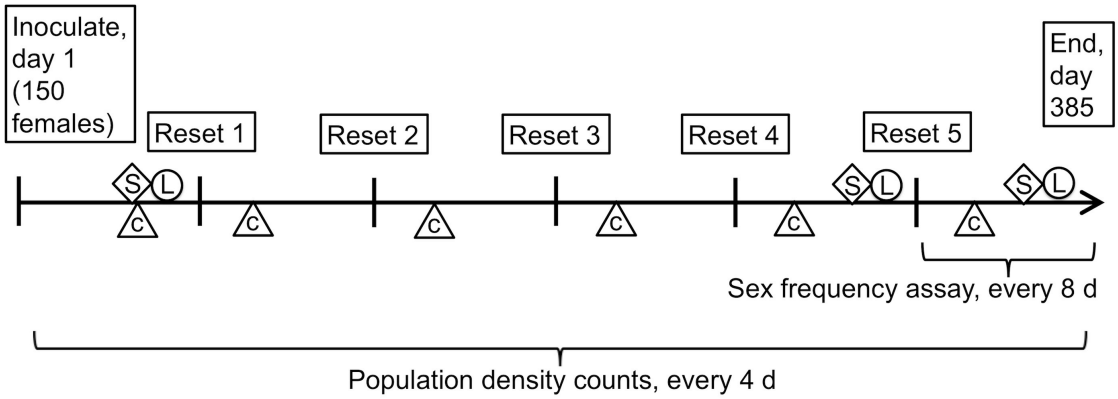

Figure 3.1. Schematic of timeline for an ephemeral chemostat. Horizontal line with arrow represents the 385-d experiment. Symbols show approximate timing of events: c, collection of diapausing embryos for use at the next reset; S, sex induction density assay; L, life history assay (lifespan, fecundity). Vertical lines denote the initial inoculation, and resets between 9-week growth seasons. (Diapausing embryo collection for reset 1 was postponed to day 44.) At each reset, all diapausing embryos in the chemostat were harvested, and used for quantification and asssessment of hatching rates after the 385-d experiment.

Sex Induction, Sex Frequency, and Diapausing Embryo Production

Bioassays testing relative propensity for sex induction were conducted at the first and final two growth seasons. Protocols were adapted from (Carmona et al. 2009), recording the female density at which males first appeared—signifying the transition to sex. Low densities suggest early induction, or a high propensity for sex. To minimize maternal effects and allow induction densities to reflect intrinsic properties of females rather than chemostat conditions, we performed a pre-experimental step following (Carmona et al. 2009): i.e., two asexual generations were maintained individually in Petri plates in low-density conditions (1 female in 25 mL medium). After two generations, a

group of females from each hydroperiod treatment was assayed for sex-induction density with sample sizes as follows: season 1, N=19 per hydroperiod; season 5, N=22 ephemeral and 35 permanent; season 6, N=30 ephemeral and 32 permanent. For the bioassay, females were allowed 10 d to produce males. Due to nonlinear dynamics in sex induction, culture volume can affect inducing densities (Carmona et al. 2011). Our use of a single, small volume (0.5 mL) precludes determination of absolute threshold densities for induction; we likely over-estimate absolute thresholds. However, use of the same conditions and volume allows comparison of relative sex induction propensity among cultures.

In the final season we monitored the frequency of sexual relative to total females every 8 d. Asexual and sexual females are indistinguishable morphologically, and can only be differentiated based on their progeny (asexual females produce females, sexual females produce males or diapausing embryos). On some dates most adult females were non-ovigerous; some may have been post-reproductive (sexual status indeterminate). Thus we analyzed sex frequency of cohorts of subitaneous female eggs laid in the chemostat. Because brachionids' sexual status is determined *in utero* (Snell et al. 2006, Gilbert 2007), the status of these eggs was determined while they formed in the chemostat. We collected ovigerous asexual females from each chemostat. Females were maintained separately in 1 mL medium in 24 well plates until their eggs hatched; 1 hatchling per female was individually transferred to fresh medium and produced male or female progeny in ~2 d. Analyses compared mean frequencies of sexual females (N=8 assays or days); each frequency was from testing 19–24 females per chemostat per assay.

At the end of each season, all diapausing embryos were collected by filtration of

settled debris at the base of each chemostat (53 μm Nitex mesh), allowed to air dry, and weighed. These were used to assess diapausing embryo production (not for ephemeral resets). From each season's collection from each chemostat, a random sample (~5 mg) of dried diapausing embryos was weighed, rehydrated in 4.75 mL 15 ppt ASW, and sub-sampled (100 μL). All embryos in the sub-sample were counted (mean number of diapausing embryos per sub-sample ± 1 SE, 105 ± 13). These counts were used to determine the density of the dried embryos (embryos / gram), which was multiplied by the total mass of dried embryos from the chemostat to yield total diapausing embryo production per chemostat per season. From ephemeral cultures, at least ~1000 embryos also had been harvested early in each season for resets so we added 1000 to final estimates of ephemeral diapausing embryo production. Because we did not count total numbers of embryos collected for resets, diapausing embryo production reported for ephemeral cultures may slightly underestimate total values.

To test hatching ability, 100 of the diapausing embryos collected from each of the 6 chemostats at the season's end were incubated to hatch. Embryos were placed in 3 mL 15 ppt ASW and incubated to hatch (22°C, 40 Watt lamp) for 1 week. All embryos were incubated to hatch simultaneously; they had been maintained in diapause (dark, 5°C) until 9.5 weeks after the end of the final (6^{th}) season.

Population Density, Individual Lifespan, and Individual Fecundity

Density of total females, sexual females carrying diapausing embryos, and males was determined from manual counts every 4 d from pooling three, 1 mL samples of the chemostat outflow (total of ~93 counts per chemostat in the 385 d). Most females were non-ovigerous so their sexual status could not be determined. To estimate asexual female

density at each density count, we subtracted the density of unfertilized and fertilized sexual females from the total female density. Fertilized sexual female density was determined from direct counts of females bearing diapausing embryos. We estimated the number of unfertilized (male-producing) sexual females based on the male density count, and mean individual fecundity of unfertilized sexual females that lay male eggs (from life history bioassays, below). In essence, we calculated the number of unfertilized sexual females required to produce the current male density. Our final calculation of asexual density (AD) was as follows: AD = total female density – (male density / daily fecundity of unfertilized sexual females) – fertilized sexual female density. This formula does not give an exact count of AD, as it does not account for factors such as the potential for more males to be produced during the day after the density count, or for sexual fertilized females not currently carrying a diapausing embryo. However, application of the same formula allowed comparison of relative AD of ephemeral and permanent cultures in this study. Any estimated asexual density ≤0 due to densities below detection was converted to 0.01 females mL^{-1} before statistical analysis and production of graphs.

During the first and the final two seasons, a life history bioassay was conducted to monitor total lifespan and fecundity of a cohort of asexual and unfertilized sexual females. We isolated ovigerous, asexual females from the outflow of each chemostat. Each ovigerous female was placed in 1 mL of medium ($5x10^5$ cells mL^{-1} *T. suecica* in ASW). The following day one neonate hatchling (F1 generation) per mother was transferred to fresh medium; the mothers were discarded. Every day until death, females of the F1 cohort were individually transferred to new medium, and their progeny (F2) were counted. For asexual females, ephemeral sample size combining chemostats was 59,

66, and 71 F1 females in the 1st, 5th, and 6th season, respectively (16≤N≤24 per chemostat per season); permanent sample size was 53, 71, and 70 in the 1st, 5th, and 6th season, respectively (17≤N≤24 per chemostat per season). For unfertilized sexual females, ephemeral sample size combining chemostats was 31, 42, and 65 F1 females in the 1st, 5th, and 6th season, respectively (8≤N≤24 per chemostat per season); permanent sample size was 41, 29, and 22 in the 1st, 5th, and 6th season, respectively (3≤N≤22 per chemostat per season). Lower sample size of sexual females reflected lower densities of sexual females in the chemostats.

Statistical Analyses

Statistics were performed at $\alpha<0.05$ in SPSS v.18. Animals were randomized across wells and plates in all bioassays. Transformations ($\log_{10}$ for counts; arcsine square root for frequencies) were made to improve normality for repeated measures analysis of variance (RM-ANOVA) and nested analysis of covariance (nested ANCOVA). Repeated measures ANOVAs were used for assays in which the level of replication was the chemostat population (i.e., sexual female frequency, total population diapausing embryo production, and female population density). We included season as the temporal within-subjects repeated factor for diapausing embryo production and day as the repeated factor for sexual female frequency and population density, with hydroperiod (ephemeral, permanent) as a between-subjects factor. Chemostats were inoculated with identical populations initially and anticipated to evolve over time across seasons, but with the type of change affected by the hydroperiod regime. Thus analysis of differences over seasons between ephemeral and permanent hydroperiods was performed by testing the significance of the hydroperiod*season or hydroperiod*day interaction in both the

ANCOVA and RM-ANOVAs. One exception was the sex frequency assay in which all measurements were made in the last season; here analysis focused on the main effect of hydroperiod. For statistical analysis of total female densities a small value (0.01, ~6 females chemostat^{-1}) was added to all densities before log transformation to account for any densities below the limit of detection.

For the sex induction bioassay, a nested ANCOVA was used rather than a RM-ANOVA because the level of replication was the individual female assayed, and females assayed in seasons 1, 5, and 6 were not repeated due to natural turnover within populations and random sampling of animals from chemostats. The nested ANCOVA was implemented as a univariate analysis of variance with chemostat as a random effect, testing effects of season as a covariate (continuous), hydroperiod treatment, the hydroperiod*season interaction, and chemostat nested within hydroperiod. Nesting chemostat within hydroperiod and inclusion of chemostat as a factor in the model was done to avoid pseudoreplication by not accounting for chemostat-specific effects. While presence of a hydroperiod*season interaction would suggest heterogeneity of slopes and necessitates caution interpreting the main effect of hydroperiod alone on rotifer traits, we note that the express purpose of the ANCOVA was to test for existence of a significant interaction.

Lifespan and fecundity did not appear to follow normal distributions and thus were not analyzed with analyses of variance. Analyses of total lifespan were done with a log-rank Mantel-Cox test implemented in the Kaplan Meier analysis of SPSS with Strata=Season. Data were pooled from the last two seasons (5, 6) to compare final outcomes of hydroperiod treatment on lifespan. Generalized linear models with Poisson

distribution, log link, and Pearson χ^2 scaling parameter were used to test for effects on fecundity with Wald's χ^2 statistics. As with the ANCOVA, analyses of fecundity tested for effects of season, hydroperiod treatment, the hydroperiod*season interaction term, and chemostat nested within hydroperiod.

Results

Sex Induction, Sex Frequency, and Diapausing Embryo Production

Rotifers in ephemeral versus permanent hydroperiod treatments evolved significantly different propensities for inducing sex across seasons (P=0.008) (Figure 3.2A, Table 3.1). The relatively higher densities for male production by permanent cultures in later seasons reflected evolution of decreased propensity to induce sex. Before inoculation in the chemostat, induction densities ranged from 2–150 females mL^{-1} (Carmona et al. 2009). Our results from season 1 are similar (10–139 females mL^{-1}). By the end of the experiment, inducing densities for some females in permanent populations exceeded initial levels by an order of magnitude (18–1610 females mL^{-1} and 18–1486 females mL^{-1} in seasons 5 and 6, respectively). Between seasons 1 and 6, mean sex induction density for permanent cultures rose from 55 to 688 (Figure 3.2A). In seasons 5 and 6, a substantial number of females in permanent systems failed to produce males (up to 87% in one permanent culture in season 5); final densities for females that did not produce males during the 10 d assay were recorded as the inducing density.

Frequency of sex in the final season, quantified as the proportion of sexual relative to total females, was higher in ephemeral versus permanent chemostats (Figure 3.2B). There was a significant difference in sex frequency between hydroperiod

treatments (P=0.024), but within this final season there was no change across days (P=0.483) or differential evolution between ephemeral and permanent cultures (P=0.977) (Table 3.1). Over time, total diapausing embryo production per season (Figure 3.3) evolved to higher levels for ephemeral than permanent populations (P=0.048) (Table 3.1). Hatching frequency of the diapausing embryos did not show differential evolution between hydroperiods across seasons (P=0.338) (Table 3.1), but overall declined in later seasons (P=0.016).

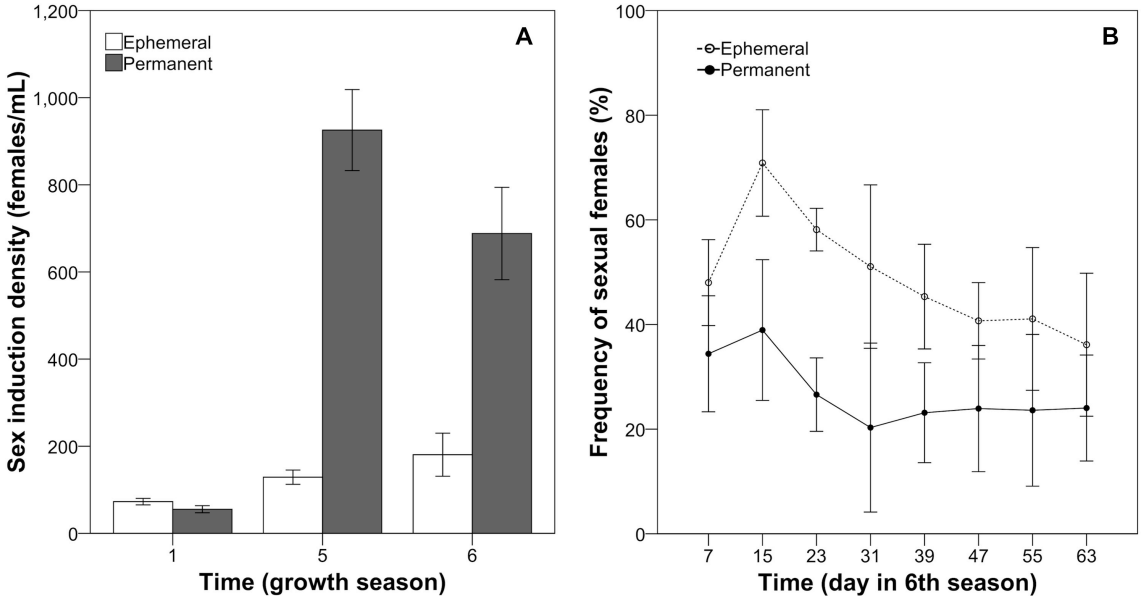

Figure 3.2. Higher sex propensity in ephemeral chemostats. Error bars are ± 1 SE. **(A)** Mean ± 1 SE female density for sex induction (first appearance of males) in the first and final two 9-week seasons, showing differential evolution of ephemeral and permanent cultures (P=0.008). Higher densities in permanent cultures for later seasons reflect evolution for lower sex induction propensity. (**B**) Frequency of sexual relative to total females was significantly higher in ephemeral versus permanent cultures in the 6th growth season (P=0.024). Circles represent mean sex frequency from assays every 8 d. Interpolating lines between data points are included to aid visualization.

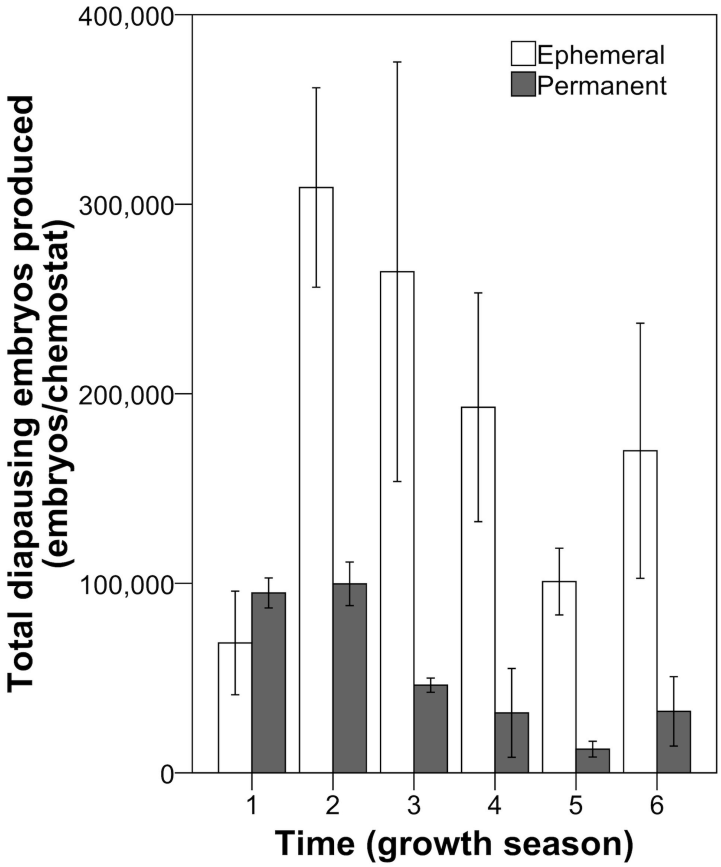

Figure 3.3. Total diapausing embryo production during each season in ephemeral and permanent cultures. Bars represent mean numbers of embryos (± 1 SE). Differential evolution in ephemeral and permanent hydroperiods was significant, with a trend towards higher production by ephemeral cultures in later seasons, and lower production by permanent cultures (P=0.048).

Population Density, Individual Lifespan, and Individual Fecundity

Total female density was higher for permanent populations in later days, and hence seasons, (P<0.001) (Figure 3.4A, Table 3.1). Evolution also led to higher mean asexual densities for permanent compared to ephemeral cultures in later days (P=0.001) (Figure 3.4B, Table 3.1), with percent differences between hydroperiods ≥76% in seasons 3–6. When a Bonferroni correction is applied due to potential redundancy in testing both asexual and total female density such that α=0.025, conclusions do not change: both total and asexual female density became significantly lower over time in the ephemeral versus permanent hydroperiod. Although the decrease in ephemeral density at a reset (i.e., re-inoculation with hatchlings of the 800 diapausing embryos) may have contributed to the results, rapid population growth makes it unlikely that resetting alone explains the lower size of ephemeral cultures. By the 4th density count following each reset, at least one of the ephemeral cultures had attained a density in the range of densities for the permanent cultures.

Lifespan and fecundity did not evolve in response to hydroperiod. Data pooled from the last two seasons revealed that the difference in lifespan between ephemeral and permanent cultures was not significant for asexual or unfertilized sexual females (P≥0.176) (Table 3.2). Mean lifespan in days (± 1 SE) for asexual females was 11.0 (± 0.3) in ephemeral, and 10.5 (± 0.2) in permanent chemostats. For unfertilized sexual females, lifespans were 8.5 (± 0.3) in ephemeral, and 8.3 (± 0.3) in permanent chemostats. Generalized linear model tests of Wald's χ^2 statistic of total lifetime fecundity showed no evolutionary response to hydroperiod treatment across seasons for asexual or unfertilized sexual females (P≥0.081) (Table 3.2). Pooling data from the final

two seasons, for asexual rotifers the mean number of progeny per female (± 1 SE) was 18.3 (± 0.3) in ephemeral, and 17.1 (± 0.4) for permanent cultures. For sexual females in the final two seasons, mean progeny per female was 13.0 (± 0.4) for ephemeral, and 13.2 (± 0.4) for permanent cultures.

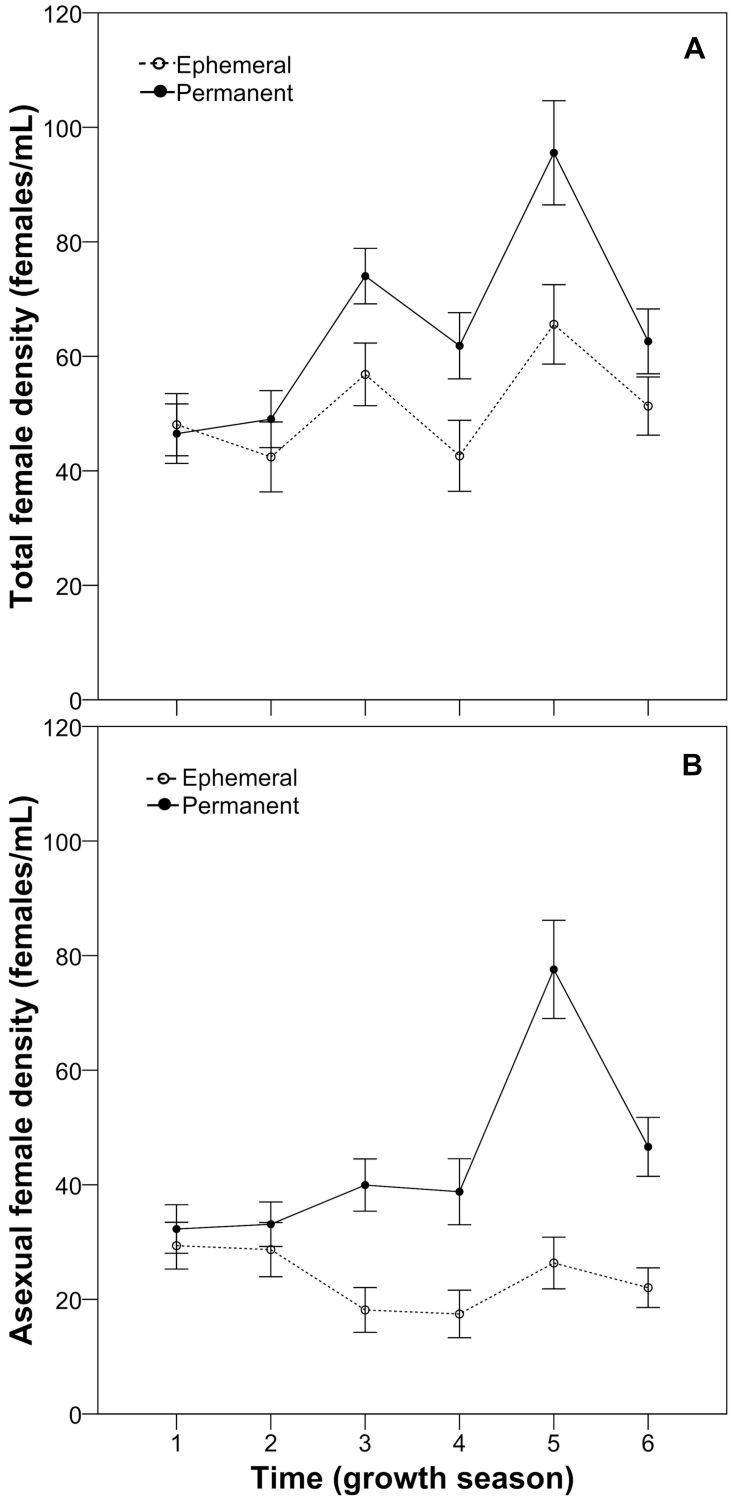

Figure 3.4. Cost of sex incurred as reduced population growth in the more sex-prone ephemeral cultures. Circles represent mean female density per season (± 1 SE). Interpolating lines between data points are included to aid visualization. Female density was higher for permanent than ephemeral cultures in later seasons, as seen both for (**A**) total female density ($P<0.001$), and (**B**) asexual female density ($P=0.001$).

Table 3.1. Summary of RM-ANOVA for the influence of hydroperiod treatment on sex frequency, diapausing embryos, and population density, and ANCOVA of sex induction density. Time represented growth season in sex induction, diapausing embryo production, and hatching bioassays. Time represented day of the assay or count in sex frequency (6^{th} season) and female density (data from all seasons) analyses. Degrees of freedom are given for the hypothesis (df_H) and error (df_E).

Bioassay	Seasons	Hydroperiod*Time			Hydroperiod			Time			Chemostat		
		F	df_H/df_E	*P*	F	df_H/df_E	*P*	F	df_H/df_E	*P*	F	df_H/df_E	*P*
Sex induction propensity	1, 5, 6	7.265	1/149	0.008	0.540	1/9.136	0.481	19.236	1/149	<0.001	8.264	4/149	0.001
Sex frequency	6	0.222	7/28	0.977	12.384	1/4	0.024	0.955	7/28	0.482	—	—	—
Diapausing embryo production	1-6	2.744	5/20	0.048	11.894	1/4	0.026	3.194	5/20	0.028	—	—	—
Diapausing embryo hatching	1-6	1.216	5/20	0.338	0.443	1/4	0.542	3.689	5/20	0.016	—	—	—
Total female density	1-6	2.336	92/368	<0.001	1.991	1/4	0.231	8.892	92/368	<0.001	—	—	—
Asexual female density	1-6	1.616	92/368	0.001	4.216	1/4	0.109	3.899	92/368	<0.001	—	—	—

Table 3.2. Summary of log-rank (Mantel-Cox) tests of lifespan (data pooled the last two seasons), and generalized linear models of fecundity (seasons 1, 5, 6).

Bioassay	Rotifer type	Hydroperiod*Time			Hydroperiod			Time			Chemostat		
		χ^2	df	*P*	χ^2	df	*P*	χ^2	df	*P*	χ^2	df	*P*
Lifespan	Asexual	—	—	—	0.926	1	0.336	—	—	—	—	—	—
	Sexual	—	—	—	1.829	1	0.176	—	—	—	—	—	—
Fecundity	Asexual	3.055	1	0.081	0.961	1	0.327	134.784	1	<0.001	28.221	4	<0.001
	Sexual	0.611	1	0.434	0.932	1	0.334	1.317	1	0.251	3.601	4	0.463

Discussion

Here we provide experimental evidence that ephemeral habitats requiring dormancy for survival, contrasted to permanent environments, impose differential selection pressure leading to rapid evolution (~84 generations, or 385 d) of sex propensity and dormancy, with an ecological impact on population density. Our selection regime was designed to mirror the pressures imposed by hydroperiod in nature by requiring or eliminating the need for dormancy, itself the outcome of sex in brachionid rotifers, but did not specifically select for individual traits such as sexual female frequency. We observed multiple responses at the individual (e.g., sex induction density) and population level (e.g., total diapausing embryo production). The requirement of diapausing embryo production for survival in ephemeral cultures was sufficient for evolution of higher sex frequency and dormancy relative to permanent populations.

In nature, aquatic habitat permanence correlates with gradients in community composition and adaptive life history traits (Wellborn et al. 1996, Simon et al. 2002, Brock et al. 2003, Jocque et al. 2010). Our laboratory experiment demonstrates that rapid evolution may allow for hydroperiod adaptation *in situ*, suggesting a causal mechanism for these correlations. Standing genetic variation may be important to rapid adaptations; initial trait variation can play a decisive role in whether eco-evolutionary dynamics maintain heritable variation in prey defense (Becks et al. 2010). Previous studies have proposed rapid evolution occurred through clonal selection, or changes in the frequency of clones and genotypes (Fussmann et al. 2003, Fussmann et al. 2007b, Turcotte et al. 2011). In contrast, we suggest our results occur through creation of new genetic material or combinations, and not solely a shift in initial genotypes or clonal lines. Females in

permanent populations in later seasons induced sex at densities higher than we observed in the first season, and higher than that reported by (Carmona et al. 2009) for any lineage in our founding populations.

The new, evolved sex-induction densities we observed could have occurred through recombination or segregation leading to novel arrangements of the genetic material. However, these meiotic processes seem unlikely in permanent cultures due to our removal of the sexually produced diapausing embryos, and the extent of the change (about an order of magnitude increase). It is known that inheritance of the *op* locus in *B. calyciflorus* results in inability to respond to the cue for sex induction via Mendelian inheritance; loss of sex is recessive (Stelzer et al. 2010, Scheuerl et al. 2011). Yet *op* heterozygotes do not differ in sex induction density from homozygous dominant rotifers (Scheuerl et al. 2011); thus recombination at this locus cannot fully explain the evolution of decreased sex induction propensity in permanent hydroperiods. *De novo* mutations (*sensu lato*) seem a more plausible explanation, although verification of exact mechanisms will require detailed genetic analyses. Be it from re-arrangements or mutations, the likely role of new genetic material in rapid evolution of a basal metazoan represents a significant extension of earlier reports of clonal selection.

Unlike some studies that have demonstrated complete loss of sex in continuous cultures of *Brachionus calyciflorus* rotifers (Bennett and Boraas 1989, Fussmann et al. 2003, Stelzer 2007, Becks and Agrawal 2010, Stelzer et al. 2010, Fussmann 2011), we demonstrate a decline in sex frequency in permanent *B. plicatilis* cultures. Our emphasis on evolution of sex frequency is congruent with studies reporting variation in sex propensity among clones for both rotifers and cladocerans (Schröder and Gilbert 2004,

Tessier and Cáceres 2004, Gilbert and Schröder 2007, Carmona et al. 2009). In permanent systems at later seasons, we observed initiation of male production by females at densities higher than any observed initially (>900 females mL^{-1} for some females in seasons 5 and 6), consistent with evolution of lower propensity for sex rather than complete loss of induction ability. Slight increases in inducing densities for ephemeral chemostats across seasons could reflect mild inbreeding depression due to the low (N=15) number of distinct lineages (genotypes) in each original population (Tortajada et al. 2009). Alternatively this may reflect adaptation to laboratory conditions, or genetic slippage from sex (Lynch and Deng 1994).

Although ephemeral systems maintained higher frequencies of sexual females in the final season, we do not report a significant difference in the change in sex frequency between ephemeral and permanent hydroperiods within a growing season. Neither was there a change in sex frequency independent of hydroperiod within the season, perhaps reflecting the somewhat short (9 week) season duration. Lower responsiveness to sex induction stimuli immediately after the reset may explain the increase in sex frequency between days 7 and 15 (Figure 3.2B) in ephemeral cultures. Brachionid diapausing embryo hatchlings and the first few generations of descendants often show reduced propensity for sex (Gilbert 2002).

Diapausing embryo production increased in ephemeral and decreased in permanent cultures. This may reflect the higher frequency of sexual females in ephemeral cultures, and/or evolution of fecundity for sexual, fertilized females. The results support the finding of a negative correlation between hydroperiod of ponds in nature, and *B. plicatilis* diapausing embryo production (Campillo et al. 2011). The initial large increase

in diapausing embryo production by ephemeral cultures between the first and second season may be due to the very strong selection pressure imposed on ephemeral populations by only using diapausing embryo hatchlings for re-colonization after resets. Decreased production by permanent cultures was not evident until later seasons. Delaying sex induction until a higher population density is reached can increase total diapausing embryo production (Serra et al. 2005); larger populations can produce more diapausing embryos because more females are available to reproduce. Yet despite their lower female density, ephemeral populations still produced more diapausing embryos than permanent cultures in later seasons. We found no differential evolution of diapausing embryo hatching success between hydroperiod treatments.

Our results are consistent with the theme of eco-evolutionary dynamics, with evolution of rotifer density-dependent sex induction affecting the ecological dynamic of population density (Stelzer 2012). This experimental evolution study also reinforces theoretical predictions that higher proportions of sexual offspring increase the cost of sex (Stelzer 2011). In the final season, asexual female density was about twice as high in permanent compared to ephemeral populations, supporting the historical idea of a twofold cost of males and sex to population growth (Maynard Smith 1978). Life history assays of cohorts showed no significant difference in fecundity or lifespan between hydroperiods for asexual or unfertilized sexual females. Thus we suggest that differences in population growth reflect higher allocation to sex (and dormancy) versus evolution of fecundity. Lower total female density in ephemeral cultures indicates the cost of sex to asexual density represents more than just a tradeoff between proportions of asexual and sexual females. Low asexual female density may further reduce population size by

slowing population growth.

It should be noted that the different numbers of generations adds another distinction between ephemeral and permanent populations. The portion of ephemeral populations that survived a reset existed as a diapausing embryo for the final 6 weeks for seasons 1–5; thus permanent cultures experienced ~84 generations, whereas ephemeral cultures would have experienced ~37 generations with the remaining time in dormancy. The greater number of generations in permanent cultures, their larger population size, and absence of the population bottleneck that occurred in ephemeral cultures at resets, could have fostered the ability of natural selection to act in permanent populations. Perhaps this promoted fixation of mutations, e.g., for lower sex propensity. Though it could be argued that one should compare equivalent numbers of generations for evolution, our study was designed to test effects of the environmental driver of hydroperiod. In nature, one might expect ephemeral pond populations to spend greater time in dormancy.

Applied to natural populations, results of this study suggest the potential for rapid evolution of dormancy to facilitate evolutionary rescue (Kinnison and Hairston 2007) from stressors such as altered hydroperiod, an anticipated effect of climate change (Brooks 2004). Furthermore, rapid evolution and adaptation could help explain the dispersal-gene flow paradox of aquatic organisms: namely, cryptic speciation and high levels of diversification among neighboring communities, despite the potential for dispersal and homogenization via gene flow (De Meester et al. 2002). Finally, the generation of new phenotypes through mutations or re-arrangements such as recombination is an intriguing addition to clonal selection for future studies of the mechanism of rapid evolution in eukaryotes.

Acknowledgements

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CHAPTER 4

EVOLVABILITY OF LIFESPAN AND REPRODUCTION

(This chapter will be submitted to the journal BMC Ecology.)

Abstract

Here we perform one of the first investigations of evolvability of lifespan and reproduction in metazoans, examining both extrinsic and intrinsic factors. We tested effects on senescence of an environmental variable (simulated lake hydroperiod, the length of time an aquatic habitat is inundated), female reproductive physiology (sexual or asexual), and time in a benign culture environment (minimal if any external mortality). To do this we established permanent and ephemeral chemostat cultures of the rotifer *Brachionus plicatilis* s.s. and maintained the cultures for 385 d, or 6 simulated growth seasons. Although there was no significant difference in lifespan between chemostat hydroperiod treatments (ephemeral versus permanent), rotifers at the experiment's end lived longer, suggesting that maintenance in laboratory conditions leads to decelerated aging. Asexual females showed a 26% increase in lifespan (23% decrease in rate of aging) and 56% increase in fecundity, whereas these traits did not change significantly in sexual females. In later seasons asexual females remained reproductive longer, and had a longer post-reproductive period, than in earlier generations. The increased longevity and fecundity of asexual but not sexual females may reflect regulation of evolvability through epigenetic differences, and/or through endocrine signaling pathways related to reproductive physiology. Contrary to the typically negative relationship postulated by the antagonistic pleiotropy and disposable soma theories of aging, we found a positive

correlation between fecundity and lifespan, with both showing significant increases over time. Overall, our study demonstrates the importance of external environmental conditions and reproductive physiology to the evolutionary potential of lifespan and fecundity.

Introduction

Research aimed at extending animal longevity through lifestyle changes such as dietary restriction (Lee et al. 1999, Grandison et al. 2009, Meydani et al. 2011), or drugs and supplements (Kenyon 2010, Le Couteur et al. 2012), presupposes that ability for life extension exists if the right conditions are met. Based on success stories of increased lifespan in research with laboratory animals, it has been suggested that animals possess a latent potential for living longer (Kenyon 2010). Efforts to study the evolution of senescence in wild populations (Monaghan et al. 2008, Ricklefs 2008) may reveal how senescence has evolved in nature in response to different factors. The ability of selective pressures to drive an evolutionary response in senescence requires a sufficiently flexible target for modification or adaptation. The capacity for heritable changes in response to selection pressure, or the ability to evolve, comprises an organism's evolvability (Pigliucci 2008). Much as understanding mechanisms such as hormesis (Le Couteur et al. 2012) may aid efforts to extend lifespan via non-heritable mechanisms (e.g., dietary restriction), understanding forces influencing evolvability may aid interpretation of the reasons for different rates of aging and life histories among species and populations.

Some of the features proposed to enhance evolvability also affect aging (senescence). In addition to standing levels of genetic variation, mechanisms guiding the way in which genotypes map onto phenotypes can impact levels of evolvability (Pigliucci

2008). These mechanisms include modularity and robustness. Subdivision into modular networks may promote evolution by allowing natural selection to act upon one trait without influencing others, much in the same way that meiotic recombination may enhance natural selection by disrupting linkage disequilibrium. Existence of networks and aging pathways (Southworth et al. 2009, Fortney et al. 2010, Peysselon and Ricard-Blum 2011) may provide the modular organization scheme for evolvability of aging. Likewise, epigenetic mechanisms are thought to underlie part of the aging process (Bandyopadhyay and Medrano 2003, Fraga and Esteller 2007), and may contribute to evolvability (Jamniczky et al. 2010, Johnson and Tricker 2010). Such epigenetic mechanisms can be heritable across generations (Youngson and Whitelaw 2008). Hence, the framework for controls of evolvability of senescence may already be in place in organisms, but the extent and basis of such a framework has yet to be revealed, and few studies of the evolvability of aging exist, c.f. (Goldsmith 2008).

Here we examine the evolvability of senescence in rotifers (Figure 4.1), basal metazoans with a long history of use in aging studies (Enesco 1993, Snell and Stelzer 2005, Wallace and Snell 2010). Rapid turnover (typical lifespans up to a few weeks) (Wallace and Smith 2009, Wallace and Snell 2010) allows studies of evolutionary dynamics for these zooplankton in a tractable timescale. To assess factors involved in evolvability of senescence—particularly lifespan and reproduction—we established six flow-through cultures (chemostats) of *Brachionus plicatilis* s.s. rotifers. Brachionids are cyclical parthenogens that reproduce as asexual (amictic) females until induced to produce sexual (mictic) daughters; sexual daughters produce male progeny, or if fertilized make resting eggs (diapausing embryos) (Wallace et al. 2006, Wallace and

Smith 2009, Wallace and Snell 2010) (Figure 4.1). We tested effects on senescence of (1) an environmental variable (simulated lake hydroperiod, the length of time an aquatic habitat holds water), (2) female reproductive physiology (sexual or asexual), and (3) time in a benign laboratory culture environment (minimal if any extrinsic mortality factors). Results are discussed in light of the role of these factors in evolvability of aging and reproduction. Although some natural populations exhibit faster development rates or shortened lifespans in ephemeral hydroperiods (Valdesalici and Cellerino 2003, Schröder et al. 2007, De Block et al. 2008), ephemeral versus permanent cultures did not differ in lifespan or fecundity. However our findings show positive correlations of lifespan and reproduction, with both increasing over time in chemostat culture for asexual, but not sexual, females.

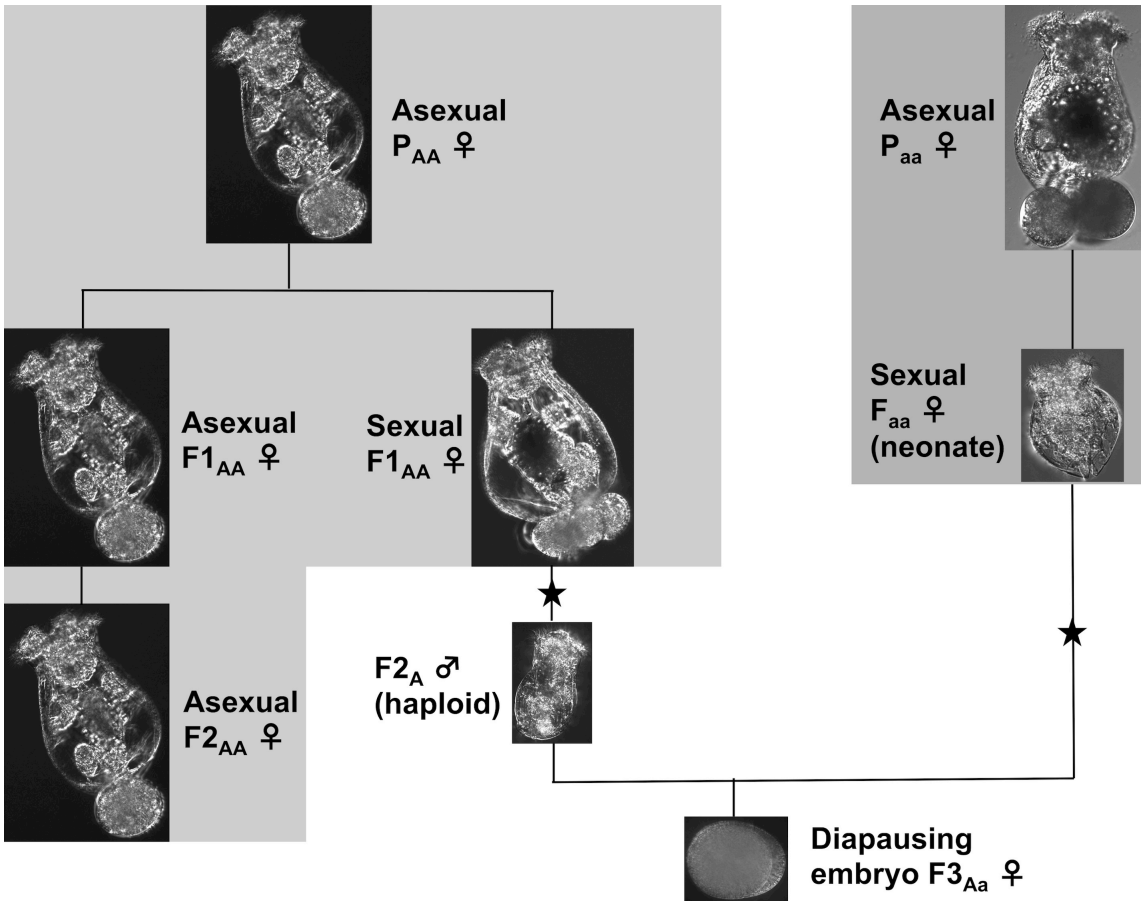

Figure 4.1. *Brachionus plicatilis* pedigree from two parental (P) asexual females through three progeny generations (F1-F3). Gray shading encompasses females with the same genotype (i.e., a P1 female and her asexually produced progeny). A single locus with two alleles is represented in the homozygous (AA or aa) and heterozygous (Aa) state to aid tracking of genotypes. Females are diploid; males are small and haploid. A diapausing embryo is produced when a male fertilizes a young (neonate) sexual female, and will hatch after obligate dormancy to yield an asexual female. Unfertilized sexual females produce males. Stars depict where meiosis occurs to produce progeny; all other reproduction is ameiotic.

Methods

Cultures and Hydroperiod Treatments

Chemostat culture treatment and maintenance is described in a companion paper investigating evolution of sex and dormancy in response to hydroperiod treatment (chapter three). For discussion of setup of rotifer chemostat systems in general, see (Stelzer 2009). In brief, 15 distinct lineages of *Brachionus plicatilis* s.s. Müller, 1786 monogonont rotifers were isolated from water samples from the pond Poza Sur (Spain) and maintained as separate, asexually propagated lineages (Carmona et al. 2009). Lineage subcultures were provided by Dimas-Flores and used to initiate six replicate chemostat populations, each inoculated with 10 asexual clonemates of each of the 15 lineages (N=150 animals per chemostat). Cultures were maintained for 385 d in Cellift™ bioreactors (Ventrex), each containing 570 mL medium: 1 million cells mL^{-1} of the alga *Tetraselmis suecica* in 15 ppt artificial seawater (ASW, Instant Ocean® sea salts). Flow rate was 150 mL d^{-1}, and chambers were kept at 22°C with continuous lighting. Location of chambers was randomized, and re-randomized every 9 weeks.

The six chemostats were randomly assigned to ephemeral or permanent hydroperiod treatments. Although sample size (N=3 each) was low due to resource availability, it was sufficient to detect significant evolution in sex propensity and dormancy (chapter three). The 385-d timeframe was divided into six growth seasons; at the end of each 9-week season ephemeral chambers were reset to mimic desiccation-refilling of ephemeral pools. For a reset, the chamber's contents were emptied, and it was re-inoculated with hatchlings of diapausing embryos (~800) produced in that chemostat in the season immediately prior to the reset. In ephemeral cultures population growth

occurred asexually during the season, but induction of sex and production of diapausing embryos during the season was required to survive each reset (N=5). Concomitant to ephemeral resets, every 9th week permanent cultures were cleaned to remove biofilm on the chamber walls and settled debris (including diapausing embryos, which sink and remain dormant until hatching is induced). Cleaned permanent chambers were refilled with their active populations and old medium, minus debris and diapausing embryos. Thus permanent cultures propagated asexually; sexual reproduction occurred but did not contribute to population growth due to removal of diapausing embryos.

Bioassays and Analysis

In the latter half of the 1st and the final two (5th and 6th) growth seasons, we conducted life history bioassays to assess total female lifespan and fecundity. Protocols followed those outlined in chapter three, but here we performed more extensive analyses of lifespan and reproduction, including factors independent of hydroperiod. Ovigerous females (P generation) were isolated from chemostat outflow and placed individually in 1 mL medium ($5x10^5$ cells mL^{-1} *T. suecica* in 15 ppt ASW) in the well of a 24 well plate. To establish a cohort from the F1 generation, the next day one neonate progeny from each female was transferred individually to a second plate in 1 mL medium; the mother was discarded. Every day, cohort females were transferred to fresh medium and their progeny (F2) were counted and discarded. For asexual females, sample size combining chemostats was 59, 66, and 71 in the 1st, 5th, and 6th season, respectively ($16 \leq N \leq 24$ per chemostat per season); permanent sample size was 53, 71, and 70 in the 1st, 5th, and 6th season, respectively ($17 \leq N \leq 24$ per chemostat per season). For sexual females, ephemeral sample size combining chemostats was 31, 42, and 65 in the 1st, 5th, and 6th season,

respectively (8≤N≤24 per chemostat per season); permanent sample size was 41, 29, and 22 in the 1st, 5th, and 6th season, respectively (3≤N≤22 per chemostat per season). Inability to distinguish cohort females as asexual (yielding female progeny) or sexual (male progeny) until they reproduced led to unequal sample sizes. Although sample size was low per chemostat, there was no effect of chemostats (see Results; $P>0.05$), and thus individuals could be pooled across chemostats for statistical analysis. During bioassays, location of test females from the six chemostats was randomized within and among 24 well plates.

Statistical analyses were performed in IBM SPSS v.18 at $\alpha=0.05$. Separate Cox regressions were performed as a proportional hazards (mortality) model analyzed with Wald's χ^2 statistic (Cox 1972, Fleming and Lin 2000) to test effects of the covariate time (growth season), hydroperiod, and the season*hydroperiod interaction on total lifespan, as well as pre-reproductive, reproductive, and post-reproductive lifespan. The reproductive phase was distinguished by presence of live progeny in the medium (versus unhatched eggs). Total fecundity per female was assessed via Generalized Linear Models with Poisson distribution, log link function, and Pearson χ^2 scale parameter. Lifespan-fecundity correlations were tested with non-parametric Spearman's rank correlation (two-tailed). A Multivariate General Linear Model (GLM) was used to test effects of season, hydroperiod, and hydroperiod*season on population summary statistics, specifically cohort generation time, T; net fecundity, Ro; and the rate of aging, ω.

Generation time and net fecundity were computed with PopTools (http://www.poptools.org). The rate of aging was calculated using the Weibull aging function as $\omega = \alpha^{1/(1+\beta)}$ (Ricklefs 2008); this is an alternative to the Gompertz model and

mortality rate doubling time, and is less sensitive to initial mortality rates (Ricklefs and Scheuerlein 2002). To compute ω, we first used SPSS for nonlinear regression to estimate the values of α and β from the formula $l_x = \exp(-m_o x - \frac{ax^{\beta+1}}{\beta+1})$ as in (Ricklefs 1998), where l_x represents the proportion of females surviving to age x, and m_0 is the initial mortality rate. Initial values were input as $m_0 = 0$, $\alpha = 0.0001$, and $\beta=3$; see (Ricklefs 1998) for comparison of parameters in mammals and birds.

Results

Separate Cox regressions on asexual and sexual females' showed lengthening of lifespan over time (six simulated growth seasons) for asexual but not sexual females (Figure 4.2). Results are based on cohort life history assays in the 1st and final two growth seasons (5th, 6th). Identity of the individual chemostat culture chamber did not significantly affect lifespan (asexual females: Wald's χ^2 statistic=9.690; df=5; P=0.085; sexual females: χ^2=4.246; df=5, P=0.515). Thus chemostats were pooled to test for effects of hydroperiod treatment (ephemeral or permanent) and the covariate time (growth season). For asexual females, there was a positive relation between lifespan and growth season (P<0.001) but no influence of permanent or ephemeral hydroperiod (Table 4.1). Mean asexual lifespan in days (± 1 SE) increased 26% over six seasons: season 1, 8.4 (± 0.3); season 5, 11.0 (± 0.2); season 6, 10.5 (± 0.3). However for sexual females, we did not find significant changes in lifespan with time or hydroperiod. For sexual females, mean lifespan in days (± 1 SE) did not change significantly: season 1, 8.1 (± 0.3); season 5, 8.5 (± 0.4); season 6, 8.4 (± 0.3).

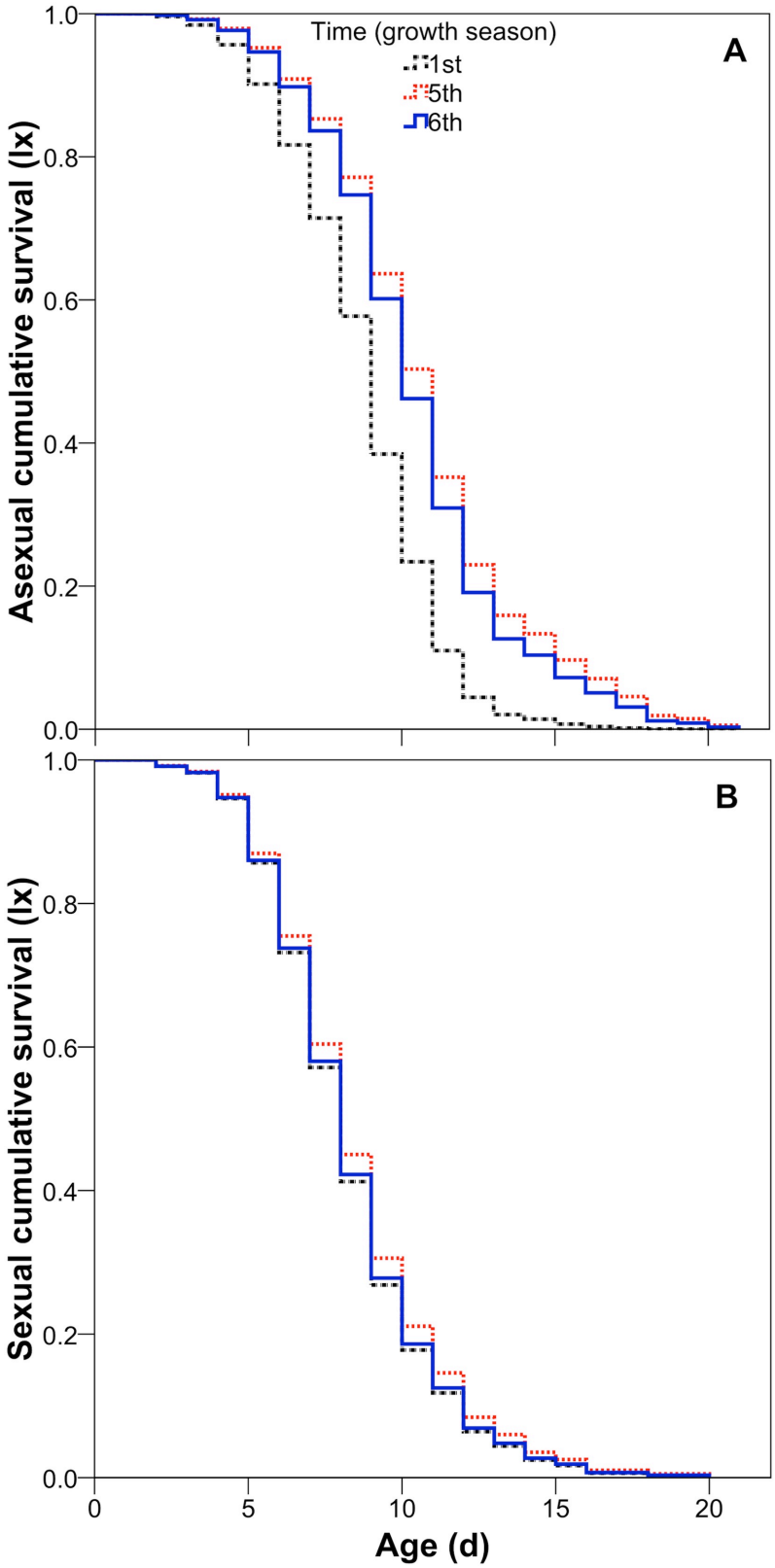

Figure 4.2. Survivorship curves of asexual (panel **A**) and unfertilized sexual (panel **B**) females. Curves depict the proportion (l_x) of females surviving to age x for the 1st and final two 9-week growth seasons (5th and 6th).

Table 4.1. Asexual female lifespan increased significantly with time in culture (season), as analyzed by Cox regression. For each term, df=1.

Life stage	Rotifer Type	Season		Hydroperiod		Hydroperiod*Season	
		Wald's χ^2	*P*	Wald's χ^2	*P*	Wald's χ^2	*P*
Total lifespan	asexual	32.203	**<0.001**	1.357	0.244	0.433	0.511
	sexual	0.088	0.767	0.151	0.697	0.040	0.841
Pre-reproductive lifespan	asexual	19.004	**<0.001**	0.151	0.698	0.364	0.547
	sexual	0.108	0.742	0.610	0.435	0.255	0.614
Reproductive lifespan	asexual	6.896	**0.009**	0.003	0.959	0.258	0.612
	sexual	0.039	0.844	0.444	0.505	1.392	0.238
Post-reproductive lifespan	asexual	38.349	**<0.001**	0.994	0.319	0.297	0.586
	sexual	0.248	0.619	0.412	0.521	0.017	0.898

The increase in total lifespan of asexual females resulted from an increase in reproductive and post-reproductive phases, with a small but significant decrease in the pre-reproductive phase shown by Cox regression (Figure 4.3). Chemostat effects were not significant on any phase for either asexual or sexual females ($P \geq 0.082$); hence, chemostats were combined for analyses. For asexual females, there was a significant effect of season on the pre-reproductive, reproductive, and post-reproductive phases ($P \leq 0.009$), but no effect of hydroperiod (Table 4.1). In contrast for sexual females, there was no significant effect of any factor on pre-reproductive, reproductive, or post-reproductive lifespan ($P \geq 0.435$ for all factors and lifespan phases).

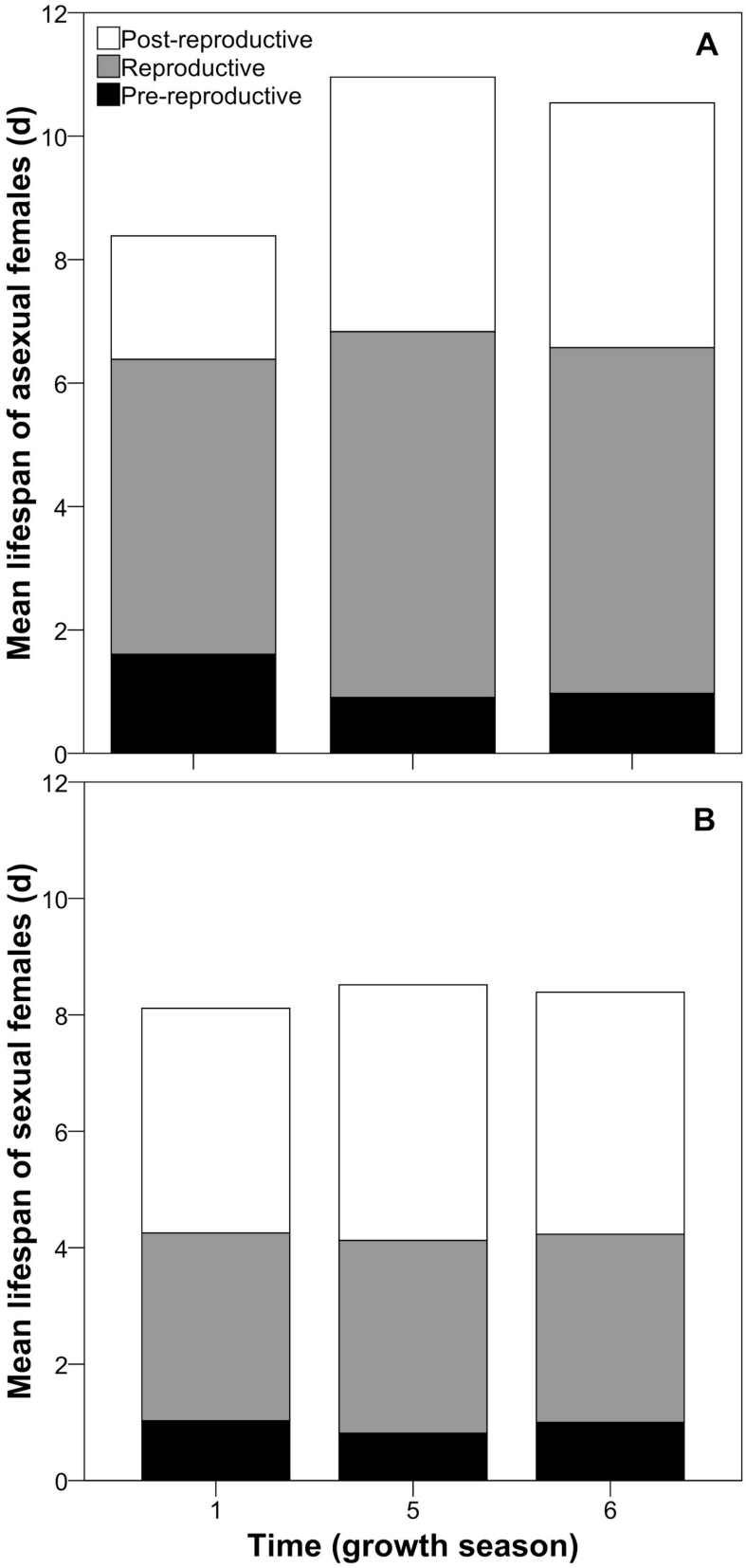

Figure 4.3. Pre-reproductive, reproductive, and post-reproductive lifespan of (**A**) asexual and (**B**) unfertilized sexual females.

Generalized linear models of asexual fecundity showed significant increases in later seasons but no effects of hydroperiod, and no evolutionary change in sexual female fecundity, as reported in a companion study on evolution of sex and dormancy in response to hydroperiod (chapter three). Here we note that asexual lifetime fecundity increased by 56% across seasons, from a mean of 11.8 progeny (± 0.5) in the 1st season to 18.4 (± 0.3) in the 6th season. We report a positive correlation between total fecundity and female lifespan for asexual females (Figure 4.4) pooling data for seasons 1, 5, and 6 (Spearman's rho=0.412, P<0.001); similar results were found for sexual females (Spearman's rho=0.237, P<0.001). After controlling for the effect of season via partial correlation analysis, the positive correlation was still significant for asexual (Spearman's partial rho=0.340, P<0.001) and sexual (Spearman's partial rho =0.238, P<0.001) females. Analyzing seasons individually revealed the correlation of fecundity and lifespan was weaker for the final two seasons compared to the initial season, with 77% lower correlation coefficients for data from the last two seasons combined (asexual Spearman's rho=0.137, P=0.023; sexual Spearman's rho=0.160, P=0.044) versus in the first season (asexual Spearman's rho=0.609, P<0.001; sexual Spearman's rho=0.402, P=0.001).

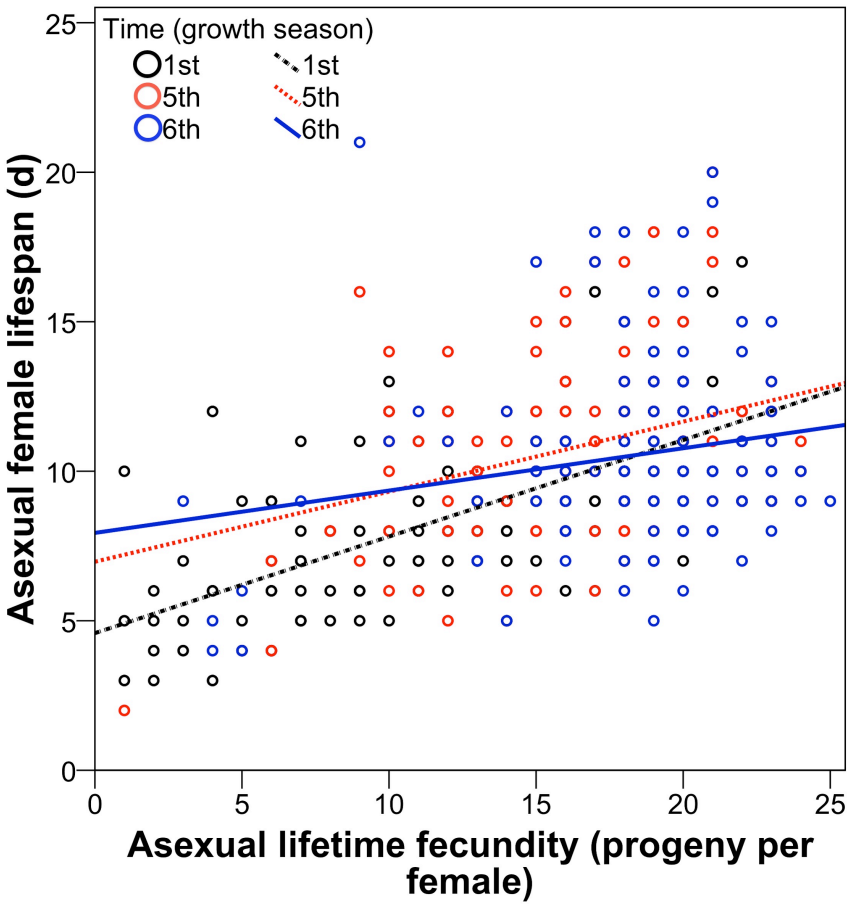

Figure 4.4. Correlation of total lifetime fecundity and lifespan for asexual females. Trendlines from separate linear regressions of data from seasons 1, 5, and 6 are included to facilitate visualization of lifespan-fecundity relationships. After controlling for the effect of season via partial correlation analysis, the positive correlation between fecundity and lifespan was still significant for asexual females (Spearman's rho=0.340, $P<0.001$).

Levene's test for homogeneity of variances revealed no change in the variance for asexual female lifespan among seasons 1, 5, and 6 (P=0.744), with standard errors of 0.28, 0.24, and 0.25, respectively. However, variance decreased significantly for lifetime fecundity of asexual females (P<0.001), with standard errors in seasons 1, 5, and 6 of 0.52, 0.35, and 0.34, respectively.

For asexual females, a multivariate General Linear Model (GLM) with growth season as a covariate testing for effects on three population summary statistics—generation time (T) (Figure 4.5), net reproductive rate (Ro) (Figure 4.6), and the rate of aging (ω) (Table 4.2)—showed a significant effect of season (season: Wilk's λ, F=11.443, df=3, P=0.003). There was no effect of chemostat, hydroperiod, or the season*hydroperiod interaction ($P \geq 0.440$ each). Post-hoc tests showed a significant decrease of T (P=0.010), increase of Ro (P=0.002), and decrease of ω (P<0.001) across seasons (Table 4.3). For sexual females, a GLM testing for effects on T, Ro, and ω again showed significant effects of season (Wilk's λ, F=4.859, df=3, P=0.033), but not chemostat, hydroperiod, or hydroperiod*season ($P \geq 0.083$). Post-hoc tests showed that season only significantly affected T, which decreased slightly across seasons (P=0.004). There was no significant effect of season on either Ro or ω ($P \geq 0.287$ each).

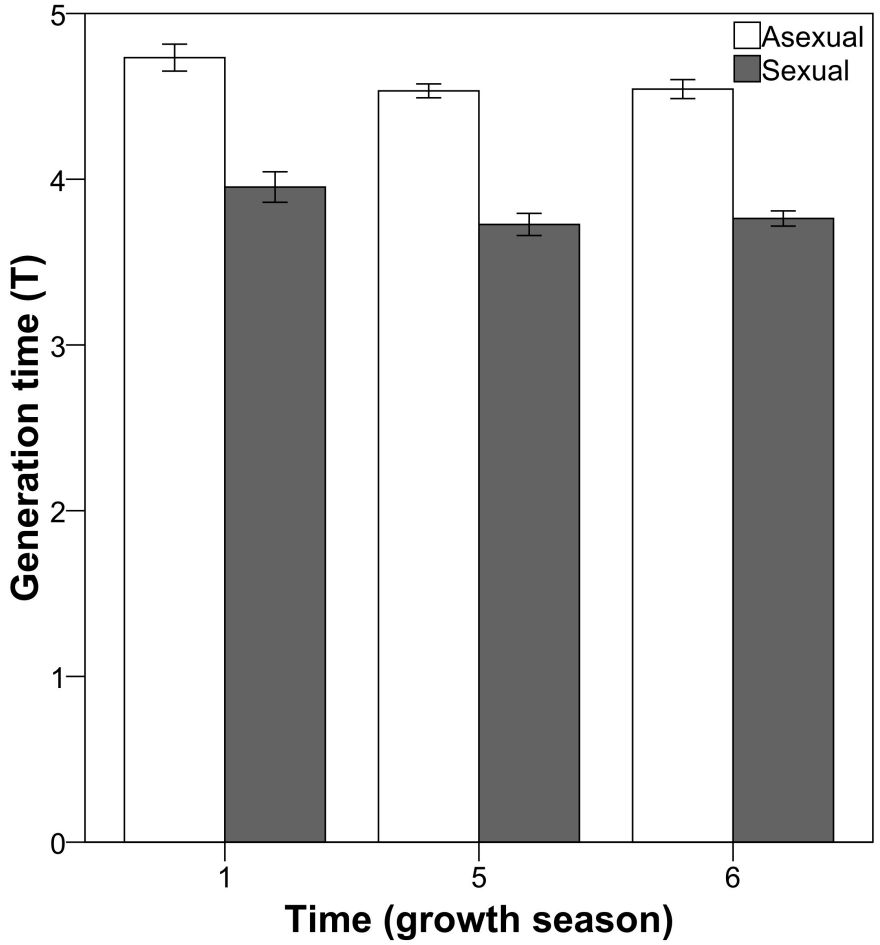

Figure 4.5. Generation time of asexual and sexual females for the first and final two seasons. Bars represent means (± 1 SE).

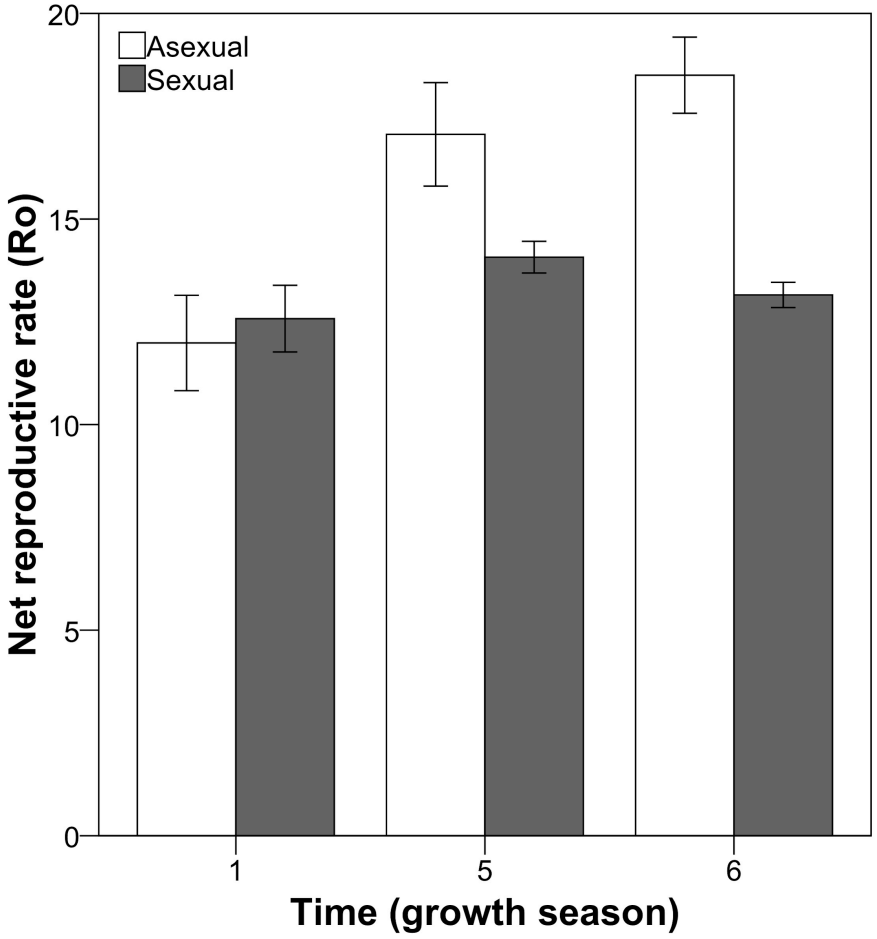

Figure 4.6. Net reproductive rate of asexual and sexual females for the first and final two seasons. Bars represent means (± 1 SE).

Table 4.2. Rate of aging (ω) of asexual and sexual female rotifers. Initial inputs for nonlinear regression estimation of parameters were m_o=0, α=1.00E-4, β=3; values of ω were computed per chemostat from estimated parameters and averaged across chemostats. Reported values are means for the six chemostats (± 1 SE). Parameter estimation and calculation of ω followed (Ricklefs 1998).

Growth season	Rotifer type	m_o	α	β	ω (d^{-1})
1	asexual	1.17E-3 (1.17E-3)	5.61E-3 (2.38E-3)	2.28 (0.31)	0.167 (0.005)
5	asexual	3.95E-3 (3.57E-3)	5.82E-5 (4.80E-5)	4.49 (0.42)	0.120 (0.003)
6	asexual	1.47E-3 (6.95E-4)	6.31E-4 (4.76E-4)	4.09 (0.70)	0.128 (0.004)
1	sexual	0.00 (0.00)	7.58E-3 (4.28E-3)	2.33 (0.39)	0.173 (0.007)
5	sexual	9.34E-3 (6.83E-3)	1.64E-3 (7.57E-4)	2.96 (0.45)	0.155 (0.010)
6	sexual	3.56E-3 (2.70E-3)	3.15E-3 (1.43E-3)	2.99 (0.52)	0.169 (0.007)

Table 4.3. Net reproductive rate and rate of aging evolved in asexual but not sexual females. These post-hoc tests were run after multivariate GLM with Wilk's λ confirmed significant impacts of season on the combination of the three rotifer life history factors (asexual: P=0.003; sexual: P=0.033).

Rotifer type	Generation time			Net reproductive rate			Rate of aging		
	F	df	*P*	F	df	*P*	F	df	*P*
Asexual	10.109	1	**0.010**	16.878	1	**0.002**	32.729	1	**<0.001**
Sexual	13.808	1	**0.004**	1.267	1	0.287	0.481	1	0.504

Discussion

Our study represents one of the first to experimentally investigate intrinsic and environmental factors affecting evolvability of the metazoan lifespan. Hydroperiod (aquatic habitat permanence) did not significantly affect lifespan. Inhabitants of temporary waters including cladocerans (Dudycha and Tessier 1999, Jocque et al. 2010) and juvenile phases of aquatic insects (Wissinger et al. 2003, De Block et al. 2008) often show faster development rates than their permanent water counterparts. *Hexarthra* sp. rotifers in extremely ephemeral desert rock pools that may fill and dry within days show faster development rates than most rotifer species (Schröder et al. 2007). Potentially the long (9 week) hydroperiod relative to the rotifer lifespan (~1–3 weeks) for our study did not impose strong enough selection to cause evolution of faster, shorter life histories. Alternatively, other traits associated with dormancy (desiccation resistance) may be more responsive to hydroperiod conditions (Gilbert and Diéguez 2010, Schröder and Walsh 2010, Campillo et al. 2011). However, time in culture (seasons) and reproductive physiology did lead to rapid evolution of life history traits. We observed a 26% increase in lifespan and a 56% increase in fecundity by asexual female rotifers over the 385 d (~84 generations) in laboratory culture. These findings have implications for understanding physiological influences on life history evolution, existence or absence of trade-offs in longevity versus fecundity, and theories for the evolution of senescence.

Physiological status as an asexual or sexual female substantially influenced the evolvability of *Brachionus plicatilis* s.s. lifespan and fecundity. Contribution of sexual versus asexual reproduction itself to evolvability appeared inconsequential, because of the absence of a difference between ephemeral cultures (which underwent 5 bouts of sex

between growth seasons) and permanent cultures (which underwent uninterrupted asexual propagation; sexually produced embryos were removed from the cultures). Yet reproductive physiological status of the females did contribute to evolvability. Initially, mean lifespans for asexual and sexual females were similar, but only asexual females showed a significant increase in mean lifespan and decrease in the rate of aging. Similarly, only asexual females showed significant increases in fecundity. Except for a slight decrease in generation time (Figure 4.5), sexual females did not show significant evolution for any of the life history parameters measured.

Studies in other taxa have shown that reproductive physiological status can affect aging, congruent with our findings. Differences in longevity or mortality risks between males and females exist for several organisms, ranging from invertebrates such as rotifers, seed beetles, and spiders to vertebrates such as birds and mammals (Clutton-Brock and Isvaran 2007, Bonduriansky et al. 2008, Ricklefs 2008, Bilde et al. 2009, Stoltz et al. 2010, Wallace and Snell 2010). Specific mechanisms by which reproductive physiology affects longevity can vary, and the relation of reproductive physiology to lifespan can be complex or indirect. However, recent studies are beginning to provide insight into sexual dimorphism; absence of an apolipoprotein D homolog in the fruit fly *Drosophila* reduces lifespan in males only (Ruiz et al. 2011). Gender also impacts the hormesis response (life extension from mild stress like heat), with male fruit flies often experiencing larger increases in lifespan (Sarup and Loeschcke 2011). In Australian redback spiders (*Latrodectus hasselti*), mated females live longer than unmated females (Stoltz et al. 2010). Beyond aging, differences between males and females exist in the extent to which parental haplotypes and rearing environment affects mass of reproductive

organs in crickets (*Teleogryllus oceanicus*) (Nystrand et al. 2011). Endocrine pathways and differential gene expression may mediate influences of reproductive status and gender on lifespan. For example, the sex hormone testosterone leads to expression of sexual traits in males but also has immunosuppressive effects and can increase sensitivity to oxidative stress (Alonso-Alvarez et al. 2007). Oxidative stress in turn may increase mortality and aging-associated damage or disease (Alonso-Alvarez et al. 2007, Muller et al. 2007, Monaghan et al. 2008). Sex steroids are known to influence oxidative stress resistance, or aging directly, in mice, *Drosophila*, and the nematode *Caenorhabditis elegans* (Baba et al. 2005, Russell and Kahn 2007, Gáliková et al. 2011).

Our study extends these reports of how longevity varies depending on reproductive status, to reveal the potential for differences in evolution of life history. This finding is similar to that from a study in beetles (*Callosobruchus maculatus*) showing differential lifespan evolution in males and females (Fox et al. 2011). It is known that asexual and sexual female rotifers differ in traits such as thermal tolerance (Snell 1986, Wallace and Snell 2010), but mechanisms underlying this trait differentiation are poorly understood. We hypothesize that endocrine signaling pathways differentiate asexual and sexual females, and that such pathways—potentially themselves involving or regulated by epigenetic mechanisms—underlie the differential evolvability reported here. Rotifers are known to respond to steroid hormones and possess associated receptors (Smith et al. 2011, Snell 2011). Future studies looking at hormonal and physiological differences between asexual and sexual females would be particularly useful to provide candidate pathways for exploration. The ability to generate animals of the same genotype but with different reproductive physiology (Figure 4.1) recommends rotifers as a particularly

useful model for studying mechanisms beyond traditional Mendelian inheritance that affect life history evolution. Genetic interactions and redundancy also may affect the outcome of mutations and genetic penetrance (Burga et al. 2011), and hence provide another potential mechanism for the differential asexual and sexual evolvability. Ongoing next-generation sequencing approaches in our laboratory are being used to investigate gene expression associated with the evolution of rotifer aging, and may help elucidate molecular controls of this evolution.

Epigenetic factors may in part explain the distinction between female types, either by regulating activity of endocrine signaling pathways as suggested above, or by more directly influencing expression of genes important to reproduction and lifespan. Reasons for the differential evolution of asexual and sexual females likely do not reflect genotypic differences. A single asexual female can give birth to both asexual and sexual daughters (Wallace et al. 2006, Fussmann et al. 2007a), resulting in identical genotypes (Figure 4.1). What remains to distinguish asexual and sexual females' reproductive phenotypes, and in turn their different evolvability, may be epigenetic factors. Lifespan appears to be at least partially heritable, perhaps due to transgenerational epigenetic inheritance (Youngson and Whitelaw 2008, Johnson and Tricker 2010). Females in both of the final two growth seasons lived longer than females in season 1, although culture (chemostat and bioassay) conditions were not changed between seasons. This suggests the increased longevity represents an evolutionary change, as opposed to phenotypic plasticity in response to varying conditions. It may be that epigenetic factors modulate expression of genes that determine sexual status of females, that these factors also (directly or indirectly) affect certain life history traits, and that only the factors present in asexual

females evolved. Alternatively, both female types may have undergone evolution in genes controlling life history traits, but different epigenetic and/or endocrine regulation of gene expression only allowed phenotypic manifestation of longer lifespan and higher fecundity in asexual females.

Besides presenting implications and hypotheses for physiological and molecular mechanisms influencing evolvability, our results have import for understanding theories of the evolution of aging. The chemostat cultures were devoid of many of the extrinsic mortality factors that would occur in natural environments, such as abiotic (e.g., salinity and temperature fluctuations) or biotic stressors (e.g., predation, starvation) (Snell 1986, Miracle and Serra 1989, Schneider and Frost 1996, Wallace et al. 2006, Wallace and Snell 2010, Kuefler et al. 2012). It has been hypothesized that removal of extrinsic, environmental mortality factors may lead to evolution of longer lifespan (Williams 1957, Williams et al. 2006, Monaghan et al. 2008). This suggestion, sometimes referred to as Williams' hypothesis (Williams et al. 2006), may explain the increased lifespan that we observed in our rotifer cultures. Not only did total lifespan increase, but also the reproductive lifespan and post-reproductive lifespan increased, with an earlier onset of reproduction and increase in fecundity of asexual females. These shifts may suggest an overall increase in fitness. Prior studies reveal the potential for an increase in several fitness components in novel environments, potentially through effects on genetic correlations (Service and Rose 1985, Ackermann et al. 2007). This idea is consistent with research suggesting that endocrine signaling (e.g., insulin pathways) influences both lifespan and health, and can be altered to delay both mortality and somatic signs of aging (Ricklefs 2008, Selman et al. 2008, Bartke 2011).

The concomitant increase in reproduction and lifespan and their positive correlation do not demonstrate the typically negative relationship proposed by the antagonistic pleiotropy and disposable soma theories of aging (Hughes and Reynolds 2005, Monaghan et al. 2008). The antagonistic pleiotropy hypothesis focuses on trade-offs in genes affecting longevity and fecundity, while the disposable soma is based on trade-offs in resource allocation to somatic maintenance versus reproduction (Monaghan et al. 2008, Kirkwood and Melov 2011). To date evidence of trade-offs in reproduction and lifespan is mixed (Anderson et al. 2011, Flatt 2011). Some studies show a trade-off (Doblhammer and Oeppen 2003, Flatt 2011), whereas in social insects and naked mole rats breeders may live longer than non-reproductive individuals (Dammann et al. 2011), and animals in captivity were found to show no trade-off in longevity and reproduction (Ricklefs and Cadena 2007). Absence of a trade-off as in our study may by default lend support to an alternative theory of senescence—Medawar's mutation accumulation hypothesis. Medawar's theory suggests that due to non-senescent (e.g., environmental) factors, few individuals live long enough for selection to be sufficiently strong in late age classes to prevent mutation accumulation, which ultimately leads to mortality (Monaghan et al. 2008). Alternatively, in nature resources (e.g., algal food) could be limiting and lead to trade-offs, in concordance with the disposable soma idea of varying allocation levels to reproduction and somatic maintenance (Ricklefs and Cadena 2007). If the disposable soma theory applies to our system, one may expect limiting food or other resources in chemostats (or wild populations) to result in the expected trade-off of lifespan and fecundity. Indeed, calorically restricted *B. plicatilis* live longer but have fewer progeny (Kaneko et al. 2011). Yet effects of caloric restriction can vary among species in the *B.*

plicatilis complex, and also may depend on methods of inducing caloric restriction (Mark Welch, pers. comm.). Additional research is needed to clarify the role of trade-offs in evolutionary trajectories, particularly as physiological trade-offs may occur in the absence of an evolutionary genetic trade-off (Flatt 2011).

The increased lifespan and fecundity of asexual females may represent clonal selection from genetic variation present in the founding population, yet this seems unlikely. In this scenario, one of the initial 15 clonal lineages (genotypes) used to inoculate the chemostats could have replaced the others. For instance, a fecund lineage also may have happened to possess a long lifespan, such that the population came to have a longer lifespan (and slower rate of aging) if this lineage came to dominate populations due to its high fecundity. A decrease in variance would be expected if a trait's evolution represented clonal replacement. Clonal selection appears possible for fecundity given that variance in fecundity decreased, but variance for lifespan did not decrease. Moreover, the maximum asexual female lifespan in the 6^{th} growth season was 24% greater than the maximum seen in the initial season (17 d initially, versus 20 d and 21 d in seasons 5 and 6, respectively). These results further suggest that lifespan evolution was not due to clonal replacement, because no observed female in the 1^{st} season lived as long as those observed in the last seasons. Finally, strength of the correlation between fecundity and lifespan decreased over time for asexual females, which appears incongruent with the hypothesis that clonal selection for increased fecundity (or lifespan) led to increased lifespan (or fecundity).

Conclusions

In summary, herein we have shown rapid evolution of slower aging rates in a

benign culture environment. Evolution of longer lifespan, slower aging, and higher fecundity in asexual but not sexual females suggests reproductive physiology is related to evolvability of aging. For humans, this may suggest the importance of considering the potential of gender to influence response to treatments aimed at life extension. Also, the observed increase in rotifers' post-reproductive lifespan is intriguing and worthy of further study, because post-reproductive individuals should be immune to selection pressures given that they do not contribute to future generations (Kirkwood and Melov 2011). This may represent an indirect effect, such as decreased extrinsic mortality factors and non-limiting resources (continuous algal food inflow) allowing for longer post-reproductive life. The benign chemostat environment could ameliorate resource trade-offs allowing for high investment in both somatic maintenance and fecundity. Fully elucidating the influences on evolvability of life history traits will require careful consideration of both extrinsic and intrinsic factors, and ways in which the environmental context may affect observed trends. Tests of mechanisms and the role of epigenetic and endocrine pathways will be particularly informative to ascertain how life history evolution proceeds.

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CHAPTER 5

MOLECULAR EVOLUTION OF THE MEMBRANE ASSOCIATED PROGESTERONE RECEPTOR IN THE *BRACHIONUS PLICATILIS* (ROTIFERA, MONOGONONTA) SPECIES COMPLEX

(This chapter has been published in the journal Hydrobiologia. The original publication is available at www.springer.com, or http://dx.doi.org/10.1007/s10750-010-0484-4.)

Abstract

Many studies have investigated physiological roles of the membrane associated progesterone receptor (MAPR), but little is known of its evolution. Marked variations in response to exogenous progesterone have been reported for four brachionid rotifer species, suggesting differences in progesterone signaling and reception. Here we report sequence variation for the *MAPR* gene in the *Brachionus plicatilis* species complex. Phylogenetic analysis of this receptor is compared with relatedness based on *cytochrome c oxidase subunit 1* sequences. Nonsynonymous to synonymous site substitution rate ratios, amino acid divergence, and variations in predicted phosphorylation sites are examined to assess evolution of the MAPR among brachionid clades.

Introduction

Considering the roles of hormones in moderating life history traits (Ketterson and Nolan 1999), studies of sex steroid hormones and their receptors may be key to understanding life cycle transitions. In *Brachionus manjavacas,* exogenous progesterone can increase mixis rates (Snell and DesRosiers 2008). Searches of a brachionid

transcriptome yielded an expressed sequence tag (EST) contig identified as a potential membrane associated progesterone receptor (MAPR) (Snell and DesRosiers 2008). The MAPR gene family is proposed to have originated from an ancestral cytochrome *b5* (Cyt *b5*), and contains Neudesin and the vertebrate-specific paralogs progesterone receptor membrane component (PGRMC) 1 and 2 (Cahill 2007). Functions of MAPR proteins vary across phyla and range from inhibition of apoptosis in ovarian granulosa cells to cholesterol synthesis and axon guidance (Cahill 2007, Rohe et al. 2009). While progesterone binding cannot be assumed by homology, it is notable that the *Brachionus* putative MAPR is the only known candidate receptor for progesterone. Chemical signals mediate brachionid rotifer mate recognition and mixis induction (Snell et al. 2006, Snell et al. 2009), but little is known of their evolution.

Through phylogenetic and substitution rate analyses and structural protein modeling we provide further evidence that this EST is a rotifer homolog of membrane associated progesterone receptors, and report on molecular evolution of the rotifer MAPR. Sex-related genes are associated with higher tendencies for positive selection (Civetta and Singh 1998). Thus, given the ability of progesterone to affect rotifer reproduction, the receptor may undergo positive selection. The fact that some rotifers in the *B. plicatilis* species complex increase diapausing embryo production in response to exogenous progesterone, while others remain unaffected at the same concentrations (Snell and DesRosiers 2008), reveals the potential for variations in receptor sequence. This could cause differential responses to the ligand. We conduct a phylogenetic analysis to assess evolution of the MAPR in the *B. plicatilis* complex, and test for positive, purifying, or neutral selection using ratios of nonsynonymous to synonymous site

substitution rates (d_N/d_S). Ratios of $d_N/d_S > 1$ suggest positive selection, ratios < 1 indicate purifying selection, and ratios = 1 imply neutral evolution. We also examine amino acid variation in the predicted protein structure.

Methods

Cultures

We studied five taxa of the larger L morphotype: *B. plicatilis sensu strictu* of Poza Sur, Spain; *B. plicatilis s.s.* of Tokyo, Japan; *B. manjavacas* of the Azov Sea, Russia; *B. plicatilis* "Austria" of Tianjin, China (hereafter "Austria"); and *B. plicatilis* "Nevada" of Little Fish Lake, Nevada, USA (hereafter "Nevada"). We examined three taxa of the smaller SS morphotype: *B. rotundiformis* of Poza Sur, Spain; *B. rotundiformis* of the Adriatic Sea, Italy; and *B. rotundiformis* of Hawaii (obtained from the Oceanographic Institute of Hawaii; exact collection site unknown). Taxonomy and morphotype classification follows prior descriptions (Gómez et al. 2002, Snell and Stelzer 2005, Fontaneto et al. 2007). Diapausing embryos were hatched at 25ºC in 15 ppt artificial seawater (ASW, Instant Ocean). For each taxon, we used a single hatchling to initiate a clonal lineage, kept at 22ºC in 15 ppt ASW and fed *Tetraselmis suecica*. Embryos and hatchlings were kept near 2000 lux fluorescent lights.

DNA Isolation, Amplification, Cloning, and Sequencing

Roughly 100–500 clonemates from ≥ 2 replicate cultures were filtered with 68 µm Nitex mesh, then rinsed into a Petri plate with 15 ppt ASW. Rotifers were ground with a pestle in a microcentrifuge tube with 180 µL of ATL buffer of the DNeasy Blood and Tissue kit (Qiagen). DNA was extracted immediately using the DNeasy kit. PCR

amplification and sequencing were performed for both the nuclear *MAPR* and mitochondrial *cytochrome c oxidase subunit 1* (*cox1*) genes. Sequences of *cox1* for four taxa and *MAPR* homologs across eukaryotes were extracted from GenBank (accessions in Figures 5.1A, 5.2); all other sequences were obtained in this study and deposited in GenBank (HM024707–HM024718). PCR conditions are available upon request. The LCO1490 and HCO2198 primers were used to amplify *cox1* (Folmer et al. 1994), modified by eliminating the first 6 bases at the 5' end of LCO1490. Following prior identification of a progesterone receptor partial transcript from searches in an EST library (Snell and DesRosiers 2008), 5'-RACE or rapid amplification of complementary DNA ends (Frohman et al. 1988) was performed on a cDNA library to obtain the complete coding sequence for the current study. *MAPR* primers were designed from complete coding sequences from 5'-RACE of *B. manjavacas* (Russia) and *B. plicatilis s.s.* (NH1L, Japan). These were MAPR.F1 (5'-ATGCCAGAAGCGTTTGCTATGG-3'), beginning at position 1 of the coding sequence and MAPR.R1 (5'-TAACTTCGGCTGACTCTTCTTCGT-3'), ending 11 bases upstream of the stop codon.

Amplicons were PCR purified after visualizing samples via gel electrophoresis or extracted with a MinElute Gel Extraction kit (Qiagen). Products of ≥ 2 separate thermal cycling reactions were combined, ligated into pCRII-TOPO vector, and transformed into chemically competent TOP10 cells with the TopoTA Cloning Kit (Invitrogen). Colonies were subcloned, and plasmids purified using the QIAprep Spin Miniprep Kit (Qiagen). From each cloning reaction, ≥ 8 plasmids were sequenced in forward and reverse with M13 primers on an ABI 3730*xl* Genetic Analyzer with an ABI Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Assembly and Analysis

Initial editing and assembly used unix shell scripts from the Josephine Bay Paul Center of the Marine Biological Laboratory, combining *phred*, *cross_match*, and *phrap* (Ewing and Green 1998). Chromatograms were reviewed in *consed* (Gordon et al. 1998). Reads were aligned in Clustal X 2.0 (Larkin et al. 2007), and consensus sequences from replicate plasmids from the same cloning reaction were made in BioEdit (Hall 1999). We compared mRNA reads from 5'-RACE with DNA sequences to identify intronic v. coding regions.

The translated *B. manjavacas MAPR* sequence was submitted to TMHMM server v. 2.0 (Krogh et al. 2001) and Pfam (Finn et al. 2008) to identity transmembrane helix and functional domains. Protein sequences of MAPR homologs from species representing plants, fungi, and an array of metazoans were aligned with the amino acid translation of *B. manjavacas MAPR* using the Espresso module of T-Coffee (Notredame et al. 2000). Because the amino and carboxy termini were poorly conserved, phylogenies were constructed from both the full-length alignment and the high-quality region of the alignment corresponding to positions 53–161 of the *B. manjavacas* peptide using maximum likelihood (ML) and Bayesian methods with the WAG model of amino acid change with a gamma shape correction, as chosen by ProtTest (Abascal et al. 2005). For ML, four independent runs of Garli 0.96b8 (Zwickl 2006) were used to find the best tree and 1000 bootstrap replicates were examined to determine support for each node. For Bayesian inference, two independent runs of MrBayes v3.1 (Huelsenbeck and Ronquist 2001) using 4 chains and 2 million generations each converged on the same tree and parameter values; the first 1 million generations were discarded as burn-in to generate

posterior probability support for each node.

Regions between PCR primers (163 codons plus the intron for *MAPR*, 201 codons for *cox1*) were used for phylogenetic analysis among brachionids. Gene trees of *cox1* and *MAPR* were generated using MrBayes 3.1 (Huelsenbeck and Ronquist 2001), with nucleotide frequencies and parameters for the GTR + gamma model estimated independently for codon first + second positions and codon third positions (+ intron positions for *MAPR*). Two independent runs of four chains were run for 2 million generations and sampled every 100 generations; comparison of parameter estimates indicated convergence (Gelman and Rubin 1992). The first 1 million generations were discarded as burn-in and consensus trees examined with FigTree v1.2.2 (Rambaut 2009). Consensus trees and sequence alignments (without the *MAPR* intron) were input in codeml in PAML 4.0 (Yang 2007) to estimate d_N and d_S. Likelihood ratio tests supported use of codon tables to estimate codon frequency for both genes (CodonFreq=3 in the codeml control file); transition/transversion and d_N/d_S ratios were estimated from the data. Tests for selection were M0 (default codeml parameters) v. M3 (Nsites=3, ncat=3); M1a (Nsites=1) v. M2a (Nsites=2); and M7 (Nsites=7, ncat=10) v. M8 (Nsites=8, ncat=10) (Zhang et al. 2005); results were evaluated by likelihood ratio tests (Yang and Nielsen 2002). Competing tree topologies were evaluated via the Kishino Hasegawa test (Kishino and Hasegawa 1989) in codeml.

The 3-dimensional structure for MAPR was predicted by submitting the *B. manjavacas* translated coding sequence to SWISS-MODEL for automated comparative modeling (Schwede et al. 2003). Structures were visualized in Cn3D (Wang et al. 2000) after conversion to the appropriate format in VAST (Gibrat et al. 1996). Amino acid (aa)

substitutions were classified as conservative, moderately conservative, moderately radical, or radical (Li et al. 1984). MAPR sequences were scanned for PROSITE motifs with ScanProsite (de Castro et al. 2006).

Results

The rotifer *MAPR* amplified region, which began with the presumptive start codon and ended 11 bases before the stop codon, consisted of 535 bp of coding sequence (178 aa), split by an intron of 53–54 bp that began after coding position 458. One transmembrane helix was predicted and the only domain with a significant E-value found by Pfam was a cytochrome *b5*-like heme/steroid binding domain (Figure 5.1A), a domain found in MAPRs of other eukaryotes (Mifsud and Bateman 2002). A search of the NCBI nr database using blastp revealed that the most similar sequences contained this domain and were annotated as membrane associated steroid or progesterone receptors. In phylogenetic analyses of a diverse set of MAPR homologs, although support for nodes was generally poor different methods returned the same best tree topology with the *B. manjavacas* MAPR grouped deeply within the clade of metazoan MAPRs (Figure 5.1B), distinct from conserved paralogs cytochrome *b5* and Neudesin (Cahill 2007).

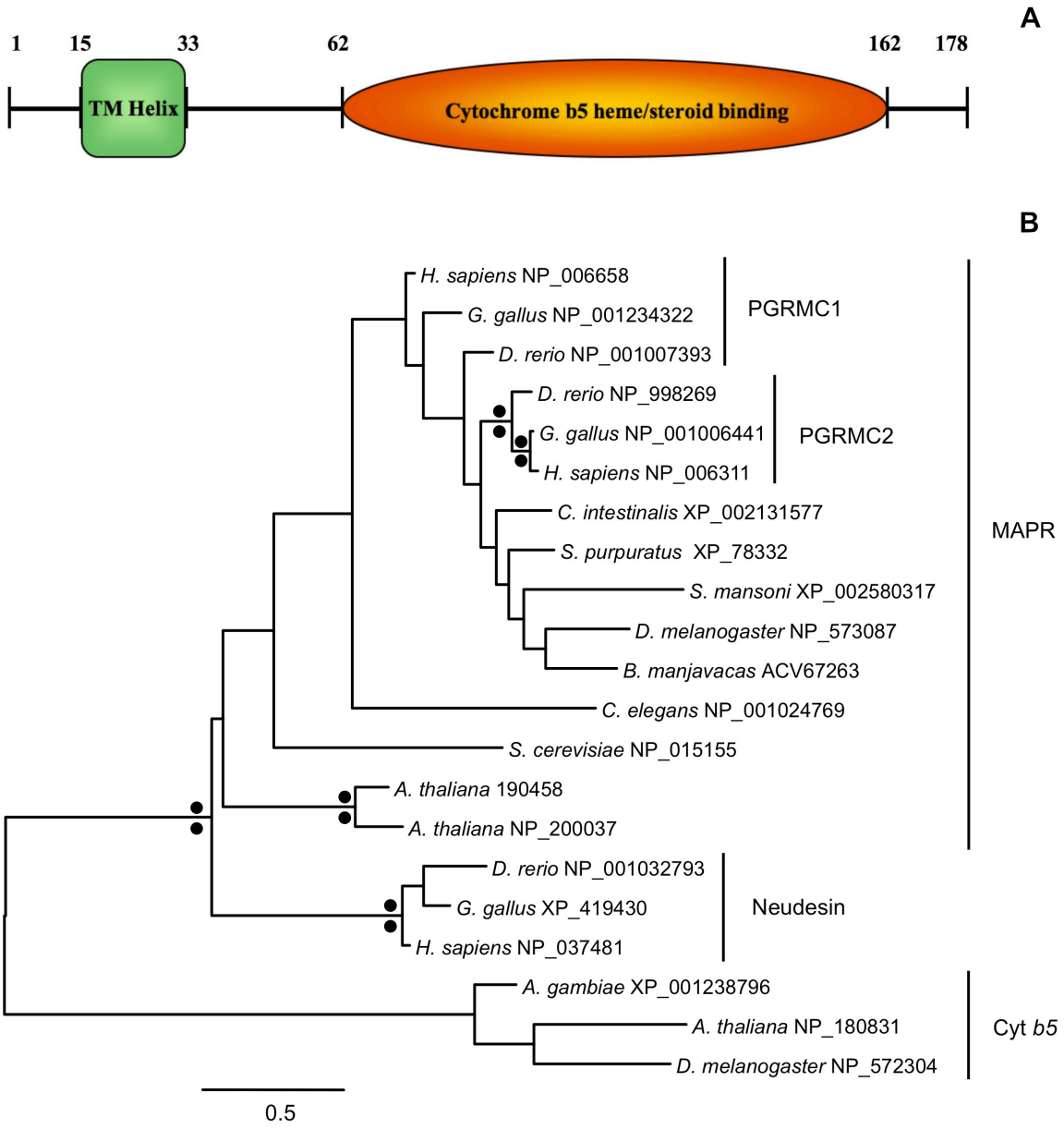

Figure 5.1. (Top, **A**) MAPR transmembrane and heme/steroid binding regions; numbers are amino acids. (Bottom, **B**) Phylogeny of MAPR proteins and paralogs; the tree is drawn with branch lengths determined by Garli (scale bar shows changes per amino acid position); the same topology was found with MrBayes and a very similar topology was found with RaxML (not shown). Solid circles above and below nodes show that greater than 70% of bootstrap datasets supported the node by Garli and greater than 95% of sampled trees after burn-in supported the node with MrBayes (posterior probability), respectively.

The gene tree of *Brachionus MAPR* sequences (Figure 5.2) was consistent with established phylogenetic relationships among *Brachionus* spp. based on *cox1* and *ITS* (Gómez et al. 2002, Suatoni et al. 2006). In contrast, the Bayesian consensus tree for *cox1* had no significant support for relative positions of "Austria," "Nevada," *B. manjavacas*, and *B. plicatilis s.s.*, and arranged the *B. plicatilis* clade as ("Austria"(*B. plicatilis s.s.*("Nevada", *B. manjavacas*))), an inaccurate arrangement based on the clades' placement in prior studies (Gómez et al. 2002, Suatoni et al. 2006). All codeml analyses using *cox1* were performed on the topology of both the *cox1* Bayesian consensus tree and the *MAPR* tree; resulting likelihoods were compared with the Kishino Hasegawa test. In no case was one topology significantly better than the other. Thus, further analysis and trees reported in this study (Figure 5.2) only use the topology from the *MAPR* consensus tree. Tests for positive selection showed no significant difference in d_N/d_S between branches or across sites, with strong purifying selection for both genes. Allowing variation of d_N/d_S across branches did not result in a significantly better model than keeping the ratio constant (for the latter model, $d_N/d_S = 0.06$ for *MAPR* and 0.0003 for *cox1*), but yielded values of d_N up to 76x higher and d_S up to 79x lower for *MAPR* v. *cox1* (Table 5.1).

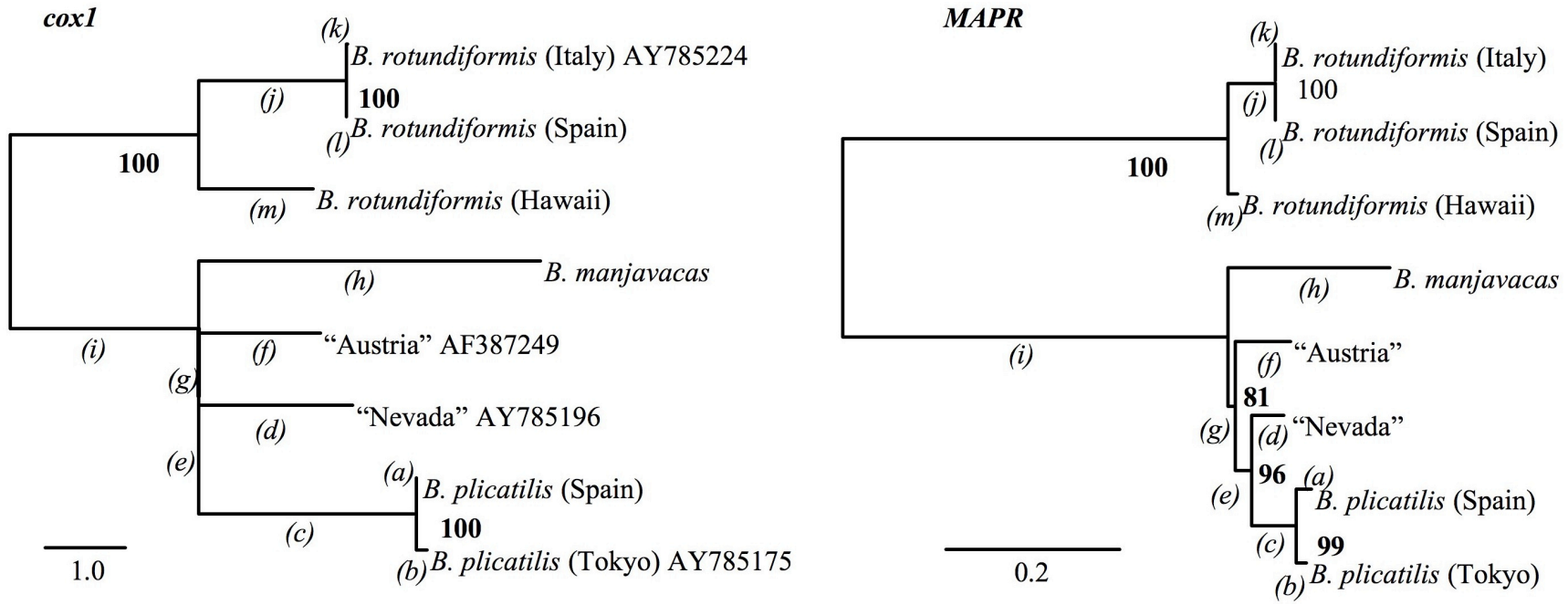

Figure 5.2. Bayesian gene trees of *cox1* and *MAPR* with branch lengths from codeml. Scale bars are number of nucleotide changes per codon. Letters identify branches in Table 5.1; numbers are Bayesian posterior probabilities (percentages). Accessions are given for *cox1* sequences taken from GenBank.

Table 5.1. Values of d_N, d_S, and d_N/d_S for *MAPR* and *cox1* tree branches, with labels as in Figure 5.2.

	coxI			***MAPR***			***coxI* : *MAPR***	
Branch	$\mathbf{d_N}$	$\mathbf{d_S}$	$\mathbf{d_N/d_S}$	$\mathbf{d_N}$	$\mathbf{d_S}$	$\mathbf{d_N/d_S}$	$\mathbf{d_N/d_N}$	$\mathbf{d_S/d_S}$
(a)	0.0000	0.0000	--	0.0000	0.0276	0.0001	--	0.00
(b)	0.0000	0.1666	0.00	0.0000	0.0173	0.0001	--	9.63
(c)	0.0023	3.4325	0.0007	0.0024	0.0714	0.0332	0.96	48.07
(d)	0.0002	2.4332	0.0001	0.0072	0.0307	0.2361	0.03	79.26
(e)	0.0000	0.0000	--	0.0024	0.0211	0.1154	0.00	0.00
(f)	0.0023	1.9178	0.0012	0.0024	0.0905	0.0266	0.96	21.19
(g)	0.0000	0.0000	--	0.0027	0.0030	0.8853	0.00	0.00
(h)	0.0005	5.4011	0.0001	0.0151	0.2354	0.0639	0.03	22.94
(i)	0.0006	5.9428	0.0001	0.0455	1.2175	0.0374	0.01	4.88
(j)	0.0002	2.3357	0.0001	0.0074	0.0576	0.1287	0.03	40.55
(k)	0.0000	0.0145	0.0001	0.0000	0.0000	--	--	--
(l)	0.0000	0.0000	--	0.0000	0.0000	--	--	--
(m)	0.0022	1.7975	0.0012	0.0046	0.0000	--	0.48	--

Structural modeling used the *Arabidopsis thaliana* MAPR homolog, PDB entry 1J03, as a template (E-value of 8.7e-30, 42% sequence identity). The model predicted a structure from amino acid 61 to 162 of MAPR (Figure 5.3). Rohe et al. (2009) reviewed four sites required for heme binding in the homolog PGRMC1 surrounding a putative ligand-binding cleft, which we found were identical to those in rotifers at analogous sites (Figure 5.3).

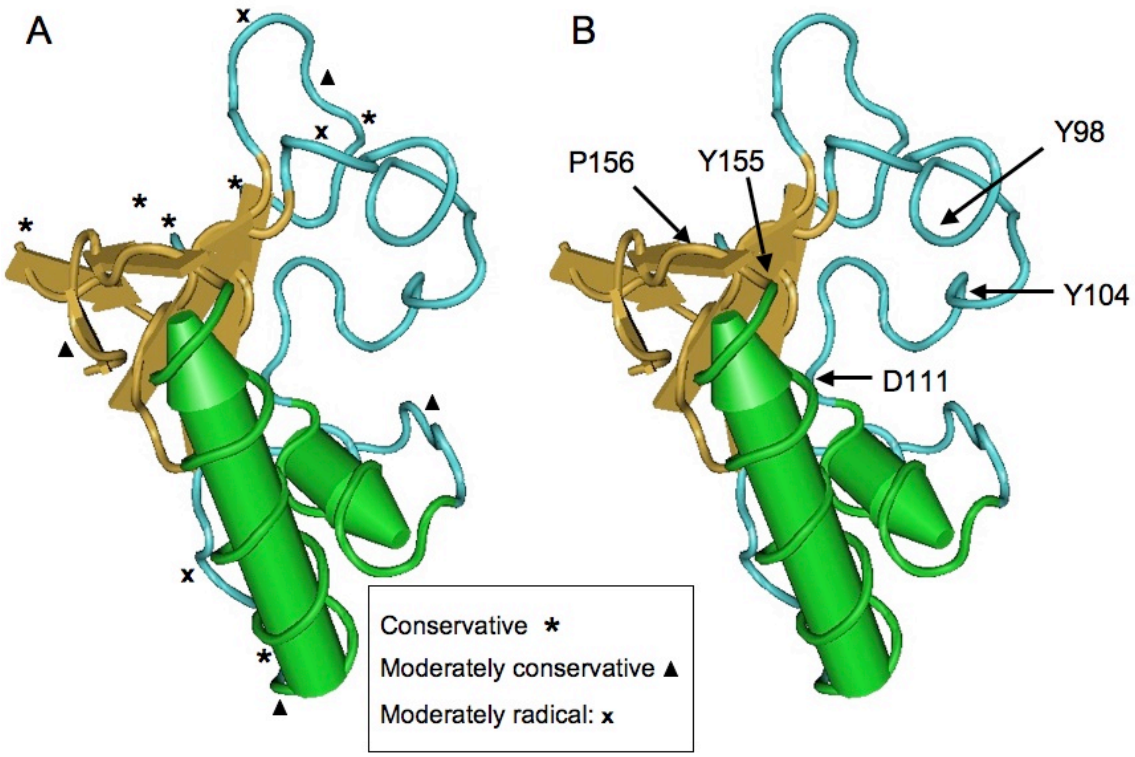

Figure 5.3. Modeled *B. manjavacas* MAPR from amino acid 61 to 162, with putative ligand-binding cleft to the right. Image **A**: amino acid differences among rotifers. Image **B**: five residues conserved among rotifers where the human analog is important to function; all but P156 match human analogs.

Scanning for PROSITE motifs yielded ≥ 10 predicted phosphorylation sites per taxon: protein kinase C phosphorylation sites, cAMP- and cGMP-dependent protein kinase phosphorylation sites, and casein kinase II phosphorylation sites. With a mere 25 sites of amino acid variation among the eight taxa, motifs varied. A phosphorylation site was predicted at 51–54 aa for the L morphotypes, while those in the three *B. rotundiformis* were slightly downstream (one at 59–62 aa, one at 59–61 aa). Only *B. manjavacas* had a phosphorylation site predicted at 64–67 aa, and only *B. rotundiformis* of Spain and of Italy had phosphorylation sites at 93–96 aa.

Discussion

We report the first in-depth analysis of molecular evolution of a *MAPR* gene in any eukaryotic lineage. Purifying selection on *MAPR* suggests it has an important role conserved among rotifers. Yet, small amino acid differences may enable some functional divergence, perhaps underlying variation in life history traits regulated by a progesterone signaling pathway.

Comparing *MAPR* and *cox1* genes enhances insight of their evolution. The ability of *MAPR* to produce a gene topology consistent with the *cox1* gene trees in Gómez et al. (2002) and Suatoni et al. (2006) shows its utility as a phylogenetic marker and supports their findings. In the *MAPR* tree genetic distance (branch length, i.e., substitutions per codon) is much shorter among members of the SS morphotype and among members of the L morphotype than between these two groups, but the difference is less pronounced on the *cox1* tree. Use of more samples (rotifer lineages) may improve resolution for the *cox1* gene tree; however, the fact that the *MAPR* gene provided better resolution for relatedness of the L morphotype clades may be due to the higher relative number of

nonsynonymous substitutions for *MAPR* relative to *cox1*. Since these nonsynonymous substitutions represent amino acid changes, and thus are more likely targets for the action of selection, they may represent important differences fixed among the clades.

The branch-site test performed here suggests both genes are under purifying selection ($d_N/d_S < 1$). Thus, variation in the degree to which exogenous progesterone impacts rotifer reproduction (Snell and DesRosiers 2008) does not appear to reflect positive selection pressure on the *MAPR* gene among sites or branches. Positive selection may occur on other genes in a progesterone signaling pathway, or other, as yet unidentified, receptors may play a role in signaling. Still, the higher d_N of the *MAPR* v. *cox1*, despite its lower d_S, suggests *MAPR* is under less intense purifying selection. The lower d_S is expected, considering the typical trend for mitochondrial DNA to show higher rates of mutation than nuclear DNA (Haag-Liautard et al. 2008). The higher d_N could underlie weak positive selection on *MAPR* not detected by the branch-site test, or relaxed selection pressure relative to *cox1*, but further study is needed (e.g., with more clades). It has been suggested that some pine tree expressed sequence tags with a d_N/d_S of 0.20–0.52 are under positive selection (Palmé et al. 2008). As Palmé et al. note, a d_N/d_S above 1 is a conservative test for positive selection, and thus absence of a ratio above 1 in our study does not eliminate the potential for a weak level of positive selection below the limits of detection.

Amino acid substitutions that accrued over time may have altered MAPR function even with purifying selection. The large number of predicted phosphorylation sites supports a role in signal transduction, as reviewed by Cahill (2007). Variation in predicted sites among rotifers could allow for differential signal transduction, though

more research is required to confirm the sites' function. Four residues critical for heme binding in PGRMC1 are conserved in rotifers. It has been proposed structural elements required for binding heme also function in interactions with a binding partner that mediates progesterone signaling (Rohe et al. 2009), though study is needed to clarify roles of specific amino acids in a progesterone pathway. In rotifers, most substitutions in the heme/steroid binding domain are distal to the ligand-binding cleft and are conservative or moderately conservative. Still, such changes could affect interactions with other molecules (e.g., binding partners). In one human patient a mutation from histidine to arginine at 165 aa was found to prevent binding of cytochrome P450 7A1, and linked to premature ovarian failure (Mansouri et al. 2008). In all eight rotifers a proline exists at the site; binding of P450 7A1 in humans may be derived trait. In conclusion, evolution of the MAPR may represent an overall sequence conservation, marked by small but significant changes allowing functional divergence.

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CHAPTER 6

DISCUSSION

Brachionid Stress Tolerance

At extremes, stress imposes a risk of mortality or impairs fitness, leading to natural selection favoring organisms with mechanisms to permit survival and reproduction under stress. Thus stress can play an important role in evolutionary trajectories. It even has been suggested that stress is positively correlated with diversity at molecular and organismal levels (Nevo 2001). Here, this evolutionary driver has been explored with a particular focus on the stress response of brachionid rotifers. I show that evolutionarily conserved cellular responses (*hsp* gene expression), combined with rapid evolution of traits such as sex propensity and dormancy, confer the ability for tolerance and adaptations to environmental stress levels. Results shed light on the potential for reproductive physiology to influence life history evolvability. Also, I report that the *membrane associated progesterone receptor*, which binds a hormone (progesterone) implicated in brachionid dormancy, is under purifying selection. Considering the threat of climate change and other anthropogenic disturbances to exacerbate stress levels, knowledge of tolerance mechanisms and adaptive capacity may aid efforts at identifying vulnerable taxa and targeting conservation efforts.

Although rotifers are the study system I employ here, the underlying principles may provide avenues for enhancing general understanding of the stress response of organisms. Rotifers are found across the globe in numerous environments from wet moss to ephemeral desert rock pools (Wallace et al. 2006, Wallace and Smith 2009, Wallace

and Snell 2010). It has been suggested that aquatic microinvertebrates may possess the ability for rapid local adaptation (De Meester et al. 2002), perhaps explaining the Phylum's distribution across such diverse ecosystems. Experimental evolution studies provide a way to directly test adaptive responses to manipulated environmental stressors. Many experimental evolution studies to date have focused on bacteria, which some researchers propose are the most useful group for such studies (Buckling et al. 2009). However, assays of basal metazoans such as rotifers may reveal new concepts specific to multicellular organisms, including the potential role of endocrine hormonal systems in evolvability. Increasing understanding of rotifers and other relatively under-studied spiralian or lophotrochozoan taxa may improve fundamental knowledge of evolutionary trends, and help fill gaps in comparisons of the other Bilaterian superclades (Ecdysozoa, Deuterostomia).

Cellular and Life History Stress Responses

Stress responses occur at multiple levels, including cellular tolerance mechanisms. Here results demonstrate that *hsp40*, *hsp60*, and *hsp70* genes each are required for thermotolerance in *Brachionus manjavacas*, with *hsp40* induction occurring within 1 h of heat shock (chapter two). Typical roles of HSPs in the stress response include targeting degraded proteins for proteolysis, and binding unfolded proteins to facilitate refolding and/or prevent deleterious molecular interactions (Parsell and Lindquist 1993, Feder and Hofmann 1999, Buckley et al. 2001, Richter et al. 2010). Nonetheless, exact mechanisms by which the rotifer *hsp* genes studied here and their encoded proteins facilitate survival awaits further study. Experiments suppressing multiple *hsps* to test for synergistic effects and studies across species also would be of

interest. *In situ* research, perhaps using micro- or mesocosms of transfected rotifers in lakes or ponds, could allow observation of the role of *hsps* in more natural conditions where heat and other stressors (e.g., ultraviolet radiation) may be present simultaneously. Although rotifers are known to inhabit regions with water temperatures reaching 40°C (the bioassay temperature) due to power plant thermal effluents (Oden 1979), future assays should include tests at lower temperatures.

Extending the analysis beyond rotifers, it has been noted that heat shock responses are absent in some stenothermal organisms, whereas taxa in highly variable environments already may be expressing heat shock proteins at or near their maximal capacity (Tomanek 2010). Species experiencing moderate fluctuations in thermal regimes are suggested to display the greatest capacity for inducing a heat shock response to temperature ranges outside those currently experienced, perhaps implying greater ability to cope with stressors such as climate change and global warming (Tomanek 2010). It would be interesting to overlay a more in-depth study of *hsp* induction in a variety of lophotrochozoans with environmental tolerance and habitat preferences. Additional stress proteins worth investigation include late embryogenesis abundant and vitellogenin proteins. These thermostable proteins are found in brachionid diapausing embryos and may contribute to the greater ability of embryos versus adult females to survive stressors such as some toxins (pentachlorophenols), desiccation, and heat (Robles-Vargas and Snell 2010, Jones et al. 2012).

Besides physiological cellular responses, adaptations to promote stress survival exist at the level of life history traits. In temporary waters where the timeframe suitable for aquatic life may be short or unpredictable, many taxa show rapid progression through

the life cycle (Valdesalici and Cellerino 2003, Wissinger et al. 2003, Suhling et al. 2005, Schröder et al. 2007). Although lifespan did not evolve with respect to hydroperiod in my chemostat cultures, increased time in the chemostat irrespective of hydroperiod led to increased lifespan and fecundity for asexual females (chapters three and four). My experimental evolution study did not specifically select for greater longevity and it is hard to isolate the exact causal mechanism. Yet as discussed in chapter four this likely is a response to conditions in the benign laboratory environment. Removal of extrinsic stressors found in nature (e.g., temperature and salinity shifts) may have reduced selection maintaining tolerance mechanisms, allowing higher investment of resources in somatic maintenance and thereby increasing longevity and fecundity. Such inverse relationships between reproduction and stress tolerance are documented in research on HSPs. Heat shock proteins can facilitate stress survival, but their expression is associated with reduced fecundity (Sørensen et al. 2003). My results are consistent with the association of decreased extrinsic mortality factors in captive populations and increased lifespan (Williams 1957, Williams et al. 2006), and suggest a link between stress exposure and evolution of longevity.

Spatiotemporal migration can provide a mechanism for escaping stressful environments, via spatial dispersal to a more suitable area, or dormancy as a form of temporal dispersal (diapause or quiescence until conditions improve) (Venable and Brown 1988, Cáceres 1997). Here I noted an increase in production of diapausing embryos in rotifer cultures mimicking temporary waters (chapter three). My work builds upon prior reports of negative correlations of hydroperiod and diapausing embryo production (Campillo et al. 2011), or associations of dormancy with surviving varied

stressors such as cold temperatures in seasonal habitats (Simon et al. 2002, Ragland et al. 2010). Together with my experimental documentation of evolution of diapausing embryo production, this body of literature strongly suggests dormancy is an adaptive stress response.

Another feature of life history that may confer stress adaptation is sexual reproduction. This is supported by my finding of evolution of higher propensity for sex induction, and frequency of sex, in rotifer cultures mimicking ephemeral versus permanent hydroperiods (chapter three). Sex and meiotic recombination often are employed by organisms under unfavorable conditions such as climatic stress, potentially due in part to the fact that dormant embryos tend to be the outcome of sex, at least in species capable of producing these stress-resistant structures (Lokker et al. 1997, Simon et al. 2002, Brock et al. 2003, Grishkan et al. 2003, Agrawal 2006, Serra and Snell 2009). While the evolutionary origin of sex is another matter not addressed here, the link between sex and adaptations such as dormancy is thought to contribute to the evolutionary maintenance of sex (de Visser and Elena 2007). Independent of this association, sex may promote stress tolerance through the benefits of genetic mixing. Meiotic recombination and segregation may augment genetic variation and/or facilitate natural selection by disrupting linkage disequilibrium, thereby promoting adaptation to new and stressful conditions (Hurst and Peck 1996, Burt 2000, Otto and Lenormand 2002, Otto 2003, de Visser and Elena 2007). It has been hypothesized that low-fitness variants have higher levels of sex or recombination, potentially spurring adaptation (Hadany and Otto 2009, Zhong and Priest 2011). This is supported by work in *Drosophila*, where heat shock results in the expected negative relation between fecundity

and recombination rates (Zhong and Priest 2011). Some authors suggest sex may have evolved to counteract DNA damage from oxidative stress (Nedelcu and Michod 2003, Gross and Bhattacharya 2010); however, this idea remains contentious and sex is not necessary for DNA repair (de Visser and Elena 2007).

Strategies for stress tolerance obviously are numerous, and multiple responses may occur simultaneously. Covering all potential adaptations is beyond the scope of this study, and becomes exceedingly complex when considering the potential for phenotypic plasticity or robustness to influence stress tolerance (Ghalambor et al. 2007). Other factors for future consideration include comparing responses to short-term, high-intensity (acute) stressors versus long-term, low-intensity (chronic) stressors, the role of acclimation, and hormesis—the ability for low levels of stress to be beneficial, while high levels are harmful (Costantini et al. 2010).

Rapid Evolution, Evolvability, and Genetics of the Stress Response

Evolution is a key component of the stress response, sometimes seen via local adaptation *in situ* to ambient stress levels. Alternatively, selection or neutral processes may have caused evolution of traits and tolerance ranges independent of or prior to stress exposure. This dissertation discusses contributions of both conserved responses (e.g., HSP induction) and rapidly evolved adaptations such as levels of sex and dormancy.

Relative contributions of extant adaptations to stress tolerance, versus modification or creation of new traits via rapid evolution, is a matter of importance for conservation efforts and ecological dynamics. Knowing the requirement for pre-existing adaptations and ability to evolve *in situ* may help predict the vulnerability of species to climate change and other anthropogenic-mediated stresses, or the likelihood that

introduced species will thrive and become invasive exotics. Invasive exotics may possess greater tolerance for environmental stressors, as suggested for the invasive freshwater snail *Melanoides tuberculatus* (Weir and Salice 2012). Alternatively, invasive species may undergo rapid evolution of stress responses facilitating adaptation upon arrival (Lee 2002). Rapid evolution also creates the potential for eco-evolutionary dynamics (Schoener 2011), with the quick pace of evolution permitting feedbacks into the species' ecology. For instance, I demonstrate the potential for hydroperiod to influence evolution of dormancy and the density-dependent process of sex induction (chapter three). In turn the cost of sex resulted in a feedback to population growth, leading to smaller populations in cultures with higher sex propensities.

Rapid evolution in eukaryotes itself may occur through various mechanisms. In chapter three, the fact that mean final sex-induction densities in permanent cultures greatly exceeded those for any of the initial founding lineages implies the evolutionary change was not merely from a shift in dominance of pre-existing genotypes. I propose that the evolution in response to hydroperiod involved *de novo* mutations or re-arrangements of the genetic material (e.g., via recombination). This is distinct from prior studies of eukaryotes that typically focus on clonal lineage selection and shifts in genotype frequencies (Duffy and Sivars-Becker 2007, Fussmann et al. 2007b, Turcotte et al. 2011).

Although the evolution of lifespan and fecundity described in chapter four was not affected by the manipulated variable of hydroperiod, insight can be gleaned into mechanisms or influences on life history evolution. Remarkably, asexual and sexual females initially had similar longevity, but only asexual brachionids increased in lifespan

and fecundity across seasons. In many species males and females differ in their lifespan, and a recent report of seed beetles even suggests gender may affect evolution of longevity (Fox et al. 2011). Because a single asexual female rotifer can produce both sexual and asexual progeny via ameiotic parthenogenesis (Wallace et al. 2006), it appears that factors beyond genomic sequences likely differentiate these females—not only in their reproductive physiology, but also their life history evolvability. Repeating the chemostat experiment but performing paired life history assays with asexual and sexual females from the same mother could help verify that genomic variation between the females did not contribute to their different evolvability.

Next-generation sequencing technologies offer unprecedented power for identifying genes underpinning organisms' phenotypic differences (Wheat 2010), and for delving deeper into the molecular mechanisms of stress responses and evolvability. As a follow-up to the work presented in chapters three and four, Snell and I have worked with colleagues at the Marine Biological Laboratory (Mark Welch, Hecox-Lea) to apply next-generation technology to samples from my chemostat cultures. I have performed differential gene expression on samples of the chemostat populations at the end of the first and final seasons. Specifically I employed 3'-tag digital gene expression (3'-DGE), a tag-based analog of RNA-Seq targeting protein-coding mRNA (Asmann et al. 2009, Wang et al. 2009). From running samples on the Illumina HiSeq platform, ca. 25,060 tags (e.g., transcripts of distinct genes, or encoding different protein isoforms) were distinguished. By comparing transcript abundance between ephemeral and permanent cultures from the initial and final seasons, we can isolate tags putatively associated with the observed adaptive responses (e.g., sex frequency and diapause) to hydroperiod.

Similarly, by comparing sequences across all six cultures in the initial and final seasons, we can identify genes showing differential expression that may underlie the rotifers' increased fecundity and longevity. The bioinformatics analysis and annotation of these data are ongoing, and will be supplemented by future RNAi studies by Snell to confirm gene function.

Gene expression differences are not the only potential contributor to phenotypic differentiation, warranting additional research beyond 3'-DGE. A host of other mechanisms that may contribute to functional differentiation include post-translational modifications, epigenetics, packaging of the genetic material into eu- or heterochromatin, and sequence differences that impact protein function or translation (Orphanides and Reinberg 2002, Kudla et al. 2009). Hence not detecting differential expression of a particular gene does not demonstrate its lack of function in divergence among chemostat cultures over time. Yet tags that do show significant differential expression can serve as candidate genes for future assays of functional genetics, and the molecular controls of life history and stress tolerance.

Despite difficulty predicting specific genes associated with brachionid rapid evolution because rotifer genomic and transcriptomic research is still in its early stages (Suga et al. 2007, Denekamp et al. 2011, Lee et al. 2011), it is possible to predict some of the types of genes that likely evolved and/or showed differential gene expression among cultures and seasons. I anticipate that genes encoding stress response proteins and members of the minimal stress proteome (Kültz 2005) are up-regulated in ephemeral cultures, including some *hsp* genes. The *hsp70* studied here is expressed in *Brachionus plicatilis* diapausing embryos (Denekamp et al. 2009, Denekamp et al. 2011), and may

have been up-regulated in ephemeral cultures due to their higher diapausing embryo production in later seasons. In general, I expect that the rapid evolution of sex, dormancy, lifespan, and fecundity resulted from mutations (*sensu lato*) or re-arrangements in categories of genes typically associated with positive selection and fast evolution, such as receptors (Civetta and Singh 1998, Swanson and Vacquier 2002). Reproductive proteins often are associated with rapid sequence evolution (Swanson and Vacquier 2002). Thus specific genes associated with rotifer sex induction may have mutated, such as the receptor for the sex (mixis) induction protein (Snell et al. 2006, Kubanek and Snell 2008), or perhaps the protein ligand itself. Complete sex loss in *Brachionus calyciflorus* is associated with Mendelian inheritance of the *op* locus in the recessive state (Stelzer et al. 2010, Scheuerl et al. 2011). However sex frequency evolution appears to be a quantitative trait, and seems distinct from the more qualitative regulation of ability for sex by the *op* locus.

The evolution of multiple traits in response to hydroperiod (e.g., sex frequency and propensity, diapausing embryo production) and time in the chemostat (increased lifespan and fecundity), suggests the evolution may not just reflect changes in the specific genes controlling these traits. Rather I anticipate the rapid evolution in chemostats at least partially is due to changes in genes involved in regulatory processes that could affect multiple traits simultaneously—perhaps facilitating modular evolution of associated traits. Hormonal signaling genes may be involved in a signaling cascade with effects on transcriptional regulation. In chapter four it is hypothesized that hormones play a salient role in evolvability, especially of longevity and fecundity. Previously, it has been suggested that variation in endocrine pathways may form part of the foundation for life

history evolvability (Amdam et al. 2007). Hormonal signaling cascades and endocrine modules can exert multiple effects on suites of correlated traits, influencing processes as diverse as reproduction, osmosregulation, and development (Ketterson and Nolan 1999). Stress can decrease levels of the sex steroid hormone progesterone, thereby impairing reproduction (Wingfield and Sapolsky 2003). The ability of hormones to integrate responses to the environment, including the role of steroid hormones in stress-related and sexual behaviors (Dufty et al. 2002, Denver et al. 2009), presents an intriguing possibility for evolution of hormonal systems to impact evolution of the stress response and life history.

Differential expression of hormonal signaling genes found via my 3'-DGE study of chemostat cultures in the initial and final season (independent of hydroperiod) would be consistent with my hypothesis that hormones play a role in life history evolvability. Several studies have focused on the ability of dietary restriction or mutations to components of insulin signaling pathways to increase lifespan, but often this is associated with a delay or decline in reproduction (Kenyon 2010, Flatt 2011). Such lifespan-fecundity tradeoffs were not observed in chapter four; rather the traits were positively correlated and both increased in asexual females. This may suggest operation of a novel or less commonly known pathway in rotifers, or modification of pre-existing pathways. For instance, recently two genes (*hpa-1 and hpa-2*) were identified in *Caenorhabditis elegans* that increase lifespan via effects on the EGF pathway rather than insulin signaling, without a noticeable reduction in reproduction or impairment of development (Iwasa et al. 2010). Brachionid rotifers exposed to vertebrate hormones exhibit a range of responses with effects on dormancy and reproduction, and in *C. elegans* and *Drosophila*

both the peptide-hormone pathway of insulin signaling as well as sex steroid pathways affect life history regulation (Snell and DesRosiers 2008, Gáliková et al. 2011, Snell 2011). Yet to date rotifer hormones have not been characterized (Snell 2011), complicating the ability to predict specific pathways that may possess high evolvability or be linked to stress tolerance.

One hormonal system of particular interest is the progesterone signaling system, motivating my assays of molecular evolution of the *membrane associated progesterone receptor* in chapter five. Increased diapausing embryo production in brachionids exposed to exogenous progesterone (Snell and DesRosiers 2008), and decreased sexuality of rotifers in which interference RNA had been used to suppress the *MAPR* (Stout et al. 2010), suggests the importance of a progesterone signaling pathway to rotifer sex and dormancy. Differences in amino acids at some positions in the receptor and existence of phosphorylation sites across brachionid species may underlie functional differentiation of distinct alleles. Site-directed mutagenesis, and perhaps analyses using RNAi across species or populations with different allelic variants, could be used to test the impacts of specific amino acid substitutions. Purifying selection promoting sequence conservation of the receptor makes it unlikely that the *MAPR* underwent rapid evolution in the experimental evolution study here. Nonetheless, this does not preclude the potential for the progesterone ligand or other components of a progesterone signaling pathway to be involved in regulation of rotifer evolvability and life history. Ongoing transcriptomic (3'-DGE) comparisons of the chemostat cultures are anticipated to provide insight into the roles of progesterone and other signaling systems in rotifer evolution, physiology, and stress tolerance.

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