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Mitochondrial Inheritance and Natural Phenotypic Variation among
Caenorhabditis briggsae Populations

by

Anna Luella Coleman-Hulbert

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biology

Thesis Committee:
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2010

Abstract

Mutations affecting the mitochondrial electron transport chain cause numerous neurodegenerative disorders in humans and affect longevity in other organisms. A natural model system to study the relationship between mitochondrial function and aging within an evolutionary or population genetic context has been lacking. Natural populations of *Caenorhabditis briggsae* nematodes were recently found to harbor mitochondrial genetic variation with likely functional consequences for aging. Specifically, *C. briggsae* isolates containing high frequencies of a deletion mutation affecting the mitochondrial *NADH dehydrogenase 5 (ND5)* gene were found to have reduced reproductive fitness and lifespan and elevated levels of mutagenic superoxide. Here, rates of growth and aging and aerobic respiratory capacity were evaluated in several isolates spanning the range of mitochondrial genetic variation in this species. There is considerable variation among isolates for all measured traits, although the observed relationships between isolate-specific trait means and *ND5* deletion frequency did not always conform to my expectations. In an effort to determine whether the among-isolate phenotypic variation is due to mitochondrial rather than to nuclear genetic variation, inter-population hybrids of *C. briggsae* were created and compared to the progenitor isolates. Surprisingly, evidence for paternal mitochondrial inheritance was detected in many of these hybrid lines. Where mitochondrial genomes were maternally inherited as expected, intergenomic epistasis appears to contribute to fitness, longevity, and aging in this species.

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INTRODUCTION

Mutations affecting mitochondrial DNA (mtDNA) and function are associated with a variety of metabolic and aging-related diseases across taxa (Chinnery and Turnbull, 2000; DiMauro and Schon, 2003; Smeitink *et al.*, 2003; Taylor and Turnbull, 2005; Wallace, 2005). Nuclear and mitochondrial gene products interact to form the energy-producing mitochondrial electron transport chain (ETC), and mutations affecting these gene functions tend to present as various neurodegenerative disorders in humans (Ozawa, 1995; Pitkanen and Robinson, 1996; DiMauro and Schon, 2003; Smeitink *et al.*, 2003; Taylor and Turnbull, 2005). In general, mtDNA mutates faster than nuclear DNA (nDNA) due to: 1) increased replication compared to nDNA over the lifetime of an individual, 2) lack of repair enzymes, and 3) proximity to reactive oxygen species (ROS) that are natural by-products of aerobic metabolism in mitochondria (Lightowlers *et al.*, 1997; Ballard and Whitlock, 2004; Baer *et al.*, 2007). When mutated, mitochondrial genes tend to exist in a heteroplasmic state; that is, wild type mitochondrial genomes exist alongside mutant genomes within the same organism, cell, or organelle (Chinnery, 2000; Smeitink *et al.*, 2003; Lemire, 2005). Several defects associated with mtDNA mutations demonstrate a threshold effect of heteroplasmy whereby a certain fraction of mutated organellar genomes must be present for deleterious phenotypes to manifest (Rossignol *et al.*, 2003; Smeitink *et al.*, 2003; Ventura *et al.*, 2006).

MtDNA mutations can be inherited, or arise spontaneously within somatic tissues and accumulate over the lifetime of an individual (Solignac *et al.*, 1987; Chinnery, 2000; Tsang and Lemire, 2002; Smeitink *et al.*, 2003; Liau *et al.*, 2007), and increases in mtDNA mutations and heteroplasmy are known to occur with age in many cases (Corral-Debrinski *et al.*, 1992.; Ozawa, 1995; Melov *et al.*, 1997; Chomyn and Attardi, 2003; Krishnan *et al.*, 2008). The precise mechanism for such increases in mutation heteroplasmy is not known, although it has been suggested that, when deletions are present within a mitochondrial genome, such a molecule could exhibit a replicative advantage over the intact (wild type) genome due simply to the shorter time required to replicate a shorter deletion-bearing sequence (Yoneda *et al.*, 1992; Melov *et al.*, 1995; Moraes, 2001; Diaz *et al.*, 2002.; however, see Solignac *et al.*, 1987). In addition to this age-related prevalence of mtDNA mutations, mutations of mtDNA and mitochondrial function have been implicated as causal agents in the aging process (Bandy and Davison, 1990; Ozawa, 1995; Lee and Wei, 2007; Alexeyev *et al.*, 2004; Chomyn and Attardi, 2003; Ventura *et al.*, 2006; Sedensky and Morgan, 2006a; Sedensky and Morgan, 2006b). Mutations leading to the dysfunction of mitochondria, specifically of the ETC, and changes in ROS production can affect rates of aging and longevity across organisms (Ozawa, 1995; Chomyn and Attardi, 2003; Tsang and Lemire, 2003; Ventura *et al.*, 2006; Sedensky and Morgan, 2006a; Sedensky and Morgan, 2006b; Hiona and Leeuwenburgh, 2008).

The long-standing free radical theory for aging purports that the degenerative features of senescence are largely due to the accumulation of lipids, proteins, and DNA that have been damaged by endogenously-produced ROS (Harman, 1956; Beckman and Ames, 1998; Alexeyev *et al.*, 2004; Balaban *et al.*, 2005). Mitochondria have been deemed the primary source of endogenous ROS (Murphy, 2009), which has placed these organelles in the spotlight of much theoretical and empirical aging research. Some predict that a vicious cycle may occur within mitochondria where natural ROS production generates mtDNA damage, this damage leads to subsequent dysfunction of the organelle thus increasing ROS production, and so on (Linnane *et al.*, 1989; Bandy and Davison, 1990; Alexeyev *et al.*, 2004; although, see de Grey, 2005, and Hiona and Leeuwenburgh, 2008). To understand the interplay of mitochondrial function, ROS production, aging, and longevity, research has utilized *in vitro*, *in vivo*, and *in situ* methods on models as diverse as snakes and ‘cybrids’ (cell cultures with replaced mtDNA populations) (Ozawa, 1995; Pitkanen and Robinson, 1996; Luo *et al.*, 1997; Melov *et al.*, 1997; Inoue *et al.*, 2000; Robert *et al.*, 2007). However, the fact remains that we have a limited understanding of the evolutionary forces underlying the aging process and mitochondrial genetics.

Ease of laboratory maintenance and manipulation, and our thorough understanding of *Caenorhabditis elegans* genetics have made this nematode a powerful model organism for studies of aging and many other biological processes. Several single *C. elegans* genes involved in mitochondrial function and biogenesis (most of which have

human orthologues) have been identified through chemical mutagenesis screens as affecting metabolism and longevity. However, a major hurdle to understanding the link between mitochondrial mtDNA damage, mitochondrial dysfunction, and the aging process has been the lack of animal model systems with relevant naturally occurring mitochondrial genetic variation. Howe and Denver (2008) recently characterized a mitochondrial deletion mutation that segregates among natural populations of the soil nematode *Caenorhabditis briggsae*, a sister species to *C. elegans* for which most of the same molecular techniques and genetic tools are available. This mutation deletes over 700bp of the ETC complex I subunit *NADH dehydrogenase 5 (ND5)*. Furthermore, the deletion-bearing mtDNA genomes comprise between 0 to over 50% of the total mitochondrial genome population depending upon the *C. briggsae* natural isolate. The natural variation present among isolates of *C. briggsae* for a clinically relevant mtDNA deletion provides an excellent opportunity to characterize aging features that are correlated to a naturally occurring and presumably deleterious mtDNA mutation.

Findings from other complex I mutants indicate that the deletion affecting *ND5* in *C. briggsae* can be expected to increase ROS production and decrease ETC efficacy, and thereby impact whole organismal function (Pitkanen and Robinson, 1996; Luo *et al.*, 1997; Bai *et al.*, 2000; Kayser *et al.*, 2004; Janssen *et al.*, 2006; Park *et al.*, 2009). Accordingly, heteroplasmy in *C. briggsae* is negatively correlated with reproductive output (Howe and Denver, 2008), population growth rate, and lifespan (Estes *et al.*, in review). Additionally, *in vivo* ROS production exhibits a non-linear relationship to *C.*

briggsae heteroplasmy; however, ATP content exhibits no relationship with *ND5* deletion level (Estes *et al.*, in review). Under the threshold effect and free radical theories, once *C. briggsae* *ND5* deletion heteroplasmy reaches a certain level, physiological function should decline and rates of aging should increase due to increased ROS production. Toward the goal of testing these ideas, I have measured various physiological and aging parameters in *C. briggsae* natural isolates to evaluate the deleterious impact of the *ND5* deletion. These data are reported in Chapter 1 of this thesis. Next, to establish that observed variation for traits measured among natural *C. briggsae* isolates is not due to among-isolate nuclear genetic variation, multiple replicates of reciprocal ‘mitochondrial replacement’ lines were created by crossing *C. briggsae* from high- and low-heteroplasmy isolates. All traits were measured in hybrids as for natural parental isolates assuming that mitochondria are inherited maternally and mtDNA variation explains the observed phenotypic differences between natural *C. briggsae* isolates. These experiments are the subject of Chapter 2 of this thesis. Overall, my findings suggest that partial deletion of *ND5* may significantly impact life history and aging in *C. briggsae*, but not certain components of aerobic metabolic function, and that paternal inheritance of mitochondria and cytonuclear conflict can occur in hybrids of *C. briggsae* natural populations.

CHAPTER 1

Phenotypic Variation among Natural Isolates of *Caenorhabditis briggsae*

BACKGROUND

Mitochondria are essential for cellular energy metabolism in eukaryotes, and mutations affecting mitochondrial function have been associated with numerous human neurodegenerative disorders (Ozawa, 1995; Pitkanen and Robinson, 1996; Chinnery and Turnbull, 2000; DiMauro and Schon, 2003; Smeitink *et al.*, 2003; Taylor and Turnbull, 2005; Wallace, 2005; Sedensky and Morgan, 2006; Hiona and Leeuwenburgh, 2008). Across taxa, mitochondrial mutations are associated with altered metabolic activity, rates of aging, and longevity (Ozawa, 1995; Pitkanen and Robinson, 1996; Luo *et al.*, 1997; Melov *et al.*, 1997; Inoue *et al.*, 2000; Robert *et al.*, 2007). Since mitochondria are the primary endogenous source of reactive oxygen species (ROS) (Murphy, 2009), these small but plentiful organelles have been a key target for studies aimed at understanding aging, a ubiquitous process thought to be linked to increases in cellular ROS and mitochondrial mutations (Miwa *et al.*, 2008).

Our extensive knowledge of *Caenorhabditis elegans* biology and the genetic and genomic tools available for this nematode make it an ideal organism for understanding the causative role of mtDNA mutations and mitochondrial function in aging and

longevity (Tsang and Lemire, 2003; Sedensky and Morgan, 2006a; Sedensky and Morgan, 2006b; Ventura *et al.*, 2006). There are four main genetic pathways known to affect longevity in *C. elegans*: insulin-like signaling, dietary restriction, inhibition of protein translation, and a mitochondrial pathway (reviewed in Houthoofd and Vanfleteren, 2007). A recent RNAi screen for genes affecting longevity in *C. elegans* found that about 25% of new candidate genes (annotated with a possible function) were involved in metabolism, many with orthologues in flies, mice, and humans, and that about half of these genes correspond to subunits of the electron transport chain (ETC) (Hamilton *et al.*, 2005). In particular, RNAi leading to impaired mitochondria and increased longevity also correlate with decreased oxygen consumption and ATP content in *C. elegans* (Lee *et al.*, 2003). Progeria-like phenotypes and decreased lifespan have been observed in *C. elegans* with knockdown of mitochondrial heat shock protein 70, an import motor essential to energy generation and mitochondrial biogenesis in eukaryotic cells (Kimura *et al.*, 2007). Inhibition of oxidative phosphorylation at various ETC complexes with RNAi prior to adulthood increases *C. elegans* longevity (Dillin *et al.*, 2002; Rea *et al.*, 2007). In general, *C. elegans* mitochondria-associated gerontogenes affect ROS production and mitochondrial oxidative phosphorylation in addition to lifespan (Kayser *et al.*, 2004; Sedensky and Morgan, 2006a; Sedensky and Morgan, 2006b). Although a great degree of homology exists between ETC genes of humans and *C. elegans* (see Table 1 in Tsang and Lemire, 2003), a natural model is lacking for studying the relationship between mitochondrial mutations, ROS, and aging.

Figure 1. *Caenorhabditis briggsae* mitochondrial genome. Positions of *ND5* deletion (dashed line at top) and Ψ ND5 elements - pseudogenes originated from duplications of *ND5*. Arrows indicate primers for PCR assays (Howe and Denver, 2008).

Caenorhabditis briggsae nematodes isolated from geographically distinct populations around the world have been found to harbor a naturally-occurring heteroplasmic mtDNA deletion (Howe and Denver, 2008; Fig. 1). When present, the deletion removes nearly half of the *NADH-dehydrogenase 5* (*ND5*) gene and over 200 amino acids from the protein product, many of which are highly conserved in *C. elegans*, *Drosophila*, and humans. The *C. briggsae* *ND5* deletion is thought to occur during genomic replication due to direct repeat sequences that flank the deleted region located within *ND5* and within the upstream Ψ ND5-2 non-coding element (*cf.* Lunt and Hyman, 1997). In *C. briggsae* isolated from Kenya that lack Ψ ND5-2, no genomes harbor the partial deletion of *ND5*, whereas isolates that do harbor the non-coding pseudogene have *ND5* deletion heteroplasmy levels ranging from 0 to 50% (Fig. 2). Putative compensatory mutations within Ψ ND5-2 are found in some isolates of the temperate clade and are thought to decrease the likelihood of the deletion event (Howe and Denver, 2008). Based on studies of other complex I mutant genotypes, including other mutations within *ND5*

(Pitkanen and Robinson, 1996; Luo *et al.*, 1997; Bai *et al.*, 2000; Kayser *et al.*, 2004; Janssen *et al.*, 2006; Park *et al.*, 2009), the heteroplasmic deletion in *C. briggsae* is presumed to decrease complex I efficiency and increase ROS production. Although the truncated *ND5* gene is transcribed in *C. briggsae*, its structure, expression pattern, and degree of functionality have not been determined.

Previous studies found a negative correlation between fecundity and *ND5* deletion heteroplasmy level among *C. briggsae* isolates suggesting that the *ND5* deletion negatively impacts fitness in *C. briggsae* (Howe and Denver, 2008). Estes *et al.* (in review) found this same correlation and, furthermore, that *C. briggsae* heteroplasmy is negatively correlated to population growth rates and lifespan. Additionally, *C. briggsae* ATP content, *in vitro* ROS production, and exogenous oxidative stress resistance vary significantly with heteroplasmy; however, only the latter was significantly correlated to heteroplasmy in a negative fashion (Estes *et al.*, in review). Use of fluorogenic dyes

Figure 2. *Caenorhabditis briggsae* intraspecific phylogeny. GL = global intraspecific superclade; KE = Kenya clade; TE and TR = temperate and tropical subclades of GL. C(+) = temperate-clade isolates bearing compensatory Ψ ND5-2 alleles; C(-) = those bearing ancestral alleles. % *ND5* Del. indicates isolate-specific percentages of total mitochondrial genomes that harbor *ND5* deletions, as determined by qPCR (Howe and Denver, 2008). * The HK104 strain used here was the inbred progenitor of mutation-accumulation lines (Baer *et al.*, 2005) found to harbor high *ND5* deletion levels similar to those of HK105 (Dee Denver, pers. comm.).

revealed a non-linear relationship between whole-worm superoxide production and *C. briggsae* isolate-specific heteroplasmy; *i.e.* high- and non-heteroplasmic isolates contain more ROS than midlevel deletion isolates (Estes *et al.*, in review). Use of alternative energy-harvesting pathways or variation in oxidant scavenging among isolates may explain the lack of correlation between *C. briggsae* heteroplasmy and both ATP content and *in vitro* hydrogen peroxide production (*cf.* Rea, 2005).

To establish *C. briggsae* as a natural model system for examining interactions between aging, heteroplasmy and ETC function, several metabolic and aging-related phenotypes have been examined in nine natural isolates spanning the range of *ND5* deletion heteroplasmy in *C. briggsae* (Fig. 2). Specifically, declines in pharyngeal pumping rate (an indication of muscle deterioration) have been examined to elucidate whether the *ND5* deletion affects the aging process in *C. briggsae*. Additionally, rates of oxygen consumption have been quantified to determine if the *ND5* deletion affects aerobic respiratory capacity, and larval and adult growth rates have been examined to determine whether the *ND5* deletion in *C. briggsae* retards cell cycle progression and therefore development. Based on previous studies of other ETC mutations in *C. elegans* (Dillin *et al.*, 2002; Lee *et al.*, 2003; Kayser *et al.*, 2004), *ND5*-deletion heteroplasmy in *C. briggsae* is expected to correlate positively to aging and negatively to metabolism.

METHODS

Nematode Strains and Maintenance

To examine the impact on aging and total physiology of a naturally-occurring partial deletion of the mtDNA-encoded *ND5* protein coding gene in *C. briggsae*, nine natural isolates from geographically diverse populations were selected for study in order to capture the full range of variation in *ND5* deletion heteroplasmy level (0 to ~50%) (Fig. 2; strains were provided by D. R. Denver, OSU). Unless otherwise noted, nematodes were grown under standard laboratory conditions at 25°C on 15mm NGM plates seeded with *Escherichia coli* strain HB101 as a food source.

Pharyngeal pumping

Caenorhabditid nematodes ingest bacteria using rhythmic contractions of the pharynx (Fig. 3) – a neuromuscular organ comprising 20 muscle cells and 20 neurons that is easily viewed using light microscopy (Albertson and Thomson, 1976). As in humans, muscle deterioration occurs with age in nematodes, and sarcopenia of the pharynx has been associated with aging in *C. elegans* (Garigan *et al.*, 2002; Herndon *et al.*, 2002). Additionally, the decline of pharyngeal pumping is a strong biomarker for chronological age in *C. elegans* (Bolanowski *et al.*, 1981; Huang *et al.*, 2004). Therefore, the decline of pharyngeal pumping rates in *C. briggsae* over adulthood have been measured here as a proxy for aging.

Figure 3. Pharynx in *Caenorhabditis briggsae* adult.

Pharyngeal pumping assays were performed at room temperature (about 22°C) on 15mm NGM plates. Pharyngeal pumping rates were determined for the same 10 to 20 hermaphrodites per isolate every other day of adulthood starting two days past the fourth larval stage (L4 + 2 days; L4 stage confirmed visually by crescent at vulva) until death. Rates were determined by terminal bulb pumps per three 5-second intervals and converted to pumps per minute. Decline in pharyngeal pumping rates were calculated between days 2 and 12 and between days 2 and 20. During the reproductive period, individuals were removed from their offspring after pharyngeal pumping measures.

Growth

If the *ND5* deletion negatively affects ETC functioning in *C. briggsae*, individuals from isolates harboring high *ND5* deletion levels are expected to exhibit slower rates of growth and to attain smaller maximum body sizes than low or zero-deletion isolates (*cf.* Dillon *et al.*, 2002, and Rea *et al.*, 2007). To test this, different aspects of body size and growth rates were quantified for replicate animals from each natural isolate.

For logistical reasons, growth rates were characterized for larvae and adults separately. For larval measurements, populations were age synchronized by allowing 10-

20 gravid hermaphrodites per isolate to lay eggs on fresh 15mm NGM plates with *E. coli* OP50-1 for two hours. From these age-synchronized cohorts, 5 to 10 larvae per plate were randomly selected for measurement at 14, 26, 34 and 42 hours after the egg stage (correlating approximately to each of the four larval stages, L1-L4). At each time point, larvae were transferred to a glass slide containing a small NGM agar pad and immobilized with 10 μ l of 20mM sodium azide. Digital images were captured using a PixeLINKTM camera mounted on a Nikon Labophot and measurements were made using ImageJ (NIH, <http://rsbweb.nih.gov/ij/>). Larval growth was calculated as the difference in average length of animals between 14 and 42 hours after the egg stage, and converted to mm per hour.

Figure 4.
Caenorhabditis briggsae at fourth instar (L4). Lines for length and width measurements.

For adult measurements, all individual nematodes from the pharyngeal pumping assay were imaged live (on NGM plates) on days 0, 6 and 12 from the L4 stage. Length measurements were made as above, as well as width at the vulva (Fig. 4), and volume was calculated following Bolanowski *et al.* (1981). Growth rates for length and volume were assessed between each time point and converted to mm per day. Note that, because

only animals that survived to be measured on day 12 were used in our analyses, any laboratory selection on body size that may have been operating will not affect our results.

From the images captured to assess larval growth rates, larval stage at 42 hours after hatching was determined to assess the affect of the *ND5* deletion on cell cycle progression in *C. briggsae*. Previous study of partial ETC disruption by increasing RNAi leading to life extension found a concomitant increase in delay of larval development rates, up to the point when life span was inversely affected (Rea *et al.*, 2007). Here, ratios of L3 to L4 stage larvae at 42 hours after hatching were compared to the expected frequency (*i.e.* zero L3 larvae at 42 hours after hatching), and these ratios were compared between larvae from heteroplasmic and non-heteroplasmic isolates.

Oxygen consumption

The nature of the *ND5* deletion, along with previous findings from other ETC complex I mutants, lead me to hypothesize that aerobic metabolic function will be compromised in *C. briggsae* isolates harboring higher frequencies of deletion-bearing mtDNA genomes. For whole-animal respiration rate quantification, production of massive, age-synchronized nematode cultures was achieved following Braeckman *et al.* (2002a). Oxygen consumption was measured on synchronous populations of L4 (L1+30 hours) animals using a Clark-type electrode following Braeckman *et al.* (2002a) except that data were normalized to the oxygen content of S-basal at ~25°C as determined by an optical dissolved oxygen meter from Eureka Environmental Engineering. The protein

content of samples was quantified using a BCA kit (Pierce) following the manufacturer's instructions after preparation following Braeckman *et al.* (2002a). Additionally, worm number per sample was calculated based on separately determined values of protein content per worm per isolate. Rates of oxygen consumption were normalized both to protein content and worm number.

Statistics

Statistical analyses were performed using R (R Development Core Team, 2009; MASS package by Venables and Ripley, 2002) and Microsoft Office Excel (2007). Not all variables are normally distributed; however, analysis of residuals from linear regression models indicate no outliers and Box Cox family transformations suggest that $\lambda=1$ (*i.e.* no transformation is able to improve the distribution) for all skewed variables. One-way analyses of variance were performed to determine variation between isolates per phenotype. To test for effects of heteroplasmy level on phenotypes, isolates were divided among three categories corresponding to relative *ND5* deletion level (high, low, and none) and Tukey HSD pair-wise comparisons were made between these levels per trait (α adjusted to correct for group-wise Type I error). This categorization enables graphical simplicity and reduces the potential effect on statistical results of high variation in heteroplasmy within isolates (see Howe and Denver 2008, Supplemental Table 1). Additionally, Spearman's rank correlation analyses were performed to test for relationships between isolate-specific *ND5* deletion heteroplasmy and phenotypes.

RESULTS

Pharyngeal pumping

Pharyngeal pumping rates over adulthood varied significantly between isolates on most days and were significantly negatively correlated to *ND5* deletion until 14 days past the L4 stage (Table 1). For isolates categorized as having high levels of *ND5* deletion heteroplasmy, pharyngeal pumping rates were significantly less than those with low or no heteroplasmy over days 2 through 18 (Tukey HSD $p < 0.05$; high deletion isolates pumped

Pharyngeal Pumping

Figure 5. Adult pharyngeal pumping rates of *C. briggsae*. Natural isolate *ND5* deletion heteroplasmy levels are indicated here as high (filled squares; HK105, HK104, VT847), low (grey triangles; PB800, EG4181, JU726, AF16), and none (clear circles; ED3092, ED3101).

less than low- but not zero-deletion isolates on days 6 and 20; Fig. 5). The decline in pharyngeal pumping rate measured between 2 and 12 days or between 2 and 20 days post-L4 also varied significantly between isolates and these rates of change correlate negatively to *ND5* deletion heteroplasmy (Table 1) such that high deletion isolates have a reduced rate of decline compared to low and no deletion isolates (Tukey HSD $p < 0.05$). (Analysis of pharyngeal pumping ceased after day 20 due to decreased power from reduced sample sizes.)

Larval Growth and Development

Fourteen hours after hatching, larval length varied significantly between *C. briggsae* isolates (Table 1) with individuals from high deletion isolates being longer than those from isolates with low or no deletion (Tukey HSD $p < 0.05$). Additionally, there was significant among-isolate variance in length at 26 and 34 hours after hatching (Table 1) and isolates without heteroplasmy were significantly shorter than isolates containing any amount of deleted genomes at these time points (Tukey HSD $p < 0.05$). Length of larvae at 42 hours after hatching was positively correlated to *ND5* deletion heteroplasmy level (Table 1) such that all heteroplasmic isolates were longer than those with wildtype genomes only (Tukey HSD $p < 0.05$; Fig. 6). Similarly, the rate of growth between 14 and 42 hours after hatching was significantly different among isolates (Table 1) such that heteroplasmic isolates grew at a faster rate between these two time points than did non-deletion-bearing isolates (Tukey HSD $p < 0.05$).

ND5 deletion-bearing isolates also developed faster than non-heteroplasmic isolates. A significant proportion of larvae at 42 hours were L3 instead of the expected L4 (40 of 312 larvae total, $p < 0.0001$). Further, significantly more of the 42-hour-old larvae from heteroplasmic isolates were L4 than were larvae from non-heteroplasmic isolates ($G = 3.16$, $df = 1$, $p < 0.05$).

Figure 6. Length of *C. briggsae* larvae at 14, 26, 34, and 42 hours after hatching (which correspond approximately to L1, L2, L3, and L4). *C. briggsae* isolates are categorized by relative heteroplasmy levels; *i.e.*, high (HK105, HK104, VT847), low (PB800, EG4181, JU726, AF16), and none (ED3092, ED3101), and the letters above bars indicate statistically indistinguishable groups (Tukey HSD, adjusted $\alpha = 0.05$). Error bars indicate one standard error.

Adult Growth

At day 0 of adulthood (L4 stage), length varied significantly among isolates and was positively correlated to *ND5* deletion frequency (Table 1). Volume exhibited the same relationship to *ND5* deletion frequency (data not shown). Six days into adulthood,

the same relationship was observed for length and volume (Table 1; volume data not shown): non-heteroplasmic isolates were significantly larger and exhibited a faster rate of growth between days 0 and 6 of adulthood compared to heteroplasmic isolates (Tukey HSD $p < 0.05$). Twelve days after L4, *C. briggsae* harboring any level of *ND5* deletion were significantly smaller than those without (length and volume, Tukey HSD $p < 0.05$). In fact, heteroplasmic isolates exhibited almost no growth, or in some cases shrunk in length, between 6 and 12 days past L4, while non-deletion isolates continued to grow (Tukey HSD $p < 0.05$; Fig. 7).

Figure 7. Length of *C. briggsae* adults at 0, 6, and 12 days post-L4. *C. briggsae* isolates are categorized by relative heteroplasmy levels; *i.e.*, high (HK105, HK104, VT847), low (PB800, EG4181, JU726, AF16), and none (ED3092, ED3101), and letters above bars indicate statistically indistinguishable groups (Tukey HSD, adjusted $\alpha = 0.05$). Error bars indicate one standard error.

Oxygen consumption

Protein content per worm varied significantly among isolates ($F(8,7)=20.69$, $p<0.001$) but did not correlate to isolate-specific *ND5* deletion heteroplasmy levels (Spearman's $r_s=0.09$, $p=0.74$). Similarly, oxygen consumption rates varied significantly among isolates (per mg protein: $F(8,9)=6.54$, $p<0.01$; per worm: $F(7,8)=18.18$, $p<0.001$) but did not correlate to isolate-specific heteroplasmy levels (per mg protein: $r_s=-0.34$, $p=0.17$, Fig. 6; per worm: $r_s=0.06$, $p=0.83$). (Oxygen consumption rates in Fig. 8 are normalized to protein content, as is the convention for *C. elegans*; see discussion in Braeckman *et al.*, 2002b.)

Figure 8. Whole-organism rates of oxygen consumption of *C. briggsae*. Isolates categorized by relative heteroplasmy levels; *i.e.*, high (HK105, HK104, VT847), low (PB800, EG4181, JU726, AF16), and none (ED3092, ED3101). Error bars indicate one standard error.

	Grand Mean	SD	n	df	F-ratio	r_s
Larval growth (length in mm)						
14 hours after hatching	0.269	0.024	204	8	6.28 ***	0.12
26 hours after hatching	0.400	0.034	208	8	11.75 ***	0.13
34 hours after hatching	0.520	0.041	255	8	14.55 ***	0.26 ***
42 hours after hatching	0.707	0.060	251	8	38.06 ***	0.37 ***
Rate of growth between 14 and 42 hours after hatching (per hour)	0.016	0.002	185	8	15.97 ***	0.11
Adult growth (length in mm)						
Day 0 (L4)	0.754	0.069	153	8	18.04 ***	0.34 ***
Day 6 (past L4)	1.505	0.142	135	8	5.04 ***	0.22 *
Day 12 (past L4)	1.472	0.194	116	8	4.92 ***	-0.17
Rate of growth between days 0 and 6 (per day)	0.757	0.141	123	8	13.09 ***	-0.48 ***
Rate of growth between days 6 and 12 (per day)	-0.006	0.028	114	8	3.59 **	0.02
Rate of growth between days 0 and 12 (per day)	0.715	0.185	116	8	6.10 ***	-0.32 ***
Pharyngeal pumping (pumps/minute)						
L4+2 days	181.100	79.879	120	8	8.39 ***	-0.51 ***
L4+4 days	121.803	65.767	122	8	4.33 ***	-0.43 ***
L4+6 days	138.116	58.542	121	8	2.43 *	-0.22 *
L4+8 days	106.300	58.335	120	8	4.66 ***	-0.46 ***
L4+10 days	93.311	62.764	119	8	5.28 ***	-0.35 ***
L4+12 days	77.778	61.252	117	8	3.95 ***	-0.21 *
L4+14 days	50.000	51.867	106	8	2.36 *	-0.16
L4+16 days	39.043	43.177	92	8	1.14	-0.10
L4+18 days	34.222	40.684	81	8	4.12 ***	-0.14
L4+20 days	20.776	31.651	67	8	2.20 *	-0.21
Rate of decline between 2 and 12 days post-L4 (per day)	10.581	8.249	115	8	2.30 *	-0.32 ***
Rate of decline between L2 and 20 days post-L4 (per day)	9.070	4.459	65	8	4.45 ***	-0.55 ***

Table 1. Test statistics per phenotype measured among nine natural isolates of *Caenorhabditis briggsae*. (r_s is the Spearman's rank correlation between isolate-specific trait means and deletion frequencies. *, **, and *** denote significance at $\alpha = 0.05$, 0.01, and 0.001, respectively.)

DISCUSSION

To evaluate the effects of a naturally occurring mtDNA deletion in the nematode *C. briggsae*, several aging and physiology-related phenotypes have been measured and compared among natural isolates chosen to capture the full range of *ND5* deletion heteroplasmy level in this species.

Pharyngeal pumping

Deterioration of the pharynx muscles and decline in pharyngeal function have previously been correlated to aging in *C. elegans* (Collins *et al.*, 2008). Here, *C. briggsae* isolates harboring more *ND5* deletion-bearing mitochondrial genomes were shown to have significantly decreased rates of pharyngeal pumping over much of adulthood when compared to isolates with low levels of deleted genomes or none at all. Interestingly, the rate of decline in pharyngeal pumping is lower for high-deletion isolates than for others. These results suggest that, although *C. briggsae* harboring more deleted *ND5* subunits are perhaps more aged over adulthood than those with fewer or no deleted *ND5* products, the former are aging less rapidly.

The relationship between heteroplasmy and pharyngeal pumping in *C. briggsae* does not align with the non-linear relationship between heteroplasmy and *in vivo* superoxide production found by Estes *et al.* (in review). However, when considered alongside exogenous oxidative stress resistance, the disparity in between-isolate

differences of pharyngeal pumping rates and superoxide production may be explained. That is, *C. briggsae* present as less aged via either decreased superoxide production (as in the low-heteroplasmy isolates) or increased oxidative stress resistance (as in the non-heteroplasmic isolates). This interpretation mimics somewhat the ‘hormetic effect’ seen between exogenous oxidative stress and upregulation of certain oxidant scavenging mechanisms seen in *C. elegans* and proposed by Rea (2005) as one possible mechanism for life extension in certain *C. elegans* ‘mit mutants.’ Additionally, these results imply that a threshold effect for this particular deletion may be at play since high heteroplasmic *C. briggsae* may be less equipped to overcome the levels of superoxide associated with high levels of the *ND5* deletion. Considering that *C. briggsae* heteroplasmy does not exceed around 50% in all natural isolates studied (see Howe and Denver, 2008), perhaps eggs or larvae with greater than 50% deleted genomes are inviable due to a greater lack of tolerance combined with even higher elevated ROS levels compared to the highest deletion-bearing isolates studied here. However, without longitudinal study of stress resistance and superoxide production among *C. briggsae* isolates, the cause of variation in the rate of decline of pharyngeal pumping found here cannot be explained.

Growth

Growth rates for *C. briggsae* larvae vary markedly between isolates and correlate positively to relative amounts of heteroplasmy. Interestingly, *C. briggsae* larvae with any amount of partially-deleted genomes grow more rapidly and to a greater mean length than

do larvae from non-heteroplasmic isolates. These findings contradict patterns previously observed in *C. elegans* where increased RNAi of various ETC-associated genes was accompanied by decreased larval development and earlier developmental arrest (Rea *et al.*, 2007). This relationship was observed at levels of RNAi dilution that first triggered increased longevity, and continued as increasing interference worked to decrease longevity (Rea *et al.*, 2007). The enhanced larval development in high *ND5* deletion *C. briggsae* isolates could indicate a change in life history strategy due to elevated superoxide production leading to decreased lifespan (Estes *et al.*, in review). However, confounding such an interpretation is the same significantly increased larval growth in low heteroplasmic *C. briggsae* that produce the least amount of superoxide and live longest compared to non heteroplasmic isolates (Estes *et al.*, in review).

The reverse relationship between growth and heteroplasmy is seen over adulthood in *C. briggsae*, thus coinciding with previous results for *C. elegans* with ETC disrupted via RNAi (Dillon *et al.*, 2002; Rea *et al.*, 2007). Non-heteroplasmic isolates continue to grow in length between 6 and 12 days past maturation (as in *C. elegans*, see Croll *et al.*, 1977 and Bolanowski *et al.*, 1981) while heteroplasmic *C. briggsae* tend to shrink. Given that *C. elegans* aging is marked with a progressive decrease in muscle cell organization, loss of cell structure, and cell shrinkage due to loss of cytoplasm (Collins *et al.*, 2008), the later change in adult size of heteroplasmic *C. briggsae* is another indication that progressive aging accompanies higher levels of *ND5* deletion heteroplasmy.

The stark change in *C. briggsae* growth rate correlates to the switch from heavily anaerobic to aerobic metabolic pathways between larval and adult stages (Braeckman *et al.*, 2008). In wild-type *C. elegans*, entry to L4 is marked by a 3-fold increase in mtDNA copy number while another 16-fold increase occurs at the final molt from L4 to adult (Bratic *et al.*, 2009). Although these grand expansions of *C. elegans* mitochondrial genomes are tightly coupled to germline cell proliferation and embryogenesis, *C. briggsae* isolates harboring high *ND5* deletion heteroplasmy may suffer when relying on the aerobic ETC pathway during adulthood especially considering declines of maternally-provided factors in adults (*i.e.* mRNA; see Rea, 2005). Combined with the decline in pharyngeal pumping, adult growth rates imply that a high degree of heteroplasmy in *C. briggsae* denotes advanced aging in adulthood.

Oxygen consumption

Although oxygen consumption rates of *C. briggsae* L4 vary significantly among isolates, there exists no correlation to relative or isolate-specific heteroplasmy levels. This lack of correlation may be due to the age at measurement here which coincides with the change from primarily anaerobic metabolism in larvae to aerobic respiration in adults (Braeckman *et al.*, 2008). An additional confounding factor to analysis of the oxygen consumption measures reported here is the knowledge that different *C. briggsae* isolates develop at different rates (see above). Therefore, although each measure of oxygen consumption was performed on massive age-synchronized populations after the same

length of time cultured (± 1 hour), the values may reflect rates of oxygen consumption of *C. briggsae* at different ages. True evaluation of aerobic respiratory capacity of *C. briggsae* via oxygen consumption will require longitudinal measures taken well before, during and after metamorphosis into adulthood.

CHAPTER 2

Inter-Population Hybrids of *Caenorhabditis briggsae* Natural Isolates

BACKGROUND

As the primary energy producers in most eukaryotic cells, mitochondria are critical for the survival of most organisms and their dysfunction can lead to inefficient energy metabolism, disease, aging, and early death (Chinnery and Turnbull, 2000; DiMauro and Schon, 2003; Smeitink *et al.*, 2003; Taylor and Turnbull, 2005; Wallace, 2005). Across many taxa, strong correlations have been drawn between mitochondrial function, and aging and longevity; however, a causal link between mitochondrial function and aging has yet to be demonstrated (Rea and Johnson, 2003; Balaben *et al.*, 2005 Rea, 2005; Lee and Wei, 2007; Hiona and Leeuwenburgh, 2008; Gems and Doonan, 2009; Lapointe and Hekimi, 2010). A major hurdle to understanding the role of mitochondria in aging and disease within an evolutionary or population genetic context has been the lack of model systems containing relevant natural mitochondrial genetic variation. In the nematode *Caenorhabditis briggsae*, the occurrence of a geographically segregating heteroplasmic mutation affecting a key unit of complex I of the electron transport chain (ETC) provides a novel chance for examining the relationship between mitochondrial

dysfunction, longevity and disease without the use of mutagens, genetic manipulation, or cell culture manipulation.

In several recently isolated populations of *C. briggsae*, a deletion removing nearly half of the mitochondrial encoded *NADH-dehydrogenase 5 (ND5)* protein coding gene has been discovered (Howe and Denver, 2008; Fig. 1). When present, the partial deletion of *ND5* in *C. briggsae* occurs in a heteroplasmic state where some portion of mitochondrial genomes harbor the deletion (mutant) and the rest do not (wildtype). Among *C. briggsae* isolates, heteroplasmy of the *ND5* deletion ranges from 0 to 50% (Fig. 2). Although the mutant *ND5* gene in *C. briggsae* is transcribed, as evidenced by identification of truncated mRNA sequences, its degree of functionality is unknown. However, because the *ND5* gene product likely functions as a proton translocator in the distal part of the membrane bound domain of complex I, and because the *ND5* subunit is rate limiting to complex I assembly, the *ND5* deletion is likely to have a severe negative impact on complex I efficiency in *C. briggsae*. In support of this idea, several neurodegenerative diseases have been associated with mutations in *ND5* and other complex I subunits (Janssen *et al.*, 2006).

Reactive oxygen species (ROS) are normal byproducts of complex I activity and have been suspected as a key determinant of aging and longevity due to their highly destructive properties. Based on studies of other complex I mutants (Pitkanen and Robinson, 1996; Luo *et al.*, 1997; Bai *et al.*, 2000; Kayser *et al.*, 2004; Janssen *et al.*, 2006; Park *et al.*, 2009), the partial *ND5* deletion in *C. briggsae* is suspected to lead to

elevated levels of ROS, perhaps such that ROS will increase with heteroplasmy levels. Once heteroplasmy and ROS levels in *C. briggsae* reach levels beyond those that can be remedied via natural oxidant defense mechanisms, heteroplasmy is suspected to negatively impact lifespan and aging in *C. briggsae*. To establish *C. briggsae* as a natural model for studying the process of aging and lifespan determination, several life history characteristics have been evaluated for various isolates from around the world spanning the range of heteroplasmy in this species. Total offspring production was shown to correlate negatively with heteroplasmy in *C. briggsae* (Howe and Denver, 2008) as was lifespan and population growth rates (Estes *et al.*, in review). Interestingly, neither ATP content nor *in vitro* hydrogen peroxide (a form of ROS) production exhibit a significant relationship to deletion heteroplasmy level, yet oxidative stress resistance and *in vivo* superoxide (another ROS) production do (Estes *et al.*, in review). In Chapter 1 of this thesis, high isolate-specific heteroplasmy levels in *C. briggsae* were associated with increased rates of larval development and aging and decreased adult growth. Overall, these findings suggest that *ND5* deletion heteroplasmy in *C. briggsae* does impact life history and may indicate an adaptive response via more rapid development to adulthood in order to minimize damage from ROS prior to reproduction.

To establish that the phenotypic variance observed among natural isolates of *C. briggsae* is due in fact to *ND5* deletion heteroplasmy rather than to natural nuclear genetic variation segregating among isolates, several inter-population hybrid lines of *C. briggsae* have been created from natural isolates with high and low heteroplasmy levels.

After nine generations of crossing hybridized offspring back to males of the nuclear genetic source population, hybrids were considered to harbor only mitochondrial genetic information from one population on the complete nuclear genetic background of another population, resulting in >99% ‘mitochondrial replacement.’ Assuming the maternal inheritance of mitochondria and that natural *ND5* deletion heteroplasmy controls the phenotypic variation previously observed among natural *C. briggsae* isolates, and barring epistasis between genomes that control the measured traits, hybrid trait means should closely match those for each parental source of mitochondria.

Somewhat contrary to expectations, for most traits measured, hybrid *C. briggsae* differ not only from their parental populations but also from other replicates of the same introgressions. Surprisingly, mitochondrial genotyping of *C. briggsae* hybrid lines revealed that mitochondria were not inherited through the maternal lineage in most cases. However, inspection of results from hybrids in which maternal transmission of mitochondria was successful indicate that epistasis may be important for most traits measured here, such that conflict between mitochondrial and nuclear genes that have evolved separately in different populations leads to breakdown of fitness and other traits in these hybrids; *i.e.*, cytonuclear conflict.

METHODS

Nematode Strains and Maintenance

In order to disentangle competing effects of mitochondrial versus nuclear genetic variation on the among-isolate phenotypic differences reported in Chapter 1, *C. briggsae* experimental lines were generated to allow me to assess the effects of a mitochondrial genome on different nuclear genetic backgrounds. Hybrid experimental lines were created by reciprocally crossing two pairs of *C. briggsae* natural isolates. Each cross utilized one parental isolate with high *ND5* deletion heteroplasmy level and one with low heteroplasmy level (Fig. 9). (Natural isolates were provided by D. R. Denver, OSU.). Three replicate introgressions were performed for each pair of natural isolates, resulting in a total of 12 hybrid *C. briggsae* lines (Table 2). Nematodes were grown under standard laboratory conditions at 25°C on 15mm NGM plates and *Escherichia coli* strain HB101, unless otherwise noted.

Nematode Hybridization

Each replicate of the four *C. briggsae* introgressions was initiated by crossing one egg-laden hermaphrodite from the mitochondrial source isolate with 5-10 males from the nuclear source isolate for a given cross. Multiple males were paired with each hermaphrodite to ensure successful outcrossing. Male-rich colonies were maintained by manual enrichment of source populations every other day. Males were removed the next

day to avoid damage to hermaphrodites from excessive mating. Three days after the initial cross, plates were examined for successful male fertilization as indicated by a 1:1 offspring sex ratio. Once offspring had reached approximately the L4 stage, one hermaphrodite from a successful cross was transferred to a new plate and crossed with 5-10 males from the nuclear source population. (Typically, more than one hybrid hermaphrodite was isolated per replicate and crossed with males in this manner to ensure advancement to the next outcrossing since numerous hybrid lineages went extinct.) The nuclear DNA (nDNA) of hybrid offspring should be entirely replaced after nine successful matings of hybrid hermaphrodites to males of the nuclear source population. In other words, F10 hybrid individuals should carry only mitochondrial DNA (mtDNA) from one natural *C. briggsae* isolate and only nDNA from a second isolate.

Figure 9. *C. briggsae* natural isolates selected for hybridization. Two pairs of high and low heteroplasmy isolates (outlined on this intraspecific phylogeny where % ND5 Del. indicates isolate-specific percentages of total mitochondrial genomes that harbor ND5 deletions, as determined by qPCR (Howe and Denver, 2008)) were used as progenitors for hybrid lines. (GL = global intraspecific superclade; KE = Kenya clade; TE and TR = temperate and tropical subclades of GL. C(+) = temperate-clade isolates bearing compensatory ΨND5-2 alleles; C(-) = those bearing ancestral alleles.) * The HK104 strain used here was the inbred progenitor of mutation-accumulation lines (Baer *et al.*, 2005) found to harbor high ND5 deletion levels similar to those of HK105 (Dee Denver, pers. comm.).

Fitness and Lifespan

The partial *ND5* mtDNA deletion segregating among natural *C. briggsae* isolates has previously been negatively correlated to fitness (Howe and Denver, 2008). To establish whether this relationship is in fact due to the natural mtDNA variation among isolates rather than to nDNA variation among isolates, offspring production and lifespan of hybrid *C. briggsae* mitochondrial replacement lines were evaluated and compared to the parental lines.

For each parental natural isolate and replicate introgressed line, several adult hermaphrodites were placed on Petri dishes, allowed to lay eggs for three hours and then removed. Two days later, 10-20 L4 individuals per line were transferred to a new plate each, and again ~24 hours later over the duration of the reproductive period. After each transfer, plates remained at room temperature for ~24 hours to allow viable eggs to hatch;

Mt Origin	Nuclear Origin	Rep	Annotation	Expected <i>ND5</i> deletion %
HK104	EG4181	1	mHnE_1	~50
		2	mHnE_2	
		3	mHnE_3	
EG4181	HK104	1	mEnH_1	~0
		2	mEnH_2	
		3	mEnH_3	
HK105	PB800	1	mHnP_1	~50
		2	mHnP_2	
		3	mHnP_3	
PB800	HK105	1	mPnH_1	~5
		2	mPnH_2	
		3	mPnH_3	

Table 2. Expected *ND5* deletion heteroplasmy levels in *C. briggsae* hybrids. In crossing *C. briggsae* natural isolates with high and low *ND5* deletion levels, these expectations are based on maternal natural isolate heteroplasmy (cf. Fig. 9).

then 1mL of 0.3M sodium azide per plate was added to euthanize larvae. Plates of progeny were stored at 4°C until later enumeration which was enabled by staining *E. coli* and agar with 0.075% toluidine blue. Total lifespan of adult hermaphrodites was determined as the number of days lived since hatching.

Pharyngeal pumping

Pharyngeal pumping rate is known to decline steadily with age in *C. elegans* and, therefore, serves as a reliable biomarker of age (Collins *et al.*, 2008). *ND5* deletion heteroplasmy in natural *C. briggsae* isolates has been found to correlate negatively to adult pharyngeal pumping (Chapter 1 of this thesis), implying that increased levels of the partial *ND5*-deletion may affect rates of aging in *C. briggsae*. If, indeed, the *ND5* deletion alters rates of aging and it is maternally transmitted to offspring as expected, pharyngeal pumping in *C. briggsae* hybrids should – barring cytonuclear epistasis – closely match that for the natural *C. briggsae* isolate that contributed the mtDNA.

To compare rates of aging between hybrid replicates and parental lineages, pharyngeal pumping was assessed every other day during adulthood starting at two days past L4 (fourth instar). The same 10-20 individuals per line analyzed in the fitness and longevity assays were examined for pharyngeal pumping, except for the third replicate of each introgressed line. Pharyngeal pumping assays were performed at room temperature on NGM plates and calculated as the average number of terminal bulb pumps from three

5-second intervals, converted to pumps per minute. Decline in pharyngeal pumping rates were calculated between days 2 and 12.

Growth

As with pharyngeal pumping, if the partial deletion of *ND5* is responsible for variation in larval and adult growth between natural isolates of *C. briggsae*, hybrid growth rates should match those for the relevant mitochondrial source population. For larval measurements, nematodes were age synchronized by allowing 10-20 gravid hermaphrodites per isolate and hybrid replicate to lay eggs on fresh 15mm NGM plates with *E. coli* OP50-1 for two hours. From these age-synchronized cohorts, 5 to 10 larvae per plate were randomly selected for measurement at 14, 26, 34 and 42 hours after the egg stage (correlating approximately to each of the four larval stages, L1-L4). At each time point, larvae were transferred to a glass slide containing a small NGM agar pad and immobilized with 10 μ l of 20mM sodium azide. Digital images were captured using a PixeLINKTM camera mounted on a Nikon Labophot and measurements were made using ImageJ (NIH, <http://rsbweb.nih.gov/ij/>). Larval growth was assessed as the difference in the average lengths of animals between 14 and 42 hours after the egg stage, and converted to mm per hour.

For adult measurements, all individual nematodes from the pharyngeal pumping assay were imaged live (remained on NGM plates) on days 0, 6 and 12 from the L4 stage. Length measurements were assessed as above (Fig. 4) and growth rates in length were

calculated between 0 and 6, and 0 and 12 days then converted to mm per day. Note that, because only animals that survived to be measured on day 12 were used in this analyses, any laboratory selection on body size that may have been operating will not affect our results.

From the images captured to assess larval growth rates, larval stage at 42 hours after hatching was determined to assess the affect of introgression and inheritance of the mtDNA deletion in *C. briggsae* on cell cycle progression in *C. briggsae*. Ratios of L3 to L4 stage larvae at 42 hours after hatching were compared to the expected frequency (*i.e.* zero L3 larvae at 42 hours after hatching), and these ratios were compared between hybrid lineages and parental isolates.

Oxygen consumption

Whole-organism oxygen consumption was previously measured on age synchronized samples and compared to determine whether natural mtDNA variation affects aerobic respiration among *C. briggsae* natural isolates (Chapter 1 of this thesis). Oxygen consumption rates were found to vary significantly between natural isolates but did not correlate significantly to *ND5* deletion heteroplasmy. To determine whether this variation is due to the mtDNA variation between isolates, oxygen consumption rates of hybrid *C. briggsae* were measured and evaluated against values for the natural mitochondrial source population of each introgression.

Whole-animal respiration rates were measured according to Braeckman *et al.* (2002a) with some modification, and these data were normalized to the protein content per sample using a BCA kit (Pierce) following preparation described in Braeckman *et al.* (2002a). Data were obtained for only two replicates of each introgression, except mHK105 x nPB800 for which all replicates were measured.

Statistics

Statistical analyses were performed using R (R Development Core Team, 2009; MASS package by Venables and Ripley, 2002) and Microsoft Office Excel (2007). Differences between parental isolates were assessed using t-tests for each trait. All replicates of each introgression were compared using one-way analyses of variance for each phenotype, or t-tests when applicable. Finally, hybrid replicates from each introgression were pooled and compared to the appropriate maternal isolate using a t-test.

Genotyping

To verify maternal transmission of mitochondrial genomes and assess the *ND5* deletion heteroplasmy level in hybrid *C. briggsae*, molecular analyses of hybrid and parental strains were performed by collaborator D. K. Howe at the Center for Genome Research and Biocomputing (OSU, Corvallis, OR). Briefly, a published PCR assay (Howe and Denver, 2008) was used to assess the relative frequency of the *ND5* deletion in each replicate of all introgressions and each parental isolate. These analyses were

performed twice for each replicate of each hybrid lineage in Table 2. Additionally, sequencing of microsatellite loci within the mitochondrial cytochrome-oxidase II (COII) gene was conducted to verify maternal inheritance of mtDNA genomes.

RESULTS

Several lines of *C. briggsae* hybridized from natural isolates with high and low *ND5* deletion heteroplasmy were created in order to determine whether the phenotypic variance observed among natural isolates is due to mitochondrial genetic differences among those isolates. If so, and assuming that mitochondria are inherited maternally and *ND5* deletion frequency is transmitted to offspring, hybrid trait means should closely match those for each respective maternal progenitor.

Fitness

Daily fecundities from the parental and reciprocal hybrid strains from crossing *C. briggsae* natural isolates EG4181 and HK104 are shown in Figure 10. Fitness varied significantly between EG4181 and HK104 natural isolates (low and high heteroplasmy, respectively) with the former producing significantly more offspring overall ($t(58)=-12.19$, $p<0.001$; Table 3). The mHK104 x nEG4181 (mHnE from here on, as in Table 2) hybrids also varied significantly between replicates in total offspring production ($t(32)=7.58$, $p<0.001$; data unattainable for the third replicate, mHnE_3, due to lack of viable larvae from age-synchronized populations; Table 4). When pooled, mHnE hybrids produced significantly more offspring than the maternal source, HK104 ($t(52)=4.19$, $p<0.001$; Table 4). Hybrids of mEG4181 x nHK104 (mEnH from here on, as in table 2) derivation did not vary significantly among replicates ($F(2,29)=2.12$, $p=0.14$; Table 5)

but produced significantly less offspring when pooled than the maternal source, EG4181 ($t(70)=-6.32$, $p<0.001$; Table 5). (Note that progeny production on the fifth day of maturity was not obtained for the third replicate of each introgression.)

Daily fecundities of the parental and reciprocal hybrid strains from crossing *C. briggsae* natural isolates PB800 and HK105 are shown in Figure 11. Low heteroplasmy natural *C. briggsae* isolate PB800 produced significantly more offspring overall than high heteroplasmy isolate HK105 ($t(48)=-6.42$, $p<0.001$; Table 3). mHK105 x nPB800 hybrids (mHnP from here on, as in Table 2) varied significantly among replicates for this

Figure 10. Total reproductive output for each parental and introgressed line of natural *C. briggsae* isolates HK104 (high heteroplasmy) and EG4181 (low heteroplasmy). Total progeny per day of reproduction is indicated by bar color as denoted in the figure legend. Error bars indicate one standard error. (Data missing for mHnE_3 – see text.)

trait ($F(2,31)=8.50$, $p<0.01$; Table 6) and, when pooled, produced significantly more offspring than the putative mitochondrial source population, isolate HK105 ($t(52)=5.86$, $p<0.001$; Table 6). However, *C. briggsae* hybrids mPB800 x nHK105 (mPnH from here on, as in Table 2) did not vary significantly among replicates ($F(2,41)=1.79$, $p=0.18$; Table 7) and produced less total offspring when pooled and compared to their maternal source, PB800 ($t(72)=-4.93$, $p<0.001$; Table 7). (Note that progeny produced on the fifth day of maturity was not obtained for the third replicate of each introgression.)

Figure 11. Total reproductive output for each parental and introgressed line of natural *C. briggsae* isolates HK105 (high heteroplasmy) and PB800 (low heteroplasmy). Total progeny per day of reproduction is indicated by bar color as denoted in the figure legend. Error bars indicate one standard error.

Lifespan

Lifespan of the parental and reciprocal hybrid strains from crossing *C. briggsae* natural isolates EG4181 and HK104 are shown in Figure 12. Lifespan in days since hatching does not vary significantly between *C. briggsae* natural isolates HK104 and EG4181 ($t(43)=-1.62$, $p=0.11$; Table 3). *C. briggsae* hybrid mHnE longevity did not differ significantly between replicates ($t(16)=0.32$, $p=0.76$; data were unattainable for the third replicate, mHnE_3, due to lack of viable larvae from age-synchronized populations) or from the maternal source natural isolate, HK104, when pooled ($t(38)=1.20$, $p=0.24$; Table 4). Similarly, mEnH hybrids did not differ significantly in lifespan either among replicates of this particular introgression ($F(2,26)=2.17$, $p=0.13$) or when pooled and compared to the maternal source isolate, EG4181 ($t(47)=-1.62$, $p=0.11$; Table 5).

Figure 12. Lifespan in days since hatching for each parental and introgressed line of natural *C. briggsae* isolates HK104 (high heteroplasmy) and EG4181 (low heteroplasmy). Error bars indicate one standard error. (Data missing for mHnE_3 – see text.)

Lifespan of the parental and reciprocal hybrid strains from crossing *C. briggsae* natural isolates PB800 and HK105 are shown in Figure 13. Lifespan of natural *C. briggsae* isolate PB800 (low heteroplasmy) is significantly greater than for HK105 (high heteroplasmy) ($t(49)=-4.29$, $p<0.001$; Table 3). However, lifespan for mHnP hybrids did not vary significantly among replicates ($F(2,26)=0.72$, $p=0.50$) nor when pooled and compared to the maternal source isolate HK105 ($t(55)=-0.54$, $p=0.59$; Table 6). Reciprocal introgression replicates mPnH_1, 2 and 3 also did not differ significantly in total lifespan in days since hatching ($F(2,27)=2.12$, $p=0.14$), but did exhibit significantly decreased mean lifespan when pooled and compared to the maternal source, PB800 ($t(51)=-4.73$, $p<0.001$; Table 7).

Figure 13. Lifespan in days since hatching for each parental and introgressed line of natural *C. briggsae* isolates HK105 (high heteroplasmy) and PB800 (low heteroplasmy). Error bars indicate one standard error.

Pharyngeal pumping

Adult pharyngeal pumping rates of the parental and reciprocal hybrid strains from crossing *C. briggsae* natural isolates EG4181 and HK104 are shown in Figure 14. As previously determined, low heteroplasmy *C. briggsae* natural isolate EG4181 exhibits significantly greater pharyngeal pumping rates every other day through day 10 of adulthood, when compared to the high heteroplasmy isolate HK104 (Table 3) as well as a significantly increased rate of decline in pharyngeal pumping between days 2 and 12 of adulthood ($t(22)=-2.45$, $p<0.05$; Table 3). *C. briggsae* hybrids mHnE replicates did not

Figure 14. Adult pharyngeal pumping for each parental and two introgressed line of each reciprocal cross of natural *C. briggsae* isolates HK104 (high heteroplasmy) and EG4181 (low heteroplasmy).

differ significantly in adult pharyngeal pumping rates nor decline in this rate and displayed increased rates when pooled and compared to maternal source population HK104 on only days 2 and 4 of adulthood (Table 4). *C. briggsae* mEnH hybrid replicates 1 and 2 differed significantly for only one day of adult pharyngeal pumping and for only two days when pooled and compared to the maternal source, EG4181 (Table 5). Neither comparison between mEnH replicates nor between pooled replicates and maternal source EG4181 proved significantly different for the rate of decline in pharyngeal pumping between 2 and 12 days of adulthood (Table 5).

Figure 15. Adult pharyngeal pumping for each parental and two introgressed line of each reciprocal cross of natural *C. briggsae* isolates HK105 (high heteroplasmy) and PB800 (low heteroplasmy).

Adult pharyngeal pumping rates of the parental and reciprocal hybrid strains from crossing *C. briggsae* natural isolates PB800 and HK105 are shown in Figure 15. Natural *C. briggsae* isolate PB800 (low heteroplasmy) exhibited significantly increased pharyngeal pumping for most days of adulthood measured compared to HK105 (high heteroplasmy), although the decline in pharyngeal pumping was not significantly different between these two isolates (Table 3). Adult pharyngeal pumping of *C. briggsae* hybrids mHnP was significantly different between replicates on only one day but significantly greater than their maternal source, HK105, on four of ten days measured as was the rate of decline in pharyngeal pumping (Table 6). For mPnH hybrid *C. briggsae*, pharyngeal pumping rates did not differ significantly between replicates but were significantly decreased for all but the first two days of adulthood when compared to the maternal source isolate, PB800 (Table 7); however, due to the lack of a significant difference between the pooled hybrid pharyngeal pumping and that of PB800 on the first day of measurement (day 2), there was no significant difference in decline of pumping rates (Table 7).

Larval Growth and Development

Length of larvae at four time points during development of the parental and reciprocal hybrid strains from crossing *C. briggsae* natural isolates EG4181 and HK104 are shown in Figure 16. Only larval length at 14 hours after hatching was significantly greater in natural isolate HK104 compared to EG4181 ($t(66)=2.9$, $p<0.05$; Table 3).

Length of mHnE larvae at the first three of four time points varied significantly among replicates and was significantly greater for all replicates compared to the maternal source isolate, HK104, as was the rate of growth in length between the first and final time point (Table 4). For mEnH hybrids, length at each time point and the change in length overall was significantly different among replicates but at only one time point when compared to the maternal source, EG4181 (Table 5).

Figure 16. Length of larvae from each parental and introgressed line of natural *C. briggsae* isolates HK104 (high heteroplasmy) and EG4181 (low heteroplasmy). Bar color indicates length at 14, 26, 34 and 42 hours after hatching, as shown in the figure legend, corresponding to each of four larval stages. Error bars indicate one standard error.

Length of larvae at four time points during development of the parental and reciprocal hybrid lines from crossing *C. briggsae* natural isolates PB800 and HK105 are shown in Figure 17. Length of *C. briggsae* isolate HK105 larvae was significantly greater than for PB800 at 14 hours after hatching, but significantly less than PB800 at 26, 34 and 42 hours after hatching resulting in an overall increased rate of growth and ultimate size in PB800 larvae compared to HK105 (Table 3). Between mHnP hybrid replicates, larval length varied significantly only at 14 hours after hatching and, accordingly, the rate

Figure 17. Length of larvae from each parental and introgressed line of natural *C. briggsae* isolates HK105 (high heteroplasmy) and PB800 (low heteroplasmy). Bar color indicates length at 14, 26, 34 and 42 hours after hatching, as shown in the figure legend, corresponding to each of four larval stages. Error bars indicate one standard error.

of growth in the hybrid larvae between the first and final time point varied significantly among these hybrid replicates (Table 6). When pooled, mHnP hybrids were longer at the last three time points and grew faster than the natural isolate HK105 (Table 6). *C. briggsae* hybrid mPnH larval length varied significantly among replicates for all but the first time point and also differed in rate of growth (Table 7) however did not differ significantly from the maternal source, PB800, except for the two middle measurements (Table 7).

Adult Growth

Length of adults at three time points of the parental and reciprocal hybrid strains from crossing *C. briggsae* natural isolates EG4181 and HK104 are shown in Figure 18. Although natural isolate HK104 length on just the first day of adulthood was greater than that of natural isolate EG4181, the latter grew faster over the first twelve days of adulthood (Table 3). Replicates 1 and 2 of hybrid cross mHnE did not differ significantly in length or growth in length over adulthood and were significantly shorter and grew slower than the maternal source of these hybrids, HK104, when pooled (Table 4). Similarly, mEnH hybrid replicates 1 and 2 did not differ in length or growth in length as adults but were significantly longer than their putative mitochondrial source isolate EG4181 on the first day of adulthood resulting in a significantly decreased rate of adult growth when combined and compared to EG4181 (Table 5).

Length of adults at three time points of the parental and reciprocal hybrid strains from crossing *C. briggsae* natural isolates PB800 and HK105 are shown in Figure 19.

Natural isolate PB800 was significantly longer than HK105 at the start of adulthood, yet neither was significantly larger later in adulthood nor grew faster in length (Table 3).

Hybrid mHnP replicates were similar at the first and final measurements of adult length, but the second replicate was significantly longer than the first on day 6 resulting in an increased rate of growth in length between 0 and 6 days compared to the first replicate (Table 6). When pooled, both mHnP replicates were significantly longer than their

Figure 18. Length of adults from each parental and introgressed line of natural *C. briggsae* isolates HK104 (high heteroplasmy) and EG4181 (low heteroplasmy). Bar color indicates length at 0, 6, 12 days of maturity, as shown in the figure legend. Error bars indicate one standard error.

maternal source HK105 on the first day of adult measurements but shorter on the final day of measurements and thus grew slower over this time period than HK105 (Table 6). No significant difference in adult length and growth exists between replicates of mPnH, yet together were shorter than the maternal source PB800 and so these hybrids grew faster over adulthood compared to PB800 (Table 7).

Adult Growth

Figure 19. Length of adults from each parental and introgressed line of natural *C. briggsae* isolates HK105 (high heteroplasmy) and PB800 (low heteroplasmy). Bar color indicates length at 0, 6, 12 days of maturity, as shown in the figure legend. Error bars indicate one standard error.

Oxygen consumption

Whole-organism oxygen consumption rates of the parental and reciprocal hybrid strains from crossing *C. briggsae* natural isolates EG4181 and HK104 are shown in Figure 20. There is no significant difference between rates of young adult oxygen consumption between high heteroplasmy isolate HK104 and low heteroplasmy isolate EG4181 ($t(2)=0.70$, $p=0.56$; Table 3). Two replicates of the hybrid line mHnE also do not differ in rates of oxygen consumption ($t(2)=-0.96$, $p=0.43$) but together consume more oxygen per hour per mg protein than the natural isolate HK104 ($t(4)=-2.51$, $p=0.07$; Table 4). Hybrid mEnH replicates consume oxygen at significantly different rates

Figure 20. Whole-organism oxygen consumption of young adults for each parental and introgressed line of natural *C. briggsae* isolates HK104 (high heteroplasmy) and EG4181 (low heteroplasmy). Error bars indicate one standard error.

($t(2)=-4.80$, $p=0.04$) but not when pooled and compared to the maternal source natural isolate EG4181 ($t(4)=0.23$, $p=0.82$; Table 5).

Whole organism oxygen consumption rates of the parental and reciprocal hybrid strains from crossing *C. briggsae* natural isolates PB800 and HK105 are shown in Figure 21. The natural isolates PB800 and HK105 do not consume oxygen at significantly different rates ($t(2)=-0.96$, $p=0.44$; Table 3), despite significant differences in heteroplasmy between them. Rates of oxygen consumption vary significantly between three replicates of mHnP hybrids ($F(2,3)=29.39$, $p=0.01$) but not when combined and compared to the putative source of mitochondria from these crosses, HK105 ($t(6)=0.11$,

Figure 21. Whole-organism oxygen consumption of young adults for each parental and introgressed line of natural *C. briggsae* isolates HK105 (high heteroplasmy) and PB800 (low heteroplasmy). Error bars indicate one standard error.

$p=0.91$; Table 6). Oxygen consumption of mPnH hybrids was not significantly different between two replicates, nor between these and the putative source of mitochondria, natural isolate PB800 ($t(4)=1.58$, $p=0.19$; Table 7).

	HK104 vs. EG4181	HK105 vs. PB800
Life history		
Total reproduction	t (58) = -12.19***	t (48) = -6.42***
Lifespan (days from hatching)	t (43) = -1.62	t (49) = -4.29***
Pharyngeal pumping (pumps/minute)		
L4+2 days	t (26) = -4.60***	t (25) = -3.75***
L4+4 days	t (26) = -4.60***	t (26) = -0.75
L4+6 days	t (25) = -2.84**	t (26) = -1.64
L4+8 days	t (24) = -3.41**	t (26) = -2.42*
L4+10 days	t (24) = -3.59**	t (25) = -4.69***
L4+12 days	t (22) = -0.34	t (25) = -4.67***
L4+14 days	t (17) = -1.00	t (25) = -2.98**
L4+16 days	t (13) = -1.49	t (24) = -1.27
L4+18 days	t (10) = 0.40	t (23) = -4.54***
L4+20 days	t (7) = -1.14	t (22) = -3.11**
Rate of decline between 2 and 12 days post-L4 (per day)	t (22) = -2.45*	t (25) = -0.72
Larval growth (length in mm)		
14 hours after hatching	t (66) = 2.09*	t (38) = 2.19*
26 hours after hatching	t (56) = -0.82	t (50) = -2.96**
34 hours after hatching	t (51) = 0.90	t (66) = -4.21***
42 hours after hatching	t (52) = 0.78	t (50) = -6.27***
Rate of growth between 14 and 42 hours after hatching (per hour)	t (52) = -1.56	t (35) = -4.80***
Adult growth (length in mm)		
Day 0 (L4)	t (30) = 7.99***	t (34) = -5.56***
Day 6 (past L4)	t (28) = -0.48	t (27) = -0.43
Day 12 (past L4)	t (22) = -0.10	t (26) = -0.07
Rate of growth between days 0 and 6 (per day)	t (28) = -3.73***	t (27) = 1.63
Rate of growth between days 0 and 12 (per day)	t (22) = -2.08*	t (26) = 1.66
Oxygen consumption		
μmol/hr/mg	t (2) = 0.70	t (2) = -0.96

Table 3. Test statistics per trait from comparisons between pairs of natural isolate *C. briggsae* progenitors used for hybridization. High *ND5* deletion heteroplasmy isolates are HK104 and HK105; low *ND5* deletion heteroplasmy isolates are EG4181 and PB800. (*, **, and *** denote significance at $\alpha = 0.05$, 0.01, and 0.001, respectively.)

mHK104 x nEG4181	hybrid replicates	pooled hybrids vs. maternal parent
Life history		
Total reproduction	t (32) = 7.58***	t (52) = 4.19***
Lifespan (days from hatching)	t (16) = 0.32	t (38) = 1.20
Pharyngeal pumping (pumps/minute)		
L4+2 days	t (24) = 1.58	t (38) = 2.50*
L4+4 days	t (20) = 1.22	t (34) = 2.06*
L4+6 days	t (17) = -0.28	t (30) = 2.03
L4+8 days	t (17) = 1.28	t (30) = 0.19
L4+10 days	t (14) = 0.40	t (27) = 1.52
L4+12 days	t (11) = -0.06	t (23) = 0.79
L4+14 days	t (6) = 1.53	t (17) = -0.03
L4+16 days	t (4) = 0.92	t (12) = -0.50
L4+18 days	NA	t (7) = 0.78
L4+20 days	NA	t (5) = -0.77
Rate of decline between 2 and 12 days post-L4 (per day)	t (11) = 0.30	t (23) = 0.71*
Larval growth (length in mm)		
14 hours after hatching	F (2, 24) = 19.22***	t (59) = -2.81**
26 hours after hatching	F (2, 26) = 47.41***	t (52) = 2.10*
34 hours after hatching	F (2, 32) = 25.56***	t (58) = 3.38**
42 hours after hatching	F (2, 44) = 1.80	t (72) = 4.38***
Rate of growth between 14 and 42 hours after hatching (per hour)	F (2, 24) = 0.52	t (52) = 5.37***
Adult growth (length in mm)		
Day 0 (L4)	t (18) = 1.76	t (45) = -2.64*
Day 6 (past L4)	t (18) = 1.95	t (34) = -4.23***
Day 12 (past L4)	t (11) = -0.99	t (25) = -2.89*
Rate of growth between days 0 and 6 (per day)	t (18) = 1.03	t (34) = -4.05***
Rate of growth between days 0 and 12 (per day)	t (11) = -1.70	t (25) = -2.40*
Oxygen consumption		
μmol/hr/mg	t (2) = -0.96	t (4) = -2.51

Table 4. Test statistics per trait from comparisons between or among replicates of mHK104 x nEG4181 hybridization ('hybrid replicates'), and between the replicates pooled and the maternal isolate, HK104. (*, **, and *** denote significance at $\alpha = 0.05$, 0.01, and 0.001, respectively. NA denotes impossible analysis due to small sample sizes.)

mEG4181 x nHK104	hybrid replicates	pooled hybrids vs. maternal parent
Life history		
Total reproduction	F (2, 29) = 2.12	t (70) = -6.32***
Lifespan (days from hatching)	F (2, 26) = 2.17	t (47) = -1.62
Pharyngeal pumping (pumps/minute)		
L4+2 days	t (17) = -2.09	t (31) = -0.91
L4+4 days	t (17) = -1.19	t (31) = -0.25
L4+6 days	t (16) = -2.53*	t (30) = -1.71
L4+8 days	t (14) = 1.16	t (27) = -3.60**
L4+10 days	t (14) = -1.43	t (27) = -4.22***
L4+12 days	t (12) = -0.13	t (24) = -0.75
L4+14 days	t (8) = -0.36	t (16) = -1.84
L4+16 days	t (5) = -1.00	t (12) = -0.26
L4+18 days	t (3) = -0.77	t (9) = -1.48
L4+20 days	NA	t (6) = -1.41
Rate of decline between 2 and 12 days post-L4 (per day)	t (12) = -1.71	t (24) = -0.20
Larval growth (length in mm)		
14 hours after hatching	F (2, 20) = 12.81***	t (55) = 1.59
26 hours after hatching	F (2, 20) = 26.16***	t (54) = 0.14
34 hours after hatching	F (2, 23) = 31.44***	t (52) = 6.74***
42 hours after hatching	F (2, 36) = 35.90***	t (64) = -1.14
Rate of growth between 14 and 42 hours after hatching (per hour)	F (2, 16) = 6.311**	t (44) = 1.10
Adult growth (length in mm)		
Day 0 (L4)	t (18) = -0.91	t (33) = 9.26***
Day 6 (past L4)	t (16) = 0.06	t (30) = -0.34
Day 12 (past L4)	t (12) = -0.266	t (22) = -1.26
Rate of growth between days 0 and 6 (per day)	t (16) = 0.17	t (30) = -3.42**
Rate of growth between days 0 and 12 (per day)	t (12) = -0.07	t (22) = -3.09**
Oxygen consumption		
μmol/hr/mg	t (2) = -4.80*	t (4) = 0.23***

Table 5. Test statistics per trait from comparisons between or among replicates of mEG4181 x nHK104 hybridization ('hybrid replicates'), and between the replicates pooled and the maternal isolate, EG4181. (*, **, and *** denote significance at $\alpha = 0.05$, 0.01, and 0.001, respectively. NA denotes impossible analysis due to small sample sizes.)

mHK105 x nPB800	hybrid replicates	pooled hybrids vs. maternal parent
Life history		
Total reproduction	F (2, 31) = 8.50**	t (52) = 5.86***
Lifespan (days from hatching)	F (2, 26) = 0.72	t (55) = -0.54
Pharyngeal pumping (pumps/minute)		
L4+2 days	t (18) = 0.26	t (33) = 5.27***
L4+4 days	t (18) = -2.60*	t (33) = 2.54*
L4+6 days	t (16) = -2.03	t (31) = 2.18*
L4+8 days	t (16) = -0.80	t (31) = 2.01
L4+10 days	t (16) = -1.34	t (31) = 2.40*
L4+12 days	t (14) = -0.68	t (29) = 1.53
L4+14 days	t (8) = -0.07	t (23) = 1.12
L4+16 days	t (6) = 0.74	t (20) = -0.04
L4+18 days	t (1) = 1.73	t (15) = 1.73
L4+20 days	NA	t (13) = -0.38
Rate of decline between 2 and 12 days post-L4 (per day)	t (14) = 0.48	t (29) = 2.57*
Larval growth (length in mm)		
14 hours after hatching	F (2, 20) = 8.27**	t (45) = 1.26
26 hours after hatching	F (2, 23) = 1.97	t (51) = 2.25*
34 hours after hatching	F (2, 234) = 2.30	t (59) = 7.22***
42 hours after hatching	F (2, 35) = 2.09	t (57) = 4.46***
Rate of growth between 14 and 42 hours after hatching (per hour)	F (2, 20) = 4.45*	t (42) = 3.76***
Adult growth (length in mm)		
Day 0 (L4)	t (18) = 0.33	t (35) = 3.77***
Day 6 (past L4)	t (17) = -3.23**	t (32) = -0.97
Day 12 (past L4)	t (14) = -1.51	t (29) = -2.55*
Rate of growth between days 0 and 6 (per day)	t (17) = -2.60*	t (32) = -2.60*
Rate of growth between days 0 and 12 (per day)	t (14) = -1.35	t (29) = -3.75***
Oxygen consumption		
μmol/hr/mg	F (2, 3) = 29.39*	t (6) = 0.11

Table 6. Test statistics per trait from comparisons between or among replicates of mHK105 x nPB800 hybridization ('hybrid replicates'), and between the replicates pooled and the maternal isolate, HK105. (*, **, and *** denote significance at $\alpha = 0.05$, 0.01, and 0.001, respectively. NA denotes impossible analysis due to small sample sizes.)

mPB800 x nHK105	hybrid replicates	pooled hybrids vs. maternal parent
Life history		
Total reproduction	F (2, 41) = 1.79	t (72) = -4.93***
Lifespan (days from hatching)	F (2, 27) = 2.12	t (51) = -4.73***
Pharyngeal pumping (pumps/minute)		
L4+2 days	t (26) = 0.02	t (38) = -1.66
L4+4 days	t (25) = -0.18	t (38) = -1.10
L4+6 days	t (25) = -0.42	t (38) = -3.90***
L4+8 days	t (24) = -0.12	t (37) = -3.31**
L4+10 days	t (22) = -0.98	t (34) = -5.12***
L4+12 days	t (24) = 0.70	t (36) = -5.29***
L4+14 days	t (21) = 0.29	t (33) = -4.42***
L4+16 days	t (17) = 1.55	t (29) = -2.81**
L4+18 days	t (16) = 0.62	t (27) = -5.36***
L4+20 days	t (7) = -0.88	t (18) = -3.06**
Rate of decline between 2 and 12 days post-L4 (per day)	t (24) = -0.46	t (35) = 0.69
Larval growth (length in mm)		
14 hours after hatching	F (2, 19) = 2.63	t (36) = -0.01
26 hours after hatching	F (2, 25) = 7.38**	t (51) = 3.31**
34 hours after hatching	F (2, 25) = 31.51***	t (60) = 3.12**
42 hours after hatching	F (2, 31) = 27.53***	t (63) = 0.86
Rate of growth between 14 and 42 hours after hatching (per hour)	F (2, 18) = 22.72***	t (35) = 0.13
Adult growth (length in mm)		
Day 0 (L4)	t (28) = 0.60	t (47) = -4.80***
Day 6 (past L4)	t (25) = 1.07	t (39) = -0.10
Day 12 (past L4)	t (24) = 0.73	t (37) = 0.52
Rate of growth between days 0 and 6 (per day)	t (24) = 0.66	t (39) = 1.10
Rate of growth between days 0 and 12 (per day)	t (24) = 0.65	t (37) = 2.05*
Oxygen consumption		
μmol/hr/mg	t (2) = -2.23	t (4) = 1.58

Table 7. Test statistics per trait from comparisons between or among replicates of mPB800 x nHK105 hybridization ('hybrid replicates'), and between the replicates pooled and the maternal isolate, PB800. (*, **, and *** denote significance at $\alpha = 0.05$, 0.01, and 0.001, respectively.)

Genotyping

Qualitative assessment of banding for PCR products containing the ~700bp partial deletion of *ND5* in *C. briggsae* hybrids and natural isolates used for hybridization revealed unexpected inheritance of the shorter mtDNA sequence. In fact, for each replicate of each introgression and its reciprocal (with one exception), banding patterns were the opposite from those expected given stable inheritance of heteroplasmy levels through the maternal lineage (Table 8). These same results were observed over two separate PCR procedures. To further verify whether mtDNA had been inherited in *C. briggsae* hybrids as expected, quantitative PCR for microsatellites in the mtDNA cytochrome oxidase II (COII) gene was performed. Again, unexpectedly, results from these assays indicated that mitochondria had been inherited through the paternal lineage of most replicate hybrids lines except for those of the mPnH introgressions (Table 8). Subsequent sequencing of the deletion-sized band from only one replicate of these mPnH hybrids revealed that a deletion of novel size had accumulated in the mtDNA of this line.

Mt Origin	Nuclear Origin	Rep	Annotation	Expected ND5 deletion %	Actual ND5 deletion %	Mt Genotype
HK104	EG4181	1	mHnE_1	~50	~50	EG4181
		2	mHnE_2		~0	HK104
		3	mHnE_3		~0	EG4181
EG4181	HK104	1	mEnH_1	~0	~50	HK104
		2	mEnH_2		~50	HK104
		3	mEnH_3		~50	HK104
HK105	PB800	1	mHnP_1	~50	~5	HK105
		2	mHnP_2		~5	ND
		3	mHnP_3		~5	PB800
PB800	HK105	1	mPnH_1	~5	~50**	PB800*
		2	mPnH_2		~50**	PB800*
		3	mPnH_3		~50**	PB800*

Table 8. Expectations of heteroplasmy levels and outcomes from crossing *C. briggsae* natural isolates with high and low *ND5* deletion levels. Actual *ND5* deletion % are based on qualitative analyses of PCR and gel electrophoresis for the region of mtDNA missing when the *ND5* deletion is present. Mt genotypes were determined by isolate-specific SNPs in the COII gene. (ND=not determined.) *Inheritance of mitochondria is maternal in all replicates of this hybridization only. **While the banding pattern of *ND5* deletion region for all replicates of this cross mimic that for the paternal population, thus contradicting results of mtDNA genotyping, sequencing of the deleted region from one of these replicates indicates a deletion of novel size and location.

DISCUSSION

To establish whether natural mtDNA variation controls the phenotypic variance among isolates from natural populations of the nematode *C. briggsae*, nematodes hybridized from various pairs of natural *C. briggsae* isolates have been analyzed for morphological and physiological traits and compared to respective parental trait means. Assuming that mitochondria are inherited through the maternal lineage, *ND5* deletion frequency is transmitted to offspring at a stable frequency, and that epistasis between genomes does not contribute to the traits measured here, the hybrid *C. briggsae* phenotypes were expected to closely match those of the maternal progenitor natural isolate.

Surprisingly, inheritance of mitochondria through the paternal lineage occurred in half of *C. briggsae* hybrid introgressions. However, paternal transmission of mitochondria is not unheard of; in fact, mitochondria from sperm enter the ovum upon fertilization in most species (Cummins, 2000). Prior to departure from the male gonad, mitochondria in sperm are ubiquitinated and then destroyed post-fertilization (Sutovsky, 2003). Although there are a few natural instances of paternally-inherited mitochondria, the persistence of paternal mitochondria occurs more often in interspecific hybrids (Cummins, 2000). In such a case, whether paternal leakage of mitochondria is due to recombination between haplotypes or to evasion of the selective forces that usually eliminate paternal mitochondria remains to be determined (Cummins, 2000).

Comparison between *C. briggsae* hybrids in which mitochondria were inherited maternally (mPnH, all replicates) and the maternal progenitor (natural isolate PB800) reveals that intergenomic epistasis contributes to the phenotypic expression of most traits measured here, *i.e.* reproduction, longevity, and aging. Typically, F1 hybrids experience ‘hybrid vigor’ denoted by increased fitness relative to parental means. Also known as heterosis, this phenomenon is likely due to overdominance (intralocus interactions that increase fitness), positive epistasis (between parental alleles), or the recovery from inbreeding depression (Lynch, 1991). Most examinations of interlocus interactions provide evidence for the latter mechanism to explain hybrid vigor (Schierup and Christiansen, 1996) which was first proposed by Crow (1948) who suggested that such an increase in hybrid fitness is due to the masking of recessive deleterious alleles that may accumulate in isolated populations due to drift. In F2 hybrids and later generations, outbreeding depression can occur upon recombining recessive deleterious alleles of the same parental type and the further break up of positively epistatic alleles which may have coevolved in one or both parental types thus resulting in decreased hybrid fitness (Edmands, 1999).

Reproductive isolation can occur between allopatric populations due to differences in mutation, adaptation to the local environment, and random changes in allele frequencies. Across taxa, most work aimed at elucidating the genetic underpinnings of intrinsic postreproductive isolates has yielded evidence supporting a primary role of epistasis (Coyne and Orr, 2004). While the reduced fitness of hybrid individuals may be

attributed to epistasis between parental alleles at nuclear loci, the same phenomenon can occur between genomes (cytonuclear interactions) and, lately, much interest has been generated around this concept due to the important role of organellar genes in fitness of both plants and animals (Wolf, 2009). Since gene interactions that increase fitness can generate linkage disequilibrium resulting in the biased inheritance of those genes, the fixation of alleles that maximize fitness at such loci may result in the evolution of coadapted gene complexes (Rand *et al.*, 2004).

To date, much evidence has been accumulated to support the notion that intergenomic coadapted gene complexes play a role in the breakdown of fitness in hybrids between allopatric populations of *Tigriopus californicus*, an intertidal marine copepod. In fact, second generation hybrids vary from parents in a number of fitness related traits (Burton, 1990; Edmands, 1999), and particular introgressions result in reduced mitochondrial function (Edmands and Burton, 1999; Harrison and Burton, 2005) that correlates to fitness (Willet and Burton, 2001) and is recovered upon restoration of parental genome pairs (Ellison and Burton, 2008). However, the extent to which such cytonuclear epistasis promotes intrinsic postzygotic isolation across taxa has yet to be determined. Perhaps divergence may result from the evolution of cytonuclear coadapted gene complexes more often for organisms with more disparate rates of mutation between genomes, as in *T. californicus* (Willet and Burton, 2004; Burton *et al.*, 2006; Burton *et al.*, 2007) and *C. briggsae* (Baer *et al.*, 2007; Phillips *et al.*, 2009; Howe *et al.*, 2010).

One caveat to the statements made here regarding intergenomic epistasis in *C. briggsae* is the accumulation of mtDNA deletions of novel size in those hybrid lines which inherited mtDNA as expected (Table 8). Since the extent of newly arisen deletions in mPnH hybrids has not been defined, no speculation can be made as to whether these novel mutations may influence the variation seen among hybrid replicates, and between these hybrids and their maternal progenitor. Interestingly, these novel mutations arose in the mtDNA of a natural isolate identified as producing low levels of *in vivo* superoxide (Estes *et al.*, in review). However, the mtDNA of this same isolate (PB800) was found to accumulate numerous large heteroplasmic deletions in a mutation-accumulation experiment (Howe *et al.*, 2010) compared to one such event in *C. elegans* mutation-accumulation experiments (Denver *et al.*, 2000).

CONCLUSION

Given the importance of the mitochondrial electron transport chain (ETC) to energy production in most eukaryotes, it is no surprise that mutations affecting electron transport function result in various human disorders (Wallace, 2005) and affect aging in other organisms (Balaben *et al.*, 2005). As for other biological processes, the nematode *Caenorhabditis elegans* has proved a useful model for understanding the genetic underpinnings of lifespan determination (Hertweck, 2005). Mutations of nuclear or mitochondrial genes that contribute to mitochondrial biogenesis and function can impact longevity, fecundity, and aging in *C. elegans* (Lee *et al.*, 2003; Rea and Johnson, 2003; Balaben *et al.*, 2005; Rea, 2005; Liao *et al.*, 2007; Rea *et al.*, 2007). However, very little is understood about the direct mechanism(s) through which genetic variation that affects mitochondria controls these traits, and the evolutionary forces that regulate those relationships (Ballard and Whitlock, 2004).

To establish a model system for studying aging and longevity in an evolutionary or population genetics framework, several physiological characteristics have been examined in recently isolated *Caenorhabditis briggsae* nematodes with naturally occurring mitochondrial genetic variation relevant to the aging process. Previously, superoxide production, reproduction and population growth rates were found to be negatively correlated to heteroplasmy levels of a ~700bp deletion present in the mitochondrial genome of most *C. briggsae* isolates (Estes *et al.*, in review). This deletion

removes around half of an ETC complex I subunit (Howe and Denver, 2008), which is thought to be essential for aerobic ATP production (Janssen *et al.*, 2006). Here, aging and time to reproductive maturity were shown to correlate positively with heteroplasmy among *C. briggsae* isolates. Future work will aim at understanding exactly how this mitochondrial mutation in *C. briggsae* impacts mitochondrial function.

Experimental hybrid lines of *C. briggsae* were generated from pairs of natural isolates with high and low heteroplasmy levels in an attempt to evaluate the phenotypic effects of a mitochondrial genome from one isolate on the nuclear genetic background of another – which is necessary to show that phenotypic variation among natural isolates is due to mitochondrial genetic variance. Contrary to expectation under the assumption that mitochondria are always inherited maternally, some hybrid F10 carried only paternally-derived mitochondrial genetic information. From *C. briggsae* hybrids that inherited maternal mitochondria, intergenomic epistasis appears to impact lifespan, fitness, and aging in this species. This latter implication is unsurprising given the close relationship between many gene products from both genomes in mitochondrial function and ATP production. In the future, hybrid *C. briggsae* can be used to examine transmission and maintenance of heteroplasmy levels as well as how drift and natural selection may contribute to observed deletion heteroplasmy levels.

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