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Phenotypic consequences of sexual conflict in the context of reproductive mode polymorphism

Emma Greimann

PHENOTYPIC CONSEQUENCES OF SEXUAL CONFLICT IN THE CONTEXT OF
REPRODUCTIVE MODE POLYMORPHISM

by

Emma Greimann

A thesis submitted in partial fulfillment of the requirements
for graduation with Honors in the Biology

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Maurine Neiman
Thesis Mentor

Fall 2017

All requirements for graduation with Honors in the
Biology have been completed.

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Lori Adams
Biology Honors Advisor

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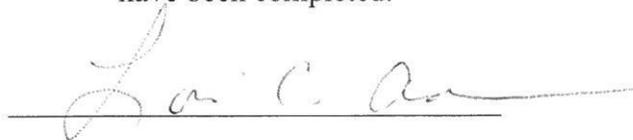
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Lori Adams, PhD.
Biology Honors Advisor

Abstract

For my Honors Research in the Neiman lab at the University of Iowa, I evaluated phenotypic variation in nuclear and mitochondrially (mt) encoded fitness-relevant traits in a New Zealand freshwater snail, *Potamopyrgus antipodarum,* within the context of sexual conflict and reproductive mode polymorphism. Sexual conflict occurs when the genetic interest of males and females diverge. That is, traits that are advantageous to one sex may be harmful to the other. My research focused on intralocus sexual conflict, involving different optima for a trait expressed in both sexes. My methodology included three assays, chosen to examine traits that are influenced by both the nuclear and mt genome. First, I used the righting assay, defined as the time that it takes for snails to return to an upright position after being flipped to the ventral side. Righting time increases with temperature in *P. antipodarum*, indicating that elevated temperatures induce stress. Second, the boldness assay quantified important elements of snail temperament and behavior, traits with important fitness and ecological consequences. Third, I used the JC-1 assay, which measures ATP synthase activity in extracted live mitochondria, to compare mitochondrial function across sexes and reproductive modes. I found that mean righting and emergence time were affected both by temperature and the interaction between lake and temperature. These results suggest that variation in nuclear and mt-encoded fitness-relevant traits are more heavily influenced by population of origin and heat stress than sex or reproductive mode. I did detect a significant interaction between both sex and reproductive mode and lake and reproductive mode on JC-1 fluorescence, suggesting that maternal inheritance and mode of inheritance affect mitochondrial function. This latter result hints that mutation accumulation might influence mitochondrial performance and that organismal function may be dependent on the interaction of nuclear and mitochondrial genomes. Because reproductive mode is in turn expected to have a

major influence on the evolution of mitonuclear interactions, my results set the stage for more

direct study of the evolutionary dynamics of these interactions in a sexual vs. asexual context.

Acknowledgements

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Introduction

Sexual Conflict

Traditional Western views of reproduction painted a harmonious view of monogamy and cooperation between sexes (Parker & Partridge, 1998). This perspective began to change in the 1900s when Charles Darwin proposed that the reality was an uneasy alliance, with both sexes indulging their own self-interests. Darwin hypothesized that some sexual dimorphisms, specifically those resulting from antagonistic interactions between males and females, evolve when optimal phenotypes differ between sexes (Rhen, 2000). This phenomenon, known as sexual conflict, occurs when the genetic interest of males and females diverge (Chapman *et al.,* 2003). That is, traits that are advantageous to one sex may be harmful to the other. Sexual conflict may drive the evolution of traits that maximize the fitness of one sex at the cost of the other because traits shared by both sexes (e.g., mating rate) may have sex-specific fitness that cannot be obtained simultaneously while encoded by a shared nuclear genome (Burke & Bonduriansky, 2017). Sexual conflict can occur within the same gene (intralocus conflict) and/or across different genes (interlocus conflict). Intralocus sexual conflict involves different optima for a trait expressed in both sexes, while interlocus sexual conflict can result in degradation of the male-female interaction (Parker & Partridge, 1998). There is evidence that sexual conflict influences differences in genome architecture between males and females, with specific alleles affecting sex-specific viability (Wright *et al.,* 2017). As such, both forms of sexual conflict have major implications for genome diversity, selection, coevolution, and speciation.

Modes and Effects of Inheritance

Traits that are inherited in the same *vs*. different manner in males and females provide a powerful context in which to examine the evolution and manifestation of sexual conflict. This expectation

is in contrast to traits that are expressed by both sexes and that share the same mode of inheritance (*i.e.,* those traits encoded by autosomal chromosomes in the nuclear genome), which should generally be subject to selection in both sexes. The implications are that traits that persist in the population are either advantageous to both sexes or are beneficial to one sex but only mildly deleterious, or neutral, to the other. Therefore, if evolution by natural selection is a dominant evolutionary force, it would be expected that autosomally determined traits are advantageous to both sexes or that the disadvantage to one sex is outweighed by the advantage to the other. One important point to keep in mind when considering the evolutionary influence of sexual conflict is that male and female fitness are not independent: negative effects of one sex on the fitness of the other sex can depress population fitness because each sex requires the other sex to reproduce.

In contrast to autosomal genes, traits controlled by sex-linked genes can persist even when the cost of the trait to one sex outweighs the benefit of the trait to the other sex (Chapman *et al*., 2003). Sex-linked genes are located on the sex chromosomes (e.g., X and Y in mammals, Z and W in birds), such that, for example, male mammals (XY) receive an X from their mother and their Y from their father, while females (XX) receive an X from both parents. Female offspring will have two copies of genes on X chromosomes, allowing compensation for harmful recessive mutations on only one of the X chromosomes and increasing the likelihood that the harmful (but silent, when heterozygous) mutation is passed on to offspring. Because males only have one copy of each sex chromosome type, a harmful mutation present on a sex chromosome is likely to affect the fitness of the individual. If this mutation is not so harmful that it prevents reproduction, it can also be passed on to offspring. The implications are that sex-linked traits can persist in populations regardless of potential costs and benefits to the respective sexes.

Maternal Inheritance and Mother's Curse

Traits that are inherited from only one parent can result in the buildup of mutations that are only harmful to the non-transmitting sex. An excellent example of this type of inheritance is provided by the mitochondrial genome (mtDNA), which is usually (but not always) inherited maternally (Barr *et al*., 2005).

The mitochondrial genome is much smaller than the nuclear genome, typically containing only 40-50 genes. 13 out of 85 components of the oxidative phosphorylation (OXPHOS) pathway in aerobic respiration are encoded by the mitochondrial genome, with the remaining 72 components encoded by the nuclear genome (Barr *et al*., 2005). Maintenance of a healthy organism requires a functioning OXPHOS pathway, meaning that the nuclear and mitochondrial genomes must work together. This kind of scenario can lead to evolutionary conflict between nuclear and mitochondrial genomes: although these genomes experience different histories of selection in males and females, the nuclear and mitochondrial encoded OXPHOS components must cooperate and physically interact to maintain organismal function.

Mitochondrial genomes are expected to have a higher load of deleterious mutations than the nuclear genome because the former accumulate deleterious mutations at a rate that is ~10-100 times higher than nuclear DNA (Moore & Reijo-Pera, 2000). This relatively high rate of mutation is especially problematic for mitochondrial genomes because mitochondria are generally inherited maternally and without recombination, with the implications that natural selection cannot effectively clear harmful mutations from mtDNA (Neiman & Taylor, 2009). These peculiarities of mitochondrial genomes are expected to lead to the accumulation of mildly deleterious mutations in mtDNA. Because mitochondria are involved in cell growth, apoptosis,

and signaling pathways, harmful mtDNA mutations can have drastic effects, ranging from infertility and cancer to degenerative neurological diseases and shortened life spans.

One expected evolutionary consequence of this type of conflict is a phenomenon known as Mother's Curse, a genetic asymmetry in which natural selection in the mitochondrial genome can only act upon females. Mother's Curse specifically predicts that while selection in females will rid the mitochondrial genome of many deleterious mutations, mutations with male-specific harmful effects can become incorporated into mitochondrial genomes (Frank & Hurst, 1996). The result is a sex-specific selective sieve where selection can only act on mutations in females (Camus *et al*., 2012). Male-specific mutations cannot be removed from mitochondrial genomes by natural selection and will tend to accumulate, so the consequences of mtDNA mutation accumulation can be especially pronounced in males. Ultimately, understanding the interaction of the nuclear and mitochondrial genome in the context of Mother's Curse may provide a partial explanation for maladaptive traits in males (Zeh, 2005).

A good example of Mother's Curse is provided by a mtDNA hypomorph of cytochrome oxidase II ("COII") that was recently identified as a "male-harming" mtDNA mutation in *Drosophila melanogaster* and other animals (Patel *et al,*. 2016). COII affects male fertility by causing defects during sperm development and does not impair other male or female functions (Patel *et al.,* 2016). As such, Mother's Curse directly affects male fitness by way of mutation accumulation in mtDNA. However, many traits require cooperation between the mitochondrial and nuclear genome. Consequently, it is important to note that mitochondrial polymorphisms can have major consequences in the male nuclear genome. Innocenti *et al*. (2011) tested whether deleterious mutations in the mitochondrial genomes could affect nuclear transcription by using experimental crosses to express five different mitochondrial DNA variants against the same

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nuclear background in *D. melanogaster*. This experiment revealed a major effect of nuclear-mitochondrial combination on nuclear gene expression, with 10% of nuclear transcripts modified relative to the wildtype transcripts when co-expressed with the mitochondrial variants in male *D. melanogaster*. This effect was largely confined to males: the same mtDNA variants had little effect on nuclear gene expression in females (Innocenti *et al.,* 2011). The genes affected in the male genome were almost exclusively male-limited transcripts associated with organs like the testes, accessory glands, and the ejaculatory duct. These expression changes also were associated with decreased male fertility and hence, reduced male relative fitness (Innocenti *et al.,* 2011). Another good example of Mother's Curse comes from Camus *et al.* (2012)*,* who found that mutation loads in the mitochondria can affect male, but not female, aging in *Drosophila melanogaster.* These studies provide a strong line of evidence that the mitochondrial genome can indeed accumulate mutations specifically and detrimentally harmful to male fitness that may also influence nuclear gene transcription.

Inheritance in asexual organisms

All of these expectations regarding the consequences of mode of inheritance assume sexual reproduction, with both males and females inheriting nuclear genome components from both parents, while mtDNA is inherited only from mothers. The situation might be quite different in asexual lineages, which are typically all female and produce offspring that inherit both their entire nuclear genome as well as the mitochondrial genome. The absence of sex may allow for the accumulation of deleterious alleles while the absence of recombination may reduce adaptability (Burke & Bonduriansky, 2017). The general absence of males, and thus, selection on genes that are important to male function, means that asexuality should also result in decreased sexual conflict because nuclear mutations that increase female fitness but decrease male fitness

will not be subject to selection. These expectations are complicated by the fact that many all-female asexual lineages with recent dioecious (separate sexes) sexual ancestors occasionally produce male offspring. Sexual conflict theory would suggest that these males should perform especially poorly because there would be little to no countervailing selection pressure to balance the selection that favors the optimization of traits important only in females. My Honors research addressed sexual conflict in sexual lineages as well as began to investigate the implications of sexual conflict in asexual lineages.

Justification for Methods

Model System

Potamopyrgus antipodarum, a freshwater snail native to New Zealand, is an ideal organism for studying questions regarding the evolution of sexual reproduction and sexual conflict. Natural *P. antipodarum* populations are characterized by coexisting sexual and asexual lineages that are otherwise phenotypically similar. Asexual *P. antipodarum* have been separately and repeatedly derived from sexual *P. antipodarum* over the last 100,000 years (Neiman *et al,.* 2012). These features of *P. antipodarum* allow for the direct comparisons among sexual and asexual individuals necessary for a direct and powerful evaluation of how sexual conflict influences male *vs.* female members of sexual and asexual lineages (Neiman *et al.,* 2012). For my Honors research, I quantified and compared the values of traits potentially affected by mitochondrial and nuclear genome composition in asexual male and female and sexual male and female *P. antipodarum*.

Temperature

It is known that righting time increases with temperature in *P. antipodarum* (Sharbrough *et al.,* 2017). Similarly, Urgiles et al. (unpublished) demonstrated that the time it takes *P. antipodarum* to emerge from their shell after the startle response also increases with temperature. These findings indicate that both the righting assay and boldness test can also be used to assess heat stress. Therefore, I applied the righting assay and the boldness test at three temperatures: 16°C (unstressful), 22°C (unstressful to stressful), and 30°C (stressful) (Sharbrough *et al*., 2017). In the context of my thesis, heat stress is both relevant and interesting because mitochondria play an important role in the stress response (Manoli *et al.,* 2007), allowing for an indirect measure of

mitochondrial function and how the mitochondrial genome may interact with the nuclear genome.

Righting Assay

Given the likelihood that nuclear-encoded genes are primarily involved in righting ability and that mitochondria play an important role in stress responses (Manoli *et al.,* 2007), this assay is meant to provide estimations of meaningful differences in phenotype for fitness-relevant traits across sexual and asexual and male and female *P. antipodarum*. For example, righting time is likely an important trait with respect to predator evasion, resilience to environmental disruptions, and maintenance of respiration and thermoregulation (Stancher *et al.,* 2006). Righting ability is also crucial with regard to mating in gastropods because effective copulation requires the ability to orient the body (Penn & Brockmann, 1995). The application of the righting assay in the context of my thesis allowed me to assay a fitness-relevant organism-level trait that is likely to reflect nuclear and mitochondrial variation.

Boldness Test

Temperament and behavior have important fitness and ecological consequences for individuals and their respective populations (Reale & Festa-Bianchet, 2002), which is why I employed an assay designed to evaluate variation in temperament and boldness in *P. antipodarum* (Fig. 1). Boldness is likely an important trait in situations where risk *vs.* reward must be considered, such as mating, feeding, and perception of predation risk, leading to differential behavior with the potential for major ecological consequences. In three-spined sticklebacks, bolder fish consume more prey than shy fish. Larger sticklebacks are also bolder and at less at risk from predation (Ioannou *et al*., 2007). In another aquatic snail, *Radix balthica*, this bold-shy continuum has been thoroughly studied (Ahlgren *et al.,* 2015), identifying individuals' consistent willingness to take

risks. Ahlgren *et al*. (2015)'s study also revealed that shell shape plays a key role in boldness; "phenotypic compensation" accounts for the fact that snails with inadequate defensive shells may be shyer, while snails with relatively protective shells can afford to take more risks (Ahlgren *et al*., 2015). In addition, boldness can be used to predict mating success in *Uca mjoebergi*, male fiddler crabs (Reaney & Backwell, 2007). In another snail, *Radix balthica*, bold individuals have higher survival (Ahlgren *et al*., 2015). Similarly boldness is heritable in bighorn sheep ewes, *Ovis canadensis,* demonstrating that boldness is a trait that can evolve under natural selection (Reale & Festa-Bianchet, 2002). For my purposes, I defined "boldness" as the amount of time elapsed before a snail emerged from its shell after being placed ventral side-up in a petri dish; after snails were treated as such, they retracted into their shell as part of a startle response (Briffa and Twyman, 2010). The less time a snail took to recover from the startle response, the bolder the snail; a relatively short emergence time indicates that the snail is ready to expose itself while re-evaluating its surroundings. Snails that take a relatively long time to recover and re-emerge after the startle response are considered to be less bold. As with the righting assay, using the boldness test in my thesis allowed me to investigate a fitness-relevant organism-level trait that is likely to reflect both nuclear and mitochondrial variation.

JC-1 Assay

I used the JC-1 assay, which measures ATP synthase activity in extracted live mitochondria (Sharbrough et al., 2017), to compare mitochondrial function across sexes and reproductive modes in *P. antipodarum*. Mitochondria are responsible for respiration and energy production within the cell. Accordingly, the JC-1 assay provides a relatively direct measure of mitochondrial function. JC-1 measures mitochondrial membrane potential and determines the strength of the electrochemical gradient mitochondria use to phosphorylate ADP to ATP. Hence, the amplitude

of membrane potential is positively correlated with the ratio of ATP to ADP in the cell, making

mitochondrial membrane potential a useful phenotype to gauge mitochondrial performance

(Sharbrough, 2016). JC-1 is used to measure membrane potential; with UV illumination, JC-1

fluoresces green when dispersed and red when aggregated. If no electrochemical gradient is

present, the JC-1 diffuses at random, such that the ratio of red:green is equal (~1). If the JC-1 is

isolated in maintained mitochondria with an electrochemical gradient, JC-1 accumulates and

fluoresces red, with an elevated red:green ratio (>1). Because higher function in this

electrochemical gradient is associated with higher mitochondrial function, the red: green ratio

represents a useful estimate of mitochondrial function, with higher ratios representing higher

function (Garner & Thomas, 1999). The development of this assay for this purpose was used by

Garner and Thomas (1999) and was then adapted to *Potamopyrgus antipodarum* (Sharbrough

2016)*. The implementation of the JC-1 assay allowed me to examine mitochondrial phenotype

through mitochondrial function, which in turn is likely affected by mutation accumulation and

mode of inheritance.

Materials & Methods

Individuals were primarily field collected (66%) from nine New Zealand lakes, though some

(34%) were reared in common garden conditions in the laboratory (Table 1). Because sexual *P.*

antipodarum are diploid while asexual individuals are either triploid or tetraploid, the potential

effect of ploidy was accounted for via flow cytometry after snail sacrifice (*i.e.,* flow cytometric

determination of nuclear genome content, e.g., Neiman *et al.,* 2011). Because definitive

determination of reproductive mode and ploidy required snail sacrifice, the field-collected snails

are from lakes known to harbor both sexual and asexual individuals, with populations at least

~10% male. I included the lab-reared snails to account for and balance the number of asexual *vs.*

sexual snails. For field-collected snails, assays were conducted blind to snail ploidy, and, thus,

reproductive mode. All snails were housed at 17°C with a 16 h light/8 h dark cycle and fed *ad*

libitum dried *Spirulina* cyanobacteria fortified with calcium, which is the standard protocol for

rearing *P. antipodarum* in our lab (*e.g.,* Zachar & Neiman, 2013).

Table 1: The number of males and females with their respective ploidy from each of the nine
New Zealand lakes that I included in my study.

Lake	Field Collected vs. Lab reared	Male		Female		Total
		Sexual	**Asexual**	**Sexual**	**Asexual**	
Ellery	Field 2016	2	2	2	10	16
Selfe	Field 2016, 2017	11	2	6	17	36
Poerua	Lab Poerua 95 3x	0	2	0	10	12
Heron	Lab Heron 72 E 3N	0	4	0	10	14
Gunn	Lab Gunn 6 4x	0	9	0	13	22
Mapourika	Field 2016, 2017	9	1	4	6	20
Kaniere	Field 2016	10	0	4	5	19

Alexandrina	Field 2017	5	0	0	0	5
Alexandrina	Lab Alex Outbred Sex	6	0	11	0	17
Rotoroa	Field 2017	13	0	9	6	28
Total		56	20	36	77	189

Righting Assay

I quantified righting ability in sexual and asexual male and female *P. antipodarum* at both stressful and relatively benign temperatures. I measured righting ability by placing snails ventral side-up in a petri dish and then recorded the time (seconds) until the snails righted themselves, up to 180 seconds, and at three different temperatures: 16°C (unstressful), 22°C (unstressful to stressful), and 30°C (stressful) (Sharbrough *et al*., 2017). I imposed the temperature treatment by exposing the snails to water at the treatment temperature for 1 hour before measurement. For 16°C treatment conditions, snails were left in the 16°C snail room for an hour before testing in their holding tanks. For 22°C conditions, snails and their holding tanks were taken into the lab and kept at 22°C for an hour before testing in an Isotemp 220 water bath. For 30°C, snails were incubated in their holding tanks in an Isotemp 220 water bath set at 30°C. Under the presumptions that nuclear-encoded genes are primarily involved in righting ability while mitochondria play an important role in heat-induced stress responses (Manoli *et al*., 2007) and that asexuals accumulate mutations at a higher rate than sexuals, I predicted that 1) sexual snails should right themselves more rapidly than asexual snails, and 2) female snails might right themselves more rapidly than male snails.

Boldness Test

I assessed boldness in sexual and asexual male and female *P. antipodarum* during simultaneous
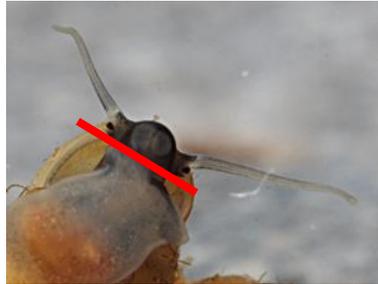
testing of the Righting Assay.

Fig. 1. The red line demonstrates what I defined as emergence: both the eyes and base of the
tentacles have emerged from the shell after a snail has been flipped to its dorsal side.
(http://www.molluscs.at/gastropoda/index.html?/ gastropoda/morphology/tentacles.html)

This boldness assay was conducted at 16°C, 22°C, and 30°C to account for and investigate the

effect of heat response on boldness in asexual and sexual males and females. Sex-based

differences in phenotype with regards to boldness can potentially be attributed to sexual conflict

as the trait is assumed to have a completely nuclear basis. By this logic, and in light of the fact

that males initiate copulation in *P. antipodarum* (Nelson & Neiman, 2011), I predicted that 1)

males should be bolder (i.e., emerge more rapidly) than females, and 2) these sex-based boldness

differences should be less pronounced, if evident at all, when asexual males are considered.

JC-1 Assay

I followed the procedure developed specifically for JC-1 in *P. antipodarum*, outlined in

Sharbrough *et al*. (2017). Because mitochondria are maternally inherited, leaving the potential

for male-harming mutations to accumulate, and given that asexual nuclear and mitochondrial

genomes are co-inherited, I predicted that 1) sexuals will have higher mitochondrial function

than asexuals, and 2), both sexual and asexual females should have higher mitochondrial

functions than their male counterparts.

Statistical Analysis

First, I used a BoxCox transformation to normalize my data. I then analyzed the data separately

for each assay with a three-way ANOVA, with the independent factors of lake, sex, and

reproductive mode and the dependent variable of righting time, head emergence time, and JC-1,

respectively. During preliminary analysis, I used Tukey post-hoc tests to determine the effect of

temperature and lake on mean emergence and mean righting time.

Flow Cytometric Determination of Ploidy/Reproductive Mode

To account for a potential effect of ploidy on fitness and to assign sexual (diploid) vs. asexual

(triploid or tetraploid) status, I followed the flow cytometry protocols outlined in, e.g., Krist *et*

al. (2014) to determine the ploidy level of each snail. Briefly, I ground snap-frozen tissue from

P. antipodarum head-halves that I dissected away and saved during preparation for the JC-1

assay in a 600 ul detergent-DAPI solution (1.4x 10-5 M). I then filtered the samples through a

30-micron mesh and ran the samples on a Beckton-Colter flow cytometer available in the

University of Iowa Flow Cytometry Facility, collecting 10,000 events (measuring fluorescence

from 10,000 cells). I used FlowJo software to process and analyze the data and assign ploidy

level.

Results

Righting Assay

To investigate a fitness-relevant organism-level trait that is likely to reflect nuclear and
mitochondrial variation, I analyzed righting time across sex, reproductive mode, and lake of
origin (Figure 2 and 3). There was a significant effect of temperature ($p < 0.001$) but not sex ($p =$
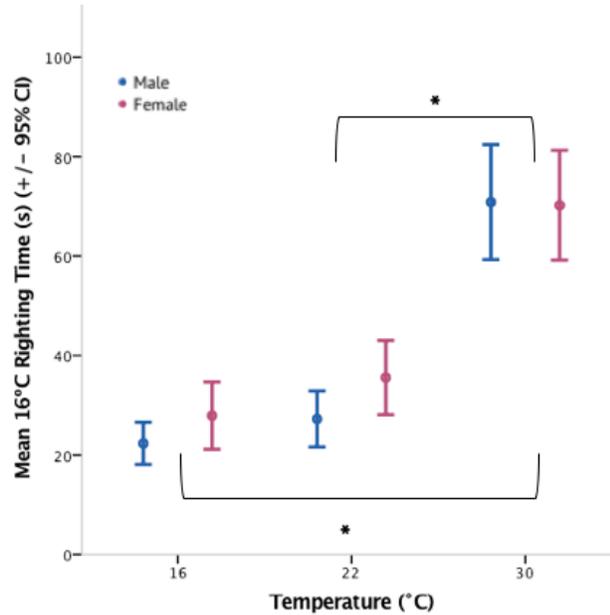0.112) on mean righting time.

Fig. 2. There was a significant effect of temperature ($p < 0.001$) on mean righting time. At
higher temperatures, snails took longer to right themselves.

There was also a significant interaction between temperature and lake with respect to mean
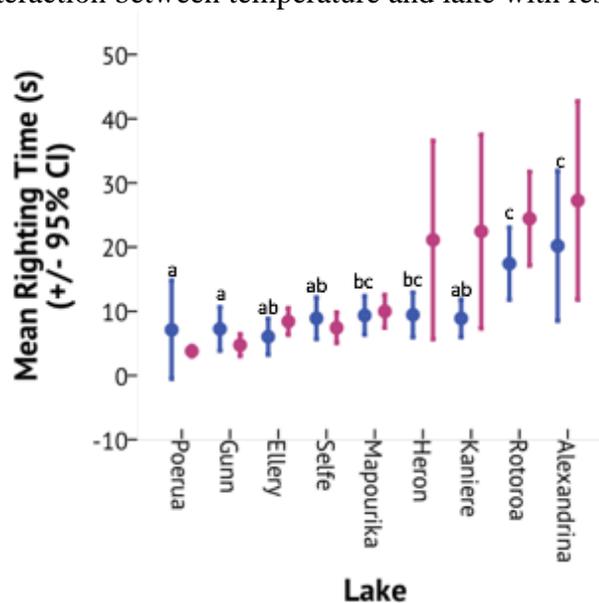righting time ($p </= 0.034$).

Fig. 3. Shared letters denote means that are not significantly different (Tukey's honestly significant
difference, $p < 0.05$ for pairwise comparisons. p (a) ≥ 0.069, p (ab) ≥ 0.064, p (bc), (c) ≥ 0.098.

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There was not a significant main effect of lake on righting time ($p = 0.089$) despite moderately

high power (0.576). There was no evidence for a significant effect of reproductive mode ($p =$

0.622) on mean righting time. Finally, there was also no evidence for a significant effect of sex

on mean righting time ($p = 0.142$).

Boldness Test

Boldness, another fitness-relevant organism-level trait that is likely to reflect both nuclear and

mitochondrial variation, was analyzed across sex, reproductive mode, and lake of origin (Figure

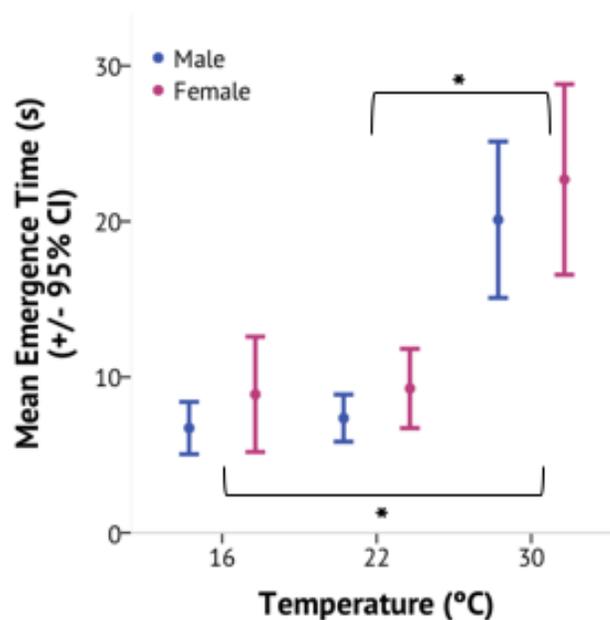4 and 5). There was a significant effect ($p < 0.001$) of temperature on mean emergence time.

Fig. 4. There is a significant effect ($p < 0.001$) of temperature on mean emergence time. Snails were less bold at higher temperatures.

There was also a significant interaction between temperature and lake ($p </= 0.021$) for mean
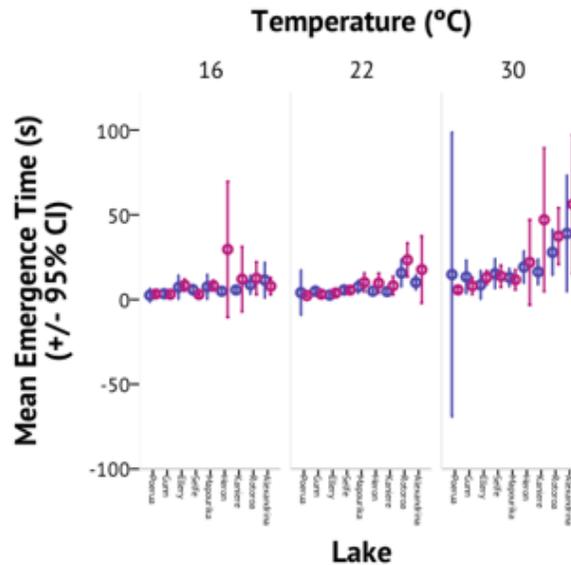
emergence time.

Fig. 5. Temperature and lake had a significant effect ($p </= 0.021$) on mean emergence time.

There was no effect of lake ($p = 0.230$), reproductive mode ($p = 0.339$), or sex ($p = 0.619$) on emergence time, though these analyses had low power (lake = 0.214, reproductive mode = 0.138, sex = 0.074).

JC-1 Assay

To compare mitochondrial function across sexes, reproductive mode, and lake of origin, I analyzed ATP synthase activity (Figure 6). There was a significant interaction between sex and reproductive mode ($p = 0.008$) and lake and reproductive mode ($p < 0.001$) with respect to JC-1 fluorescence.
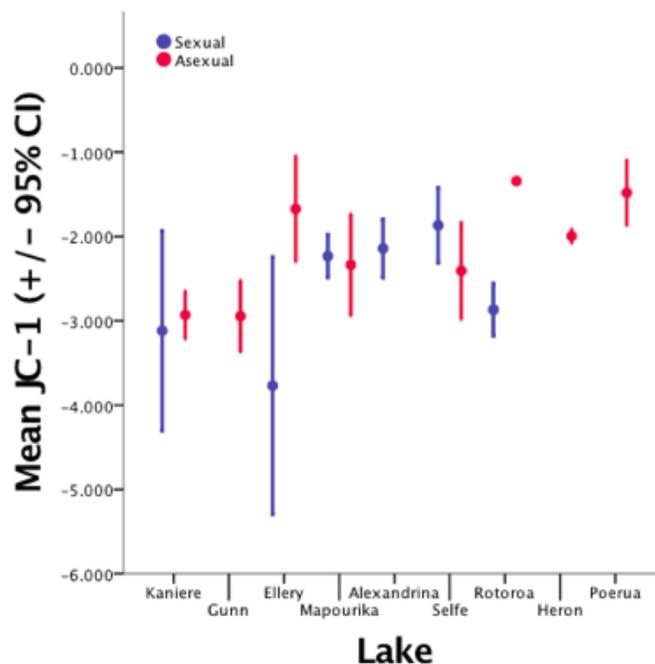

Fig. 6. There was a significant interaction between reproductive mode and lake with respect to JC-1 fluorescence ($p < 0.001$).

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There was no significant main effect of reproductive mode ($p = 0.141$, power $= 0.292$), sex alone ($p=0.334$, power $= 0.142$) or lake ($p =0.936$, power $=0.097$ power) on JC-1 fluorescence.

Discussion & Implications

Righting Assay

Mean righting time was affected both by temperature and the interaction between lake and temperature but not by lake, reproductive mode, or sex alone. These results suggest that across-lake environmental, thermal, and genetic variation are the major contributors to variation in this trait relative to reproductive mode or sex. These findings also indicate that sexual conflict, maternal inheritance, and mode of inheritance have little, if any, effect on righting time.

My temperature results are consistent with Sharbrough *et al*., (2017), who demonstrated that righting time increases with temperature in *P. antipodarum*. This connection between righting time and temperature emphasizes that this trait is largely influenced by the response of *P. antipodarum* to stressful heat conditions, though the interaction between temperature and lake that I observed suggests that heat stress may be influenced by lake of origin. More broadly, these results suggest that phenotypic variation in nuclear and mt-encoded fitness-relevant traits are more heavily influenced by population of origin and heat stress than sex or reproductive mode, with the caveat that the low power of the analyses of main effects (power $</= 0.214$) prevents strong conclusions with respect to these negative results.

Boldness Test

The boldness test results were broadly similar to the righting assay results, revealing a significant effect of temperature on mean emergence time (also see Urgiles *et al*.) and a significant interaction between temperature and lake. Again, these findings point to population-of-origin effects and suggest a weak to nonexistent role for sex or reproductive mode.

JC-1 Assay

  The JC-1 assay allowed for insight into mitochondrial phenotypic variation, which is likely related to mutation accumulation and maternal inheritance. While there were no significant main effects of reproductive mode, sex, or lake on JC-1 fluorescence, the low power of these analyses ($</= 0.29$) means that I cannot rule out a minor role for these factors. I did detect significant interactions between sex and reproductive mode and lake and reproductive mode on JC-1 fluorescence, suggesting that mitochondrial variation and mutation accumulation are affected by the combination of sex, reproductive mode, and lake of origin. Together, these findings hint that mode of inheritance might influence mitochondrial function in *P. antipodarum*.

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