TITLE:

Variation in physiological function across source populations of a New Zealand freshwater snail

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ABSTRACT

The mitochondrial and nuclear-encoded genes responsible for cellular respiration are expected to experience relatively intense purifying selection, meaning that variation in these genes will often decrease fitness. Still, extensive variation for mitochondrial haplotype and function persists in natural populations. We integrated physiological, cellular, and behavioral approaches to quantify phenotypes relevant to mitochondrial function across a diverse sample of *Potamopyrgus antipodarum*, a New Zealand snail characterized by frequent coexistence between otherwise similar sexual and asexual individuals. We found extensive across-lake variation in organismal oxygen consumption and behavioral response to heat stress coupled with elevated mitochondrial membrane potential in males vs. females. These data set the stage for applying this important model system for sex, host-parasite interactions, invasion biology, and ecotoxicology to novel tests of the relationships between mitochondrial variation and performance in natural populations.

INTRODUCTION

Mitochondrial function is a critical component of eukaryotic function and fitness [1]. Despite its importance, genes underlying oxidative phosphorylation are often polymorphic within species [1], which has important consequences for phenomena from mitonuclear incompatibilities [2] to DNA barcoding [3]. Indeed, extensive variation for mitochondrial function has been observed in a diverse array of species [4]. Although some variation in metabolic and mitochondrial traits has been linked to specific environmental correlates (e.g., altitude – [5], temperature – [6], energy source – [7]), we lack a systematic understanding of the distribution of this variation across biogeographic space.

Maternal transmission of mitochondrial genomes is expected to influence the distribution of phenotypic variation because only female-derived mutations are transmitted. This phenomenon has two primary consequences: 1) ~50% reduction in N_e relative to biparentally inherited genomes, and 2) sexually antagonistic mutations only experience effective natural selection in females [8]. This latter phenomenon, the so-called “Mother’s Curse”, is predicted to result in the accumulation of mutations that are neutral or beneficial in females, but deleterious in males [9]. The lack of widespread evidence for Mother’s Curse (but see [10]) may point to mechanisms that prevent the spread of male-specific deleterious mutations in mitochondrial genomes [11, 12]. The extent to which Mother’s Curse shapes patterns of variation in mitochondrial function therefore represents yet another important unanswered question in evolutionary biology.

Because mitochondrial function requires compatibility between nuclear and mitochondrial gene products [13], reproductive mode can also dramatically impact the

evolution of variation in mitochondrial function because only sexual reproduction allows for the regular movement of mitochondrial genomes across diverse nuclear genomic backgrounds. Sexual reproduction between distantly related parents can give rise to hybrid offspring harboring mitonuclear incompatibilities [14]. On the other hand, inbreeding and asexual reproduction can reduce the efficacy of selection on mitochondrial genomes [15, 16]. While reduced mitochondrial function and organismal fitness in hybrid lineages is well documented [2], there are few empirical tests of whether and how inbreeding or asexuality affects mitochondrial function, leaving an important gap in our understanding of the evolutionary consequences of changes in reproductive mode. Surveys of mitochondrial genomes of asexual lineages [17, 18] have revealed elevated rates of accumulation of nonsynonymous mutations in mitochondrial genomes compared to sexual lineages. Determining whether these mutations actually result in reduced function will have profound implications for our understanding of the maintenance of sex.

Potamopyrgus antipodarum, a New Zealand freshwater snail [19], is ideally suited to answer these outstanding questions regarding mitochondrial function. There is both extensive mtDNA population structure in their native range [20] and evidence for local adaptation of snails to their source lakes [21, 22]. Temperature in particular appears to be a primary determinant of the geographical distribution of *P. antipodarum* within New Zealand [23]. Because asexuality has arisen multiple times within *P. antipodarum* [20, 24], and because sexual and asexual lineages frequently coexist in nature [24], asexual lineages can be treated as repeated “natural experiments” into the evolutionary consequences of asexuality.

Here, we tested whether lake of origin, reproductive mode, or sex affect mitochondrial and behavioral function under laboratory conditions in field-collected *P. antipodarum*.

MATERIALS AND METHODS

Because definitive determination of reproductive mode requires snail sacrifice, we sampled field-collected snails from lakes known to harbor sexual and asexual individuals, with populations at least ~10% male [24]. Upon arrival at the University of Iowa, snails were housed at 16ºC on a 18hr light/6 hr dark schedule, and fed *Spirulina* algae 3x per week, as described in [25]. We arbitrarily selected adult snails from each lake collection and isolated each snail in a 0.5 L glass container with 300ml carbon-filtered H_2O. Water was changed weekly. Assay sampling details (sample size, etc.) are summarized in Table 1. Reproductive mode was determined after assay completion, following the flow cytometry protocol outlined in [24].

We measured oxygen consumption as described in [25] for 57 wild-caught snails from each of six lakes at three different water temperatures: 16ºC (not stressful, and similar to New Zealand lake temperatures), 22ºC (moderately stressful), and 30ºC (stressful) [25]. At each temperature, we assayed snails in a randomly determined order, and only snails that completed all temperature trials were included in analyses. Mean wet mass for each individual was calculated from the three separate temperature trials.

Righting behavior [25] and time to emergence following a startling stimulus (M Neiman pers. obs.) increase with temperature in *P. antipodarum,* indicating that both assays are effective proxies for heat stress. We quantified righting and emergence

times under each of the same three temperature treatments as for oxygen consumption in 46 wild-caught *P. antipodarum*.

JC-1 is a small, positively charged molecule that diffuses down the electrochemical gradient of the inner mitochondrial membrane and fluoresces green when dispersed and red when aggregated inside the mitochondrial matrix [26]. Therefore, the ratio of red: green fluorescence in freshly isolated mitochondria can serve as a proxy for mitochondrial membrane potential. We measured red: green ratios in JC-1-treated mitochondrial extracts from 46 wild-caught *P. antipodarum* as described in [25] using a Becton Dickenson LSR II flow cytometer.

We used a mixed-effects model framework to quantify the relationships between oxygen consumption and behavioral metrics with categorical variables for temperature (16º C, 22º C, 30º C), lake of origin (n = 3-6 depending on the analysis), reproductive mode (asexual, sexual), sex (male, female; only fit in models pertaining to behavior assays), and a continuous variable for mass (g; only fit in model pertaining to oxygen consumption). We modeled a term for snail identity as a random intercept to account for repeated measures on individuals across temperatures. Finally, we modeled mitochondrial membrane potential, measured as the ratio of red: green fluorescence, as a function of lake, reproductive mode, and sex using analysis of variance (ANOVA).

We developed final models using backwards selection until only predictors with *p*-values less than 0.05 remained. To test assumptions of normality and heteroscedacity of errors, we graphically inspected residuals and log- or square-root-transformed response variables when necessary. We performed all statistical analyses in R [27], fitting fixed-effect models with the lm function, fitting mixed-effects models using the

lme4 package [28], and estimating degrees of freedom for mixed-effect models using Satterthwaite's approximation via the lmerTest package [29].

RESULTS

All model-fitting results are detailed in Table 2. We found that temperature ($p < 0.0001$), mass ($p = 0.00154$), and lake of origin ($p = 0.0072$), but not reproductive mode, were significantly associated with the rate of oxygen consumption (Figure 1a). Temperature was a significant predictor of both righting ability ($p < 0.0001$, Figure 1b) and emergence time ($p < 0.0001$, Figure 1c). Lake of origin was a significant predictor of righting ability ($p = 0.0155$), but not of emergence time. Neither reproductive mode nor sex were significantly associated with behavioral responses to heat stress. Sex, but not lake of origin or reproductive mode, was a significant predictor of mitochondrial membrane potential ($p = 0.0070$, Figure 2), with higher mitochondrial membrane potential in males vs. females.

DISCUSSION

Here, we report the first evidence of population-structured variation for mitochondrial and behavioral function in *P. antipodarum*. Combined with population structure in mitochondrial genes [18, 20], this result suggests that mitochondrial function could be locally tuned in *P. antipodarum*. We also find that males have higher mitochondrial membrane potential than females. This result suggests that male *P. antipodarum* do not suffer from Mother's Curse, at least with respect to their ability to generate a proton motive force.

These data set the stage for future studies addressing multiple important evolutionary questions, including invasiveness, response to parasite infection, ecotoxicology, and the evolutionary maintenance of sex. Chief among these questions is whether and how asexuality might influence mitonuclear coevolution. While asexuality should reduce the efficacy of natural selection in both nuclear [30] and mitochondrial genomes [16, 31], stable transmission of mitonuclear genotypes may also facilitate rapid mitonuclear coadaptation and thereby local adaptation [32]. Importantly, the strong lake effect implies that extensive intrapopulation sampling is necessary for evaluating mitochondrial function in *P. antipodarum*.

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DATA Availability

Data to be archived on Dryad.

AUTHOR CONTRIBUTIONS

ESG, JTS contributed to all aspects of study, SF to statistical analyses and manuscript drafting, JLC, MN to concept, statistical design, and manuscript drafting, and JDW, SKH, MRK, JAM, and MRP to data collection and manuscript editing.

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COMPETING INTERESTS

We have no competing interests.

ETHICAL STATEMENT

Not applicable to mollusks.

163

Table 1. Summary of source populations of *Potamopyrgus antipodarum*.

Oxygen consumption assay

Lake	Latitude, Longitude	Sexual	Asexual	Male	Female
Alexandrina	-43.900476, 170.453978	14	2	-	16
Clearwater	-43.602131, 171.043917	-	4	-	4
Kaniere	-42.832886, 171.14759	16	-	-	16
Paringa	-43.713068, 169.411348	5	-	-	5
Rotoroa	-41.855414, 172.637882	-	17	-	17
Selfe	-43.237765, 171.520449	-	3	-	3

Behavior and mitochondrial membrane potential assays[1]

Lake	Latitude, Longitude	Sexual	Asexual	Male	Female
Alexandrina	-43.900476, 170.453978	3	-	3	-
Ellery	-44.046898, 168.654261	2	3	-	5
Kaniere	-42.832886, 171.14759	5	1	4	2
Mapourika	-43.315212, 170.204061	8	2	6	4
Rotoroa	-41.855414, 172.637882	4	1	-	5
Selfe	-43.237765, 171.520449	9	8	9	8

[1] – Same individual snails were used in behavioral and mitochondrial membrane potential assays

164

Table 2. Linear and mixed-effects models of select predictors on oxygen consumption, righting time, emergence time, and mitochondrial membrane potential.

Oxygen consumption[1]

Factor	χ^2	df	*p*	Non-significant predictors*
Intercept	2.700	1	0.1004	
Temperature	39.038	2	< 0.0001	Reproductive Mode
Mass	11.061	1	0.0009	
Lake of Origin	15.280	5	0.0092	

Factor[§]	χ^2	df	*p*	Non-significant predictors*
Intercept	2.164	1	0.141	
Temperature	27.336	2	< 0.0001	Reproductive Mode
Mass	4.365	1	0.0367	

Righting time[2]

Factor	χ^2	df	*p*	Non-significant predictors*
Intercept	59.205	1	< 0.0001	
Temperature	73.661	2	< 0.0001	Reproductive Mode, Sex
Lake of Origin	14.020	5	0.0155	

Factor[§]	χ^2	df	*p*	Non-significant predictors*
Intercept	94.767	1	< 0.0001	
Temperature	69.655	2	< 0.0001	Reproductive Mode, Sex
Lake of Origin	13.429	4	0.0094	

Factor[†]	χ^2	df	*p*	Non-significant predictors*
Intercept	364.550	1	< 0.0001	Reproductive Mode, Sex,
Temperature	52.370	2	< 0.0001	Lake of Origin

Emergence Time[3]

Factor	χ^2	df	*p*	Non-significant predictors*
Intercept	448.891	1	< 0.0001	Reproductive Mode,
Temperature	46.646	2	< 0.0001	Sex, Lake of Origin

Factor[§]	χ^2	df	*p*	Non-significant predictors*
Intercept	363.860	1	< 0.0001	Sex, Reproductive Mode,
Temperature	34.608	2	< 0.0001	Lake of Origin

Factor[†]	χ^2	df	*p*	Non-significant predictors*
Intercept	59.205	1	< 0.0001	
Temperature	73.661	2	< 0.0001	Reproductive Mode, Sex
Lake of Origin	14.020	5	0.0155	

Mitochondrial membrane potential[4]

Factor	Sum of Squares	df	*F*	*p*	Non-significant predictors*
Intercept	55.876	1	305.1038	< 0.0001	Reproductive Mode,
Sex	1.466	1	8.0035	0.0070	Lake of Origin
Residuals	8.058	44			

Factor[§]	Sum of Squares	df	*F*	*p*	Non-significant predictors*
Intercept	55.876	1	329.344	< 0.0001	Reproductive Mode, Lake of Origin
Sex	1.026	1	6.046	0.0183	
Residuals	6.956	41			

Factor[†]	Sum of Squares	df	*F*	*p*	Non-significant predictors*
Intercept	96.564	1	482.37	< 0.0001	Reproductive Mode, Lake of Origin, Sex
Residuals	6.406	32			

[1] – Type III Repeated-Measures Analysis of Deviance $\chi 2$ Test of O_2 consumption per hour

[2] – Type III Repeated-Measures Analysis of Deviance $\chi 2$ Test of log-transformed righting times

[3] – Type III Repeated-Measures Analysis of Deviance $\chi 2$ Test of square-root-transformed emergence times

[4] – Type III Analysis of Variance *F* Test of log-transformed ratios of red: green in mitochondrial extracts

***** – Non-significant predictors listed in order of elimination from the model

§ – Model fit only included lakes from which both sexual and asexual snails were assayed

† – Model fit only included lakes from which both male and female snails were assayed

165

166 **FIGURE LEGENDS**

167 **Figure 1. Physiological responses to heat stress for *P. antipodarum* across**

168 **source lakes**. a) Oxygen consumption/hour/gram. b) Righting time. c) Emergence time

169

170 **Figure 2. Mitochondrial membrane potential in field-collected *P. antipodarum*.**

171 Ratios of red: green fluorescence of JC-1-treated mitochondrial extracts for a) snails

172 from all six New Zealand lakes, b) male vs. female snails from all lakes, and c) male vs.

173 female snails from three New Zealand lakes with replication for sex.

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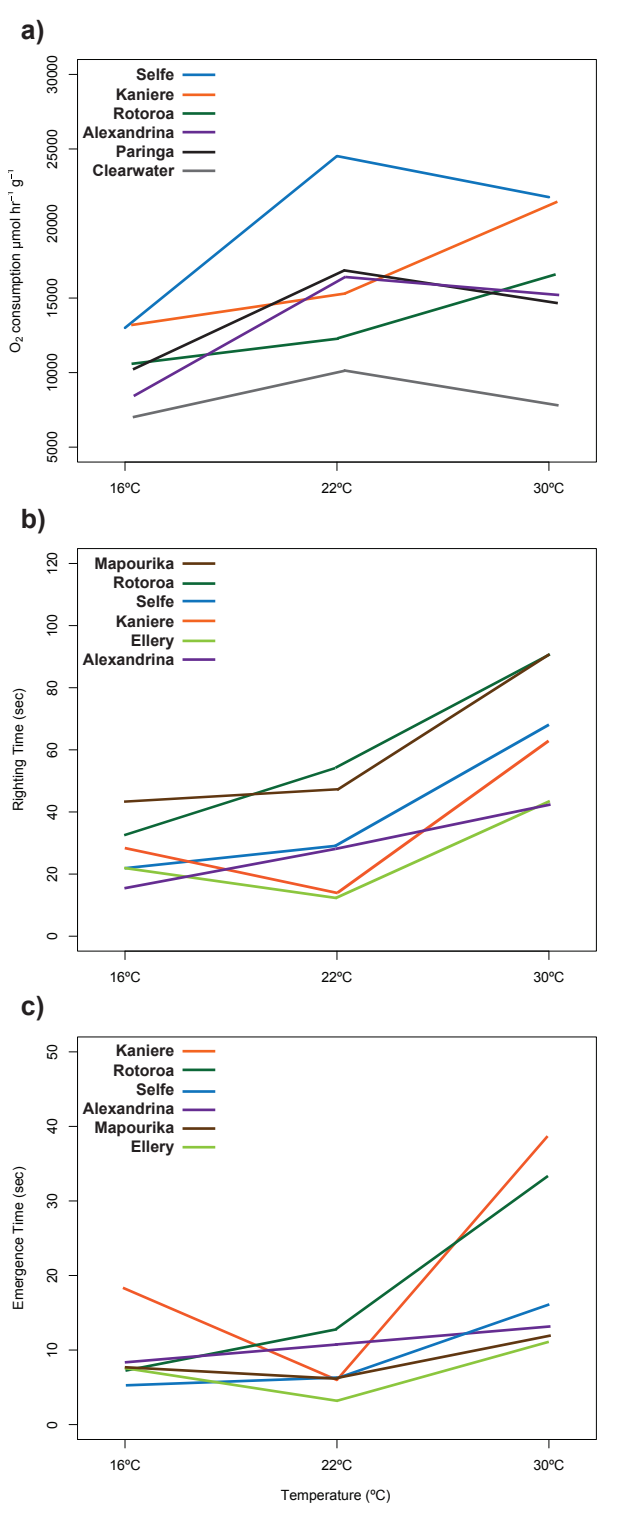
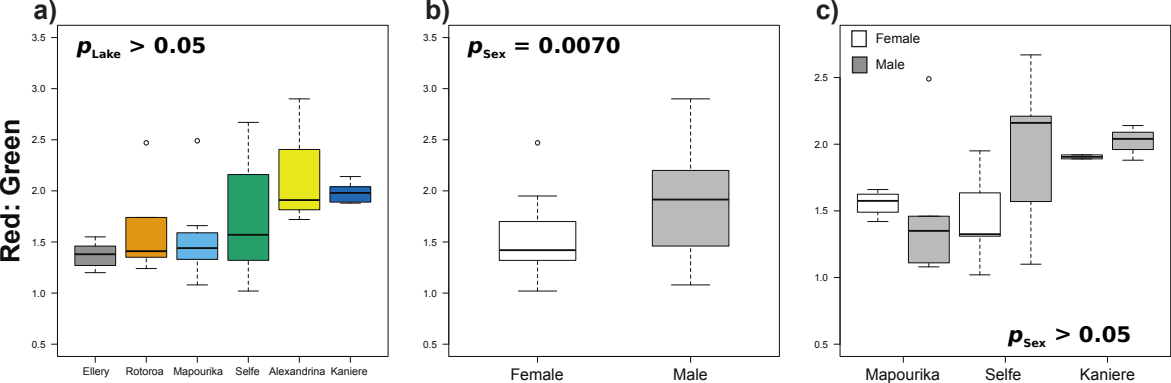

a)
Selfe
Kaniere
Rotoroa
Alexandrina
Paringa
Clearwater
O_2 consumption µmol hr^{-1} g^{-1}
5000
10000
15000
20000
25000
30000
16ºC
22ºC
30ºC
b)
Mapourika
Rotoroa
Selfe
Kaniere
Ellery
Alexandrina
Righting Time (sec)
0
20
40
60
80
100
120
16ºC
22ºC
30ºC
c)
Kaniere
Rotoroa
Selfe
Alexandrina
Mapourika
Ellery
Emergence Time (sec)
0
10
20
30
40
50
16ºC
22ºC
30ºC
Temperature (ºC)


a)
b)
c)
Red: Green
p_{Lake} > 0.05
p_{Sex} = 0.0070
p_{Sex} > 0.05
Female
Male
Ellery
Rotoroa
Mapourika
Selfe
Alexandrina
Kaniere
3.5
3.0
2.5
2.0
1.5
1.0
0.5