TITLE:

Variation in physiological function across source populations of a New Zealand freshwater snail[1]

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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 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.

KEYWORDS

asexual reproduction, JC-1, mitochondria, oxygen consumption, *Potamopyrgus antipodarum*, sexual reproduction

INTRODUCTION

The production of ATP *via* cellular respiration is a critical component of eukaryotic function and fitness (Chen *et al.*, 2007; Pike *et al.*, 2007; Dowling *et al.*, 2008; Barreto and Burton, 2013; Dowling, 2014), which likely explains why the components of the oxidative phosphorylation (OXPHOS) pathway generally appear to be evolving under purifying selection (Blier *et al.*, 2001; Montooth *et al.*, 2009; Neiman *et al.*, 2010; Castellana *et al.*, 2011; Zhang and Broughton, 2013; Havird and Sloan, 2016; Sharbrough *et al.*, 2018). By this logic, one would expect that the OXPHOS genes would tend to harbor relatively low intraspecific variation. Instead, these genes are often polymorphic within species (Ballard and Whitlock, 2004; Dowling *et al.*, 2008; Galtier *et al.*, 2009; Bock *et al.*, 2014; Dobler *et al.*, 2014; Sharbrough *et al.*, 2018), with important implications for phenomena from mitonuclear incompatibilities (*e.g.,* Ellison and Burton, 2006) to DNA barcoding (Hebert *et al.*, 2003). The effects of this genetic variation on phenotypic variation for metabolic function - and indeed, how mitochondrial function is maintained over evolutionary time despite mutational pressure (Gabriel *et al.*, 1993) - remain unclear despite extensive variation for metabolic and mitochondrial function in natural populations of a diverse array of species (see *e.g.,* Ursi *et al.*, 2003; Sadowska *et al.*, 2005; Dowling *et al.*, 2008; Nilsson *et al.*, 2009; Arnqvist *et al.*, 2010; Oellermann *et al.*, 2012; Chung *et al.*, 2017; Nespolo *et al.*, 2017; Sharbrough *et al.*, 2017). Although some variation in metabolic and mitochondrial traits has been linked to specific environmental correlates (*e.g.,* altitude – Simonson *et al.*, 2010; Storz *et al.*, 2010, temperature – Clarke and Johnston, 1999, energy source – Montooth *et al.*, 2003), we lack a systematic understanding of the distribution of this variation across biogeographic

space. We can address this important knowledge gap by evaluating mitochondrial function across species ranges, which will provide insight into how phenotypic variation is partitioned across populations and environments.

Potamopyrgus antipodarum, a New Zealand freshwater snail (Winterbourn, 1970), is ideally suited for investigating spatial patterns in mitochondrial functional variation. There is both extensive mtDNA population structure in their native range (Neiman and Lively, 2004; Neiman *et al.*, 2010; Paczesniak *et al.*, 2013) and evidence for local adaptation to biotic (Lively and Jokela, 1996; Krist *et al.*, 2000; Dybdahl and Krist, 2004; Jokela *et al.*, 2009; King *et al.*, 2009; Koskella and Lively, 2009; Bankers *et al.*, 2017) and abiotic (Lively and Jokela, 1996; Krist *et al.*, 2000; Krist *et al.*, 2014; Krist *et al.*, 2017) environmental conditions of their source lakes. Temperature in particular appears to be a primary determinant of the geographical distribution of *P. antipodarum* within New Zealand (Winterbourn, 1969). Because sexual and asexual *P. antipodarum* frequently coexist in nature (Lively, 1987) and because asexuality has arisen multiple times within *P. antipodarum* (Neiman and Lively, 2004; Neiman *et al.*, 2011), distinct asexual lineages can be treated as repeated “natural experiments” into the consequences of asexuality for mitochondrial function. Asexual production of male offspring by obligately asexual female *P. antipodarum* (Neiman *et al.*, 2012) also makes it possible to assay sex-specific mitochondrial function, even in asexual lineages whose mitochondrial genomes have been “trapped” in females for generations. These features, along with extensive and heritable variation for mitochondrial performance among asexual lineages (Sharbrough *et al.*, 2017), allow *P. antipodarum* to be used for

64 powerful tests of the contribution of sexual reproduction to the processes underlying
65 mitonuclear coevolution.

66 Here, we tested whether source population, reproductive mode, or sex (male vs.
67 female) affect mitochondrial and physiological function at organismal and organellar
68 levels under laboratory conditions in field-collected *P. antipodarum*. We found evidence
69 that metabolic rate and behavioral response to heat stress vary across *P. antipodarum*
70 from different New Zealand lakes. The importance of lake-of-origin as a determinant of
71 mitochondrial phenotype demonstrates that direct and rigorous comparisons of
72 phenotypic function between sexual and asexual lineages and between males and
73 females in *P. antipodarum* will require extensive within-lake sampling. Altogether, our
74 results confirm previous reports of extensive variation for mitochondrial function in *P.*
75 *antipodarum* (Sharbrough *et al.*, 2017), extend these results to natural populations, and
76 set the stage for powerful comparisons between fitness-relevant traits in sexual and
77 asexual snails.
78

79 **MATERIALS AND METHODS**

80 **Field collections of *P. antipodarum***

81 While the phenotypic and ecological similarity of sexual *vs.* asexual *P. antipodarum*
82 enable direct comparisons across reproductive modes, it also means that definitive
83 determination of reproductive mode requires snail sacrifice. We therefore sampled field-
84 collected snails from New Zealand lakes known to harbor sexual and asexual
85 individuals, with populations at least ~10% male (Neiman *et al.*, 2011; Paczesniak *et al.*,
86 2013; Bankers *et al.*, 2017). 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 (Zachar and Neiman, 2013). We arbitrarily selected adult snails from lake collections (each of which consisted of 100s-1000s of individuals) and isolated each snail in a 0.5 L glass container with 300ml carbon-filtered H_2O. Water was changed weekly. Two distinct samples were used in this study: (1) we assayed oxygen consumption under heat stress in 57 wild-caught females, and (2) we assayed behavioral function and mitochondrial membrane potential in 46 wild-caught male and female snails (Table 1). All functional and behavioral assays began immediately following isolation, and all assays were completed within six months of arrival at the University of Iowa.

Oxygen consumption under heat stress conditions

Because oxygen becomes limiting to ectotherms under elevated temperatures (Abele *et al.*, 2007) and because *P. antipodarum* demonstrates signs of stress at elevated (~30ºC) temperatures (*e.g.,* reduced fecundity – Dybdahl and Kane, 2005, elevated oxygen consumption and decreased righting ability – Sharbrough *et al.*, 2017), we measured oxygen consumption using an aquatic respirometer as described in (Sharbrough *et al.*, 2017) for 57 wild-caught female *P. antipodarum* from each of six lakes. Oxygen consumption was assayed at three different water temperatures: 16ºC (not stressful, and similar to New Zealand lake temperatures), 22ºC (moderately stressful), and 30ºC (stressful) (Sharbrough *et al.*, 2017). Each snail was assayed at each temperature in a randomly determined order, and only snails that survived across all three temperature treatments were included in our analyses. Wet mass for each

individual was calculated after each trial, and mean mass across all three trials was used in our final analyses.

Behavioral response to heat stress

Righting time (Sharbrough *et al.*, 2017) and time to emergence following a startling stimulus – hereafter "shyness" – (M Neiman pers. obs.) increase with temperature in *P. antipodarum,* indicating that both assays can be used to assess heat-induced stress. We quantified righting times and shyness under each of the same three temperature treatments as for oxygen consumption in 46 wild-caught *P. antipodarum* and compared behavior reaction norms across temperatures, lakes, reproductive modes, and sexes. Snails were each assayed once at each temperature, and only snails surviving all three temperature treatments were included in our final analyses.

Mitochondrial membrane potential

JC-1 is a small positively charged molecule that diffuses down the electrochemical gradient across the inner mitochondrial membrane (Garner and Thomas, 1999). Under UV illumination, JC-1 fluoresces green if dispersed and red if aggregated (e.g., when inside the mitochondrial matrix) (Garner and Thomas, 1999). As a result, the median ratio of red: green fluorescence in freshly isolated, live mitochondria stained with JC-1 can serve as a proxy for mitochondrial membrane potential. We isolated mitochondria from 46 wild-caught *P. antipodarum* and measured red: green ratios in JC-1-treated mitochondrial extracts as described in Sharbrough *et al.* (2017) using a Becton Dickenson LSR II flow cytometer. Using only the set of 46 snails that completed

behavioral assays at all three temperatures, we compared median red: green ratios across lakes, reproductive modes, and sexes.

Determination of reproductive mode

Sexual *P. antipodarum* are diploid and asexual *P. antipodarum* are polyploid (triploid or tetraploid – Wallace, 1992; Neiman *et al.*, 2011), allowing us to use flow cytometry to determine DNA content and, thus, reproductive mode of individual *P. antipodarum*. After completing all physiological and/or behavioral assays, we dissected head tissue from each individual snail, flash froze this tissue in liquid nitrogen, and stored the frozen tissue at -80ºC until flow cytometry. We then homogenized head tissue in DAPI solution, filtered this solution through a 30 µm filter, and ran the solution on a Becton-Dickinson FACS Aria II following the protocol (including a chicken red blood cell standard) outlined in Krist *et al.* (2014).

Statistical analyses

We used a mixed-effects model framework to quantify the relationships between oxygen consumption and behavioral metrics (righting time, shyness) 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), which does not assume balanced or large sample sizes in its inferences. Only main effects were considered, as the study was not designed to investigate interactions.

We developed final models using backwards selection until only predictors with *p*-values less than 0.05 remained. To test assumptions of normality and heteroscedasticity of errors, we graphically inspected residuals and log- or square-root-transformed response variables when necessary. We performed all statistical analyses in R (R Core Team, 2017), fitting fixed-effect models with the *lm* function, fitting mixed-effects models using the lme4 package (Bates *et al.*, 2007), and estimating degrees of freedom for mixed-effect models using Satterthwaite's approximation *via* the lmerTest package (Kuznetsova *et al.*, 2015).

RESULTS

All model-fitting results are detailed in Table 2.

We measured oxygen consumption in 57 female *P. antipodarum* collected from six New Zealand lakes (see Table 1) under three different temperature regimes (*i.e.,* 16ºC, 22ºC, and 30ºC). We found that temperature ($p < 0.0001$), mass ($p = 0.00154$), and lake of origin ($p = 0.0072$), but not reproductive mode, moderated the rate of oxygen consumption (Fig. 1a, Table 2). This result indicates that snails collected from different lakes exhibit distinct respiratory responses to laboratory-controlled heat stress treatments.

Using a second sample of 46 male and female *P. antipodarum* from six New Zealand lakes (see Table 1), we tested whether lake-of-origin, reproductive mode, sex,

and temperature were associated with righting time and shyness under heat stress conditions. We found that temperature was a significant predictor of both our behavioral assays of righting ability ($p < 0.0001$, Fig. 1b) and of shyness ($p < 0.0001$, Fig. 1c). Lake of origin was a significant predictor of righting time ($p = 0.0155$) but not of shyness. Neither reproductive mode nor sex were significantly associated with either behavior in response to heat stress. These data indicate that elevated temperatures stress *P. antipodarum* sufficiently to alter behavior, and that righting ability differs across New Zealand lake populations of *P. antipodarum.*

We next assayed mitochondrial membrane potential in live mitochondrial extracts using the fluorescent dye JC-1 in this same set of 46 field-collected snails. We found that sex, but not lake of origin or reproductive mode, was a significant predictor of mitochondrial membrane potential ($p = 0.0070$, Fig. 2), with higher mitochondrial membrane potential in males *vs*. females. We had relatively low power to detect differences in mitochondrial membrane potential across lakes (power = 0.427) or sexes (power = 0.365). Together these data indicate that while mitochondrial membrane potential differs across sexes in *P. antipodarum*, larger sample sizes are necessary to compare mitochondrial membrane potential across lakes and reproductive modes.

DISCUSSION

Extensive work in the *P. antipodarum* system has documented lake-specific phenotypes and local adaptation for resistance to infection by the trematode parasite *Microphallus livelyi* (Lively and Jokela, 1996; Krist *et al.*, 2000; Dybdahl and Krist, 2004; Jokela *et al.*, 2009; King *et al.*, 2009; Koskella and Lively, 2009; Bankers *et al.*, 2017), life history

traits such as growth rate and size (Larkin *et al.*, 2016), and response to nutrient limitation (Krist *et al.*, 2014; Krist *et al.*, 2017). Here, we report the first evidence of population-structured variation for mitochondrial and behavioral function in *P. antipodarum*. Combined with population structure for mitochondrial genetic variation (Neiman and Lively, 2004; Neiman *et al.*, 2010; Neiman *et al.*, 2011; Paczesniak *et al.*, 2013), this result suggests the intriguing possibility that mitochondrial function is locally tuned in *P. antipodarum*.

These data set the stage for downstream studies addressing multiple important evolutionary questions. Chief among these questions is whether and how asexuality might influence mitonuclear coevolution. While the elevated linkage disequilibrium expected to result from asexuality should reduce the efficacy of natural selection in both nuclear (Fisher, 1930; Muller, 1964; Hill and Robertson, 1966; Birky and Walsh, 1988; Kondrashov, 1993; Charlesworth, 2012) and mitochondrial genomes (Gabriel *et al.*, 1993; Normark and Moran, 2000; Neiman and Taylor, 2009), stable transmission of mitonuclear genotypes may also facilitate rapid mitonuclear coadaptation and thereby local adaptation (Neiman and Linksvayer, 2006). There are few empirical tests of whether and how asexuality affects mitochondrial performance, leaving an important gap in our understanding of the evolutionary consequences of changes in reproductive mode. Surveys of mitochondrial genomes of asexual lineages (Paland and Lynch, 2006; Johnson and Howard, 2007; Neiman *et al.*, 2010; Henry *et al.*, 2012; Sharbrough *et al.*, 2018) have revealed elevated rates of putatively harmful mutations in mitochondrial genomes compared to sexual lineages. Absent nuclear compensation for mitochondrial function (*e.g.,* Harrison and Burton, 2006; Osada and Akashi, 2012), we predict

mitochondrial genomes carried by asexual lineages will therefore be associated with reduced mitochondrial performance. Evaluating whether these mutations actually result in reduced function will have profound implications for our understanding of the maintenance of sex. Importantly, the strong lake effect observed here means that the effects of reproductive mode and sex on mitochondrial and behavioral function can only be rigorously evaluated with extensive within-lake sampling.

Asexuality, and its implications for mitonuclear coevolution are especially relevant considering the invasion success of some asexual *P. antipodarum* lineages. Indeed, invasive lineages of *P. antipodarum* are entirely asexual (Wallace, 1992); however, only a few lineages of asexual *P. antipodarum* have successfully colonized novel environments to become invasive (Stadler *et al.*, 2005; Dybdahl and Drown, 2011; Levri *et al.*, 2017). The extensive but heterogeneous colonization success around the world by *P. antipodarum* therefore suggests that there exists substantial variation for invasive potential among asexual lineages, making this snail model a useful system in which to test hypotheses relating to traits associated with successful invasion (*e.g.,* Dybdahl and Kane, 2005). Notably, the indirect evidence that a particular mitochondrial haplotype is spreading among asexual lineages (haplotype 1A –Paczesniak *et al.*, 2013), is particularly intriguing in this context, as it may indicate an association between mitochondrial haplotype and migration in asexual *P. antipodarum*. The relationship between mitochondrial performance and stress documented here and in (Sharbrough *et al.*, 2017), combined with the co-transmission of mitonuclear genotypes in asexual lineages outlined above raises the possibility that invasive *P. antipodarum* may exhibit particularly good mitochondrial performance in response to environmental stress.

Maternal transmission of mitochondrial genomes precludes inheritance of all mitochondrial mutations originating in males, with two primary consequences: 1) ~50% reduction in N_e relative to a scenario in which cytoplasmic genomes are inherited biparentally, and 2) sexually antagonistic mutations only experience effective natural selection in females (Frank and Hurst, 1996; Gemmell *et al.*, 2004). 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 (Camus *et al.*, 2012). The lack of widespread evidence for mother's curse (but see Innocenti *et al.*, 2011; Patel *et al.*, 2016; Camus and Dowling, 2017) may point to mechanisms that prevent the spread of male-specific deleterious mutations in mitochondrial genomes (*e.g.,* paternal leakage – Kuijper *et al.*, 2015, inbreeding – Wade and Brandvain, 2009, kin selection – Wade and Brandvain, 2009, nuclear-encoded restorers of male function – Delph *et al.*, 2007; Dowling *et al.*, 2007). We found that male *P. antipodarum* have higher mitochondrial membrane potential than females, leading us to speculate that male *P. antipodarum* do not suffer from mother's curse type mutations that decrease their ability to generate a proton motive force. Importantly, mitochondrial membrane potential depends upon protons pumped by OXPHOS complexes comprised of both nuclear and mitochondrial gene products (*i.e.,* complexes I, III, and IV Hatefi, 1985), meaning that we should expect to detect reduced mitochondrial membrane potentials if male-harming mutations accumulate in *P. antipodarum* mitochondrial genomes. Still, sex-specific optima for mitochondrial membrane potentials make this prediction difficult to evaluate. Asexual *P. antipodarum* occasionally produce males (Neiman *et al.*, 2012), such that nuclear and mitochondrial genomes that have been "trapped" in females for

generations are suddenly expressed in a male context. These asexual males are expected to exhibit particularly poor mitochondrial performance because selection against male-harming mutations is completely ineffective in asexual lineages, making them uniquely well suited to evaluate the efficacy of selection against mother's curse in nature.

CONCLUSIONS

Our results point toward population-specific mitochondrial and behavioral function that is related to temperature, which has implications for climate change response and colonization success in this globally invasive species. This lake effect necessitates extensive sympatric sampling when comparing mitochondrial function in *P. antipodarum*. Together, our results establish *P. antipodarum* as a model system for evaluating mutational hypotheses for sex *via* comparisons of mitochondrial performance in sexual *vs.* asexual snails and for evaluating the strength and efficacy of selection against mother's curse in sexual populations.

DATA ACCESSIBILITY

Oxygen consumption, behavioral, and mitochondrial membrane potential data will be made freely accessible upon acceptance.

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.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Physiological responses to heat stress for *P. antipodarum* across source lakes. a) Oxygen consumption/hour/gram. b) Righting time. c) Shyness.

Figure 2. Mitochondrial membrane potential in field-collected *P. antipodarum*. Ratios of red: green fluorescence of JC-1-treated mitochondrial extracts for a) snails from all six New Zealand lakes, b) male *vs.* female snails from all lakes.

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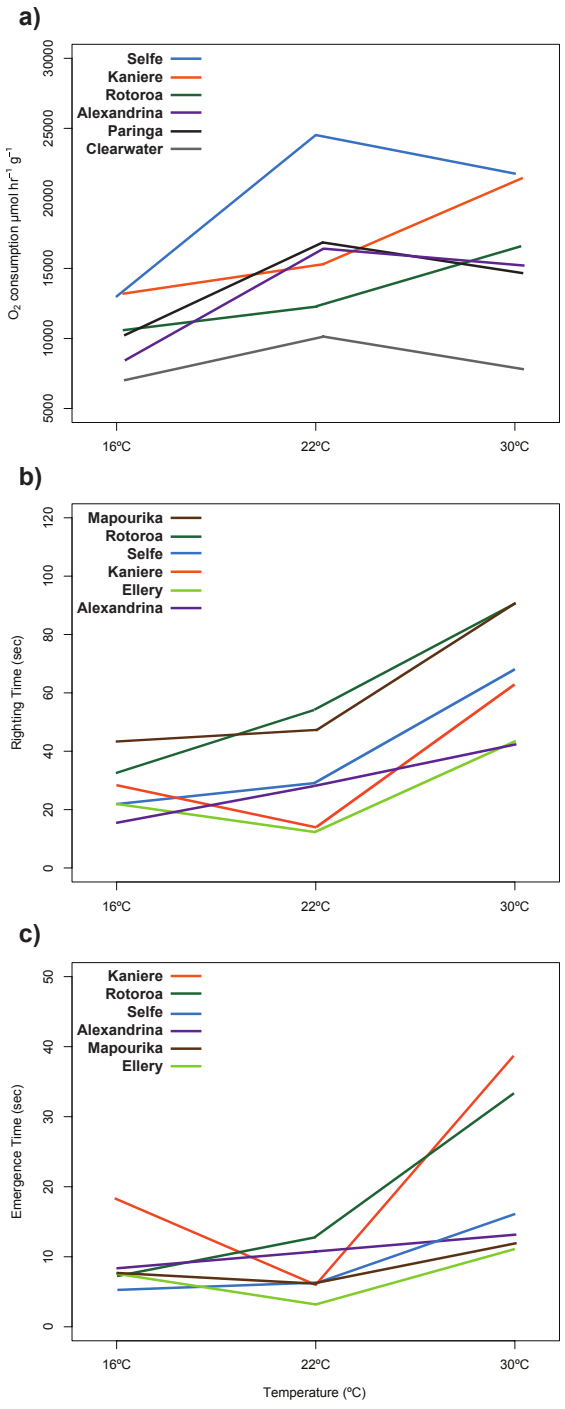

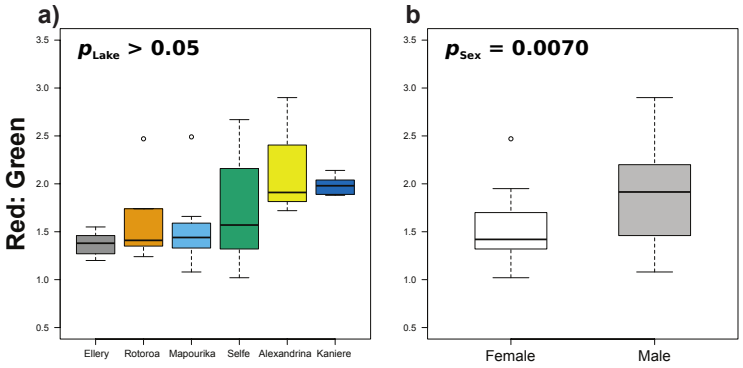

Table 1. Summary of source populations of *Potamopyrgus antipodarum* collected from New Zealand lakes.

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

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

Oxygen consumption[1]

Factor	χ^2	df	*p*	Non-significant predictors[6]
Intercept[2]	2.700	1	0.1004	Reproductive Mode
Temperature	39.038	2	< 0.0001***	
Mass	11.061	1	0.0009***	
Lake of Origin	15.280	5	0.0092**	

Righting time[3]

Factor	χ^2	df	*p*	Non-significant predictors[6]
Intercept[2]	59.205	1	< 0.0001***	Reproductive Mode, Sex
Temperature	73.661	2	< 0.0001***	
Lake of Origin	14.020	5	0.0155*	

Shyness[4]

Factor	χ^2	df	*p*	Non-significant predictors[6]
Intercept[2]	448.891	1	< 0.0001***	Reproductive Mode, Sex, Lake of Origin
Temperature	46.646	2	< 0.0001***	

Mitochondrial membrane potential[5]

Factor	Sum of Squares	df	*F*	*p*	Non-significant predictors[6]
Intercept	55.876	1	305.104	< 0.0001***	Reproductive Mode, Lake of Origin
Sex	1.466	1	8.004	0.0070**	
Residuals	8.058	44			

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

[2] – Snail ID was fit as a random intercept

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

[4] – Type III Repeated-Measures Analysis of Deviance $\chi 2$ Test of square-root-transformed shyness

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

[6] – Non-significant predictors listed in order of elimination from the model

* – $p < 0.05$

** – $p < 0.01$

*** – $p < 0.001$