Integrative and Comparative Biology

Integrative and Comparative Biology, volume 60, number 2, pp. 275–287
doi:10.1093/icb/icaa066

Society for Integrative and Comparative Biology

SYMPOSIUM

Phenotypic Variation in Mitochondria-Related Performance Traits Across New Zealand Snail Populations

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From the symposium "Building Bridges from Genome to Phenome: Molecules, Methods and Models" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2020 at Austin, Texas.

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Synopsis Mitochondrial function is critical for energy homeostasis and should shape how genetic variation in metabolism is transmitted through levels of biological organization to generate stability in organismal performance. Mitochondrial function is encoded by genes in two distinct and separately inherited genomes—the mitochondrial genome and the nuclear genome—and selection is expected to maintain functional mito-nuclear interactions. The documented high levels of polymorphism in genes involved in these mito-nuclear interactions and wide variation for mitochondrial function demands an explanation for how and why variability in such a fundamental trait is maintained. *Potamopyrgus antipodarum* is a New Zealand freshwater snail with coexisting sexual and asexual individuals and, accordingly, contrasting systems of separate vs. co-inheritance of nuclear and mitochondrial genomes. As such, this snail provides a powerful means to dissect the evolutionary and functional consequences of mito-nuclear variation. The lakes inhabited by *P. antipodarum* span wide environmental gradients, with substantial across-lake genetic structure and mito-nuclear discordance. This situation allows us to use comparisons across reproductive modes and lakes to partition variation in cellular respiration across genetic and environmental axes. Here, we integrated cellular, physiological, and behavioral approaches to quantify variation in mitochondrial function across a diverse set of wild *P. antipodarum* lineages. We found extensive across-lake variation in organismal oxygen consumption and behavioral response to heat stress and differences across sexes in mitochondrial membrane potential but few global effects of reproductive mode. Taken together, our data set the stage for applying this important model system for sexual reproduction and polyploidy to dissecting the complex relationships between mito-nuclear variation, performance, plasticity, and fitness in natural populations.

Introduction

The production of adenosine triphosphate (ATP) via cellular respiration is a critical component of eukaryotic performance and fitness (Pike et al. 2007; Barreto and Burton 2013a; Dowling 2014). The unique genetic architecture of the ATP-generating oxidative phosphorylation (OXPHOS) enzyme complexes has significant implications for the evolution of energy metabolism. OXPHOS is unusual in that the protein subunits that comprise the majority of the pathway are encoded by two separate genomes (i.e., the nuclear and mitochondrial) with different inheritance regimes (i.e., biparental vs. uniparental, respectively) (Rand et al. 2004). The coevolutionary dynamics of "mito-nuclear" interactions have been the subject of intense study (Ellison and Burton 2006; Osada and Akashi 2012; Barreto and Burton 2013b; Sloan et al. 2014; van der Sluis et al. 2015;

Advance Access publication June 26, 2020

Adrion et al. 2016; Camus and Dowling 2018; Yan et al. 2019), but the consequences of coevolution between genomes for organismal performance and fitness have only been evaluated in a handful of species and for a handful of mito-nuclear genotypes (Ellison and Burton 2006; Dowling et al. 2007; Hoekstra et al. 2013; Paliwal et al. 2014; Touzet and Meyer 2014). Because these mito-nuclear interactions have implications for a variety of evolutionary processes including inter-species (Meiklejohn et al. 2013) and inter-population (Ellison and Burton 2006) incompatibilities, introgression (Beck et al. 2015; Sharbrough et al. 2017a; Sloan et al. 2017), and local adaptation (Clarke and Johnston 1999; Montooth et al. 2003; Cheviron and Brumfield 2009; Simonson et al. 2010; Storz et al. 2010; Luo et al. 2013), among others (Hebert et al. 2003; Ursi et al. 2003; Sadowska et al. 2005), understanding how epistasis between nuclear and mitochondrial genomes contributes to the maintenance of variation in natural populations represents a critical open question in evolutionary biology (Hill et al. 2019).

Teasing apart how mito-nuclear genotype is connected to phenotype and how mitochondrial function is maintained over evolutionary time is not just a practical obstacle. The separate inheritance of nuclear and mitochondrial variation during sexual reproduction also presents a challenge for natural selection in shaping co-adapted mito-nuclear genotypes (Neiman and Linksvayer 2006). Particularly high-fitness mito-nuclear genotypes may be lost from populations, as selection is expected to favor the fixation of mitochondrial genomes that have the highest mean fitness across the suite of nuclear variants in the population (Clark 1984). The overwhelming preponderance of sexual reproduction in nature would thus seem to imply that the reliable transmission of particular mito-nuclear combinations is not all that important. Yet, sex linkage and sex-specific fitness effects provide a particular set of conditions that enable the maintenance of mito-nuclear variation in sexual populations (Rand et al. 2001). Investigations are thus warranted that can reveal how reproductive mode may affect the maintenance of mito-nuclear variation and the relationship between mito-nuclear genetic variation, mitochondrial function, organismal performance, and fitness.

Asexual reproduction, in which nuclear and mitochondrial genomes are co-inherited from mother to daughter, offers a useful contrast for understanding the consequences of sexual reproduction for mitochondrial function. In particular, mitochondrial genomes that are "trapped" in lineages that have transitioned to asexuality are inextricably linked to their nuclear genomic background. Because asexual lineages are expected to accumulate deleterious mutations in both the nuclear and mitochondrial genomes (Muller 1964; Hill and Robertson 1966; Neiman and Taylor 2009), asexual lineages should exhibit reduced mitochondrial performance relative to sexual lineages. However, co-inheritance of nuclear and mitochondrial genomes also allows epistatic variation to become visible to natural selection, such that asexual lineages may experience both relatively intense and efficient selection favoring compensatory co-evolution between nuclear and mitochondrial loci. The extent to which asexual lineages exhibit reduced vs. improved mitochondrial performance compared to close sexual relatives will be essential for understanding how epistatic mito-nuclear variation contributes to mitochondrial function and organismal fitness.

Potamopyrgus antipodarum is a New Zealand freshwater snail (Winterbourn 1970) with frequent co-existence and competition among obligately sexual and obligately asexual lineages (Lively 1987). Sexual and asexual *P. antipodarum* are so similar that they can only be distinguished by their relative genome sizes (asexuals are polyploid and sexuals are diploid) (Jokela et al. 1997). Because asexuality has arisen on multiple separate occasions 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 sexual reproduction for mitochondrial performance (Neiman et al. 2010; Sharbrough et al. 2018). *Potamopyrgus antipodarum* populations also feature extensive population structure in mitochondrial DNA sequences (Neiman and Lively 2004; Neiman et al. 2010; Paczesniak et al. 2013), and asexual lineages harbor heritable variation for mitochondrial function (Sharbrough et al. 2017b). Notably, the occasional production of male offspring by obligately asexual female *P. antipodarum* (Neiman et al. 2012) makes it possible to evaluate the phenotypic consequences of mitochondrial mutations that are neutral or beneficial when present in females but deleterious when present in males. In particular, selection against male-harming mutations is expected to be relaxed in asexual lineages, whose mitochondrial genomes have been evolving in females for generations, with the downstream prediction that asexual males should have an especially poor mitochondrial function. Together, these features make *P. antipodarum* uniquely well suited for understanding how the separate inheritance of nuclear and mitochondrial

genomes contributes to mitochondrial function in natural populations.

The observation that mitochondrial genomes of asexual *P. antipodarum* lineages accumulate putatively harmful mutations more readily than do their sexual counterparts (Neiman et al. 2010; Sharbrough et al. 2018) combined with the heritable variation for mitochondrial function present in asexual lineages (Sharbrough et al. 2017b) leads to the expectation that asexual *P. antipodarum* will exhibit reduced mitochondrial performance compared to sexual lineages. Here we tested that prediction as well as whether sex (i.e., male vs. female) and population (i.e., lake-of-origin) affect mitochondrial function and organismal performance under laboratory conditions using wild-caught *P. antipodarum*.

Materials and methods

Field collections of *P. antipodarum*

The phenotypic and ecological similarity of sexual vs. asexual *P. antipodarum* enables direct comparisons across reproductive modes, but also means that definitive determination of reproductive mode (via flow cytometry—see Supplementary File S1) requires snail sacrifice. Our first step was therefore to collect snails from New Zealand lakes known to harbor both sexual and asexual individuals (Neiman et al. 2011; Paczesniak et al. 2013; Bankers et al. 2017). These snails were collected in January 2015 and December 2016 and transported by hand in damp paper towels to the University of Iowa. Upon arrival, snails were housed at 16°C with an 18 L: 6 D photoperiod and fed *Spirulina* algae three times per week, as previously described (Zachar and Neiman 2013). We arbitrarily selected adult snails from lake collections (each of which consisted of hundreds to thousands of individuals) and isolated each snail in a 0.5 L glass container with 300 mL carbon-filtered H_2O. Water was changed weekly. All functional assays began immediately following isolation and were completed within six months of arrival at the University of Iowa.

To maximize our ability to sample and compare sexual and asexual individuals from the same lake, we first assayed oxygen consumption under heat stress (details below) in 158 wild-caught females from six lakes (Fig. 1 and Table 1) and used destructive sampling to determine reproductive mode in snails that survived all three temperature trials. The recent discovery of asexually produced males in *P. antipodarum* (Neiman et al. 2012) and recognition of their unique potential to provide insight into the frequency of male-harming mitochondrial mutations motivated us to include both males and females in our next experiment. Here, we assayed behavioral function in response to heat stress and mitochondrial membrane potential in 46 wild-caught snails (Fig. 1 and Table 1).

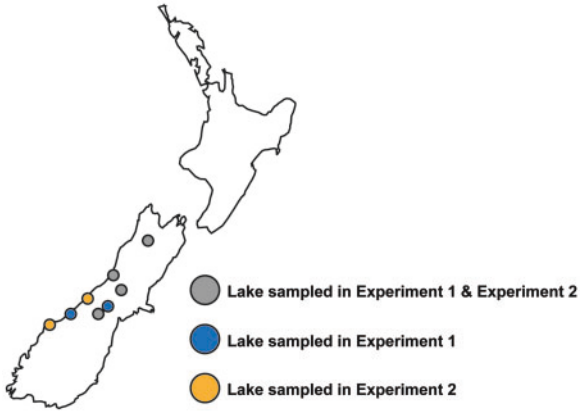

Fig. 1 Sampling *P. antipodarum* from New Zealand lakes. Samples from four lakes were used in both experiments, samples from two lakes were used in the oxygen consumption experiment only, and samples from a different two lakes were used for the behavioral and mitochondrial membrane potential experiments only.

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 (~30°C) temperatures (e.g., reduced fecundity (Dybdahl and Kane 2005), elevated oxygen consumption (Sharbrough et al. 2017b), and decreased righting ability (Sharbrough et al. 2017b), we measured oxygen consumption using an aquatic respirometer as previously described (Sharbrough et al. 2017b) for 158 wild-caught female *P. antipodarum* from each of six lakes (see Supplementary File S1 for a brief description). Oxygen consumption was assayed at three distinct water temperatures 16°C (not stressful, and similar to New Zealand lake temperatures), 22°C (moderately stressful), and 30°C (stressful) (Sharbrough et al. 2017b). Each snail was assayed at each temperature in a randomly determined order, and only the 61 snails (38.6%) that survived across all three temperature treatments were included in our analyses. We were unable to determine a single source of mortality for those snails that did not survive all three temperature trials, although exposure to high temperatures combined with hypoxic conditions inside the respiration chamber may have played a role. *Potamopyrgus antipodarum* is also

Table 1 Summary of source populations of *P. antipodarum* sampled from New Zealand lake collections

Lake	Latitude, longitude	Sexual	Asexual	N/A[a]	Male	Female
Oxygen consumption assay						
Alexandrina	−43.900476, 170.453978	12	3	11	—	26
Clearwater	−43.602131, 171.043917	—	3	17	—	20
Kaniere	−42.832886, 171.14759	16	—	12	—	28
Paringa	−43.713068, 169.411348	4	—	20	—	24
Rotoroa	−41.855414, 172.637882	—	16	22	—	38
Selfe	−43.237765, 171.520449	—	3	19	—	22
Total	–	32	25	101	0	158
Behavior and mitochondrial membrane potential assays[b]						
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
Total	—	31	15	0	22	24

[a]N/A refers to snails that did not survive all three temperature trials.
[b]Same individual snails were used in behavioral and mitochondrial membrane potential assays.

difficult to keep alive in laboratory conditions when isolated from other snails, and our temperature treatment procedure combined with recovery periods required that snails spent many weeks in isolation. 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 (Supplementary File S2) and time to shell emergence following a startling stimulus increase with temperature in *P. antipodarum* (Sharbrough et al. 2017b), indicating that both assays can be used to assess heat-induced stress. We quantified righting times and time to emergence under each of the same three temperature treatments used 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.

Mitochondrial membrane potential

Mitochondrial membrane potential is the electrochemical gradient that spans the inner mitochondrial membrane and drives ATP synthesis. As such, the magnitude of mitochondrial membrane potential is an indicator of cellular energy production and aerobic ATP production.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 ratio of red: green fluorescence in freshly isolated live mitochondria stained with JC-1 can serve as a proxy for mitochondrial membrane potential, with a ratio $\gg$1.0 indicating high membrane potential. After allowing the 46 wild-caught *P. antipodarum* snails used in our behavioral experiments to recuperate for 1–4 weeks, we sacrificed the snails and separated snail head and bodies with forceps. We saved head tissues for flow cytometric determination of reproductive mode and enriched for mitochondria from bodies according to a previously described protocol (Sharbrough et al. 2017b) (see Supplementary File S1 for a brief description).

We divided mitochondrial enrichments into three equal samples and placed each in separate tubes containing assay buffer (Supplementary File S1): the first sample was left untreated to measure autofluorescence, the second sample was stained with JC-1 to measure live mitochondrial membrane potential, and the third sample stained with JC-1 and treated with carbonyl cyanide m-chlorophenyl hydrazone (CCCP), a mitochondrial membrane decoupler. We

collected forward scatter, side scatter, FL1 (green), and FL2 (red) on a Becton Dickenson LSR II flow cytometer from several hundred to several thousand mitochondrial particles per sample and filtered out debris using FloJo version 10.0.8 software. After ensuring that autofluorescence was low and that CCCP-treated reactions displayed distributions typical of decoupled mitochondrial membranes, we compared median red: green ratios from each JC-1+/CCCP-treated sample across lakes, reproductive modes, and sexes.

Statistical analyses

Using a mixed-effects model, we tested whether mass, temperature (16°C, 22°C, and 30°C), lake-of-origin ($n = 6$), reproductive mode (sexual or asexual), and interactions of mass × temperature, mass × lake-of-origin, mass × reproductive mode, temperature × lake-of-origin, temperature × reproductive mode, mass × temperature × lake-of-origin, and mass × temperature × reproductive mode were good predictors of oxygen consumption per hour. Lake-of-origin was treated as a fixed effect primarily because the sampled lakes span a wide latitudinal gradient in New Zealand, setting the stage for follow-on work to address whether variation in heat responses might be explained by local adaptation. A term for snail identity was fit as a random intercept and a random slope was fit for the continuous variable of mass to account for repeated measures on individuals across temperatures (factorial) and mass (continuous) (Schielzeth and Forstmeier 2009; Arnqvist 2020). We employed a similar framework for emergence time and righting behavior, except we also included terms for sex (male or female) and a temperature × sex interaction, as we had both male and female snails in the sample used in these analyses. 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). Full details on our statistical methods can be found in Supplementary File S1.

Results

Prevalence of reproductive modes and sexes in two samples of wild-caught *P. antipodarum*

Our first sample was comprised of 158 female *P. antipodarum* collected from six New Zealand lakes (Lakes Alexandrina, Clearwater, Kaniere, Paringa, Rotoroa, and Selfe) (Fig. 1). In 61 snails from this sample, we were able to measure whole-snail oxygen consumption rates in a closed-system aquatic respirometer at 16°C, 22°C, and 30°C. We determined reproductive mode for 57 out of these 61 snails. The final sample included 32 sexual snails, 25 asexual snails, and 4 snails of unknown reproductive mode (Table 1). Only one of our sampled lakes included multiple sexual and asexual snails (Lake Alexandrina) (Table 1).

Our second sample was comprised 22 male and 24 female *P. antipodarum* collected from six New Zealand freshwater lakes (Lakes Alexandrina, Ellery, Kaniere, Mapourika, Rotoroa, and Selfe) (Fig. 1). This sample included 31 sexuals and 15 asexuals, with multiple sexual and asexual individuals sampled within each of three different lakes (Lakes Ellery, Mapourika, and Selfe). Lakes Mapourika and Selfe also had replication for sex, with both male and female sexuals sampled from both lakes. However, only in Lake Selfe did we sample male and female snails within each mode of reproduction (Table 1). The 46 snails present in this second sample were used for behavioral and mitochondrial membrane potential assays.

Lake-of-origin effects on mitochondria-related performance traits

From each of six lakes, we measured individual snail reaction norms for oxygen consumption across temperature treatments (Supplementary Fig. S1a). Variation in oxygen consumption rates was explained by significant effects of snail wet mass ($\chi^2 = 11.46$, df = 1, $P = 0.0007$), temperature treatment ($\chi^2 = 47.43$, df = 2, $P < 0.0001$), and lake-of-origin ($\chi^2 = 12.90$, df = 5, $P = 0.0243$) (Table 2). Snail wet mass had a positive effect on the oxygen consumption rate (correlation slope estimate = 6124.34) (Supplementary Fig. S2). *Post hoc* pairwise *t*-tests revealed that snails consumed significantly more oxygen at 22°C than at 16°C (t ratio = −4.19, df = 128, $P = 0.0001$), at 30°C than at 16°C (t ratio = −6.83, df = 129, $P < 0.0001$), and at 30°C than at 22°C (t ratio = −2.70, df = 129, $P = 0.0078$) (Fig. 2a). This pattern appeared most strongly in Lakes Kaniere, Rotoroa, and Selfe, but the lake-of-origin × temperature interaction was not a significant predictor of oxygen consumption. *Post hoc* pairwise *t*-tests of mean reaction norms across lakes-of-origin revealed that snails from Lake Kaneire and Lake Selfe exhibited especially high rates of oxygen consumption per hour. In sum, snails from different lakes have different organismal metabolic rates, with oxygen consumption generally increasing across this thermal range from benign to stressful temperatures.

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

Oxygen consumption model	Factor[a]	χ^2	*Df*	P-value	Non-significant predictors[b]
A	Intercept[c]	4.38	1	0.0364	Lake-of-origin × Mass × Temperature; Mass × Reproductive mode × Temperature; Lake-of-origin × Mass; Mass × Temperature; Mass × Reproductive mode; Reproductive mode × Temperature; Reproductive mode; Lake-of-origin × Temperature
	Mass	11.46	1	0.0007	
	Temperature	47.43	2	<0.0001	
	Lake-of-origin	12.90	5	0.0243	
Emergence time[e]					
Model	**Factor**	χ^2	**Df**	***P*-value**	**Non-significant predictors**
B	Intercept[d]	138.14	1	<0.0001	Reproductive Mode × Sex × Temperature; Sex × Temperature; Lake-of-origin × Temperature; Reproductive mode × Temperature; Reproductive mode × Sex; Reproductive mode; Sex
	Temperature	48.53	2	<0.0001	
	Lake-of-origin	11.34	5	0.0450	
Righting time[f]					
Model	**Factor**	χ^2	**Df**	***P*-value**	**Non-significant predictors**
C	Intercept[d]	238.80	1	<0.0001	Reproductive mode × Sex × Temperature; Reproductive mode × Temperature; Sex × Temperature; Lake-of-origin × Temperature; Lake-of-origin
	Temperature	73.36	2	<0.0001	
	Reproductive mode	4.48	1	0.0343	
	Sex	2.49	1	0.12	
	Reproductive mode × sex	6.52	1	0.0107	
Mitochondrial membrane potential[g]					
Model	**Factor**	**Sum of Squares**	**df**	***F***	***P*-value**	**Non-significant predictors**
D	Intercept	3.911	1	64.53	<0.0001	Reproductive mode × Sex; Reproductive mode; Lake-of-origin
	Sex	0.42	1	6.84	0.0122	
	Residuals	2.67	44	—		

[a]Type III Repeated-Measures Analysis of Deviance χ^2 test of O_2 consumption per hour per gram.
[b]Semi-colon delimited list of nonsignificant predictors in order of elimination from the model (i.e., worst predictor listed first).
[c]A random slope was fit for snail wet-mass, and a random intercept was fit for Snail ID.
[d]Snail ID was fit as a random intercept.
[e]Type III repeated-measures analysis of deviance χ^2 test of Box-Cox-transformed emergence time ($\lambda = -0.2845$).
[f]Type III repeated-measures analysis of deviance χ^2 test of log-transformed righting times.
[g]Type III analysis of variance F test of log-transformed ratios of red:green in mitochondrial extracts.

Using a second sample of 46 male and female *P. antipodarum* from six lakes (Fig. 1), we measured individual snail reaction norms for emergence time following a startling stimulus and for righting time across temperature treatments (Supplementary Fig. S1b and c). Variation in emergence time was explained by significant effects of temperature ($\chi^2 = 48.53$, df = 2, $P < 0.0001$) and lake-of-origin ($\chi^2 = 11.34$, df = 5, $P = 0.0450$), with no significant lake-of-origin × temperature interaction (Table 2). *Post hoc* *t*-tests of temperature effects averaged across lakes revealed that snails emerged from their shells significantly more slowly at 30°C than at either 16°C (t ratio = 6.42, df = 90, $P < 0.0001$) or 22°C (t ratio = 5.55, df = 90, $P < 0.0001$), with no significant difference in emergence time at 16°C vs. 22°C (t ratio = 0.87, df = 90, $P = 0.66$) (Fig. 2b). Despite the overall effect of lake-of-origin on emergence time, *post hoc* pairwise comparisons of lakes averaged over temperature could not distinguish between specific lakes ($P > 0.05$ for all comparisons). Temperature was also a significant predictor of righting behavior ($\chi^2 = 73.36$, df = 2, $P < 0.0001$), and *post hoc* pairwise analyses revealed that snails take significantly longer to right themselves at 30°C than at 16°C (t ratio = 8.04, df = 90, $P < 0.0001$), and than at 22°C (t ratio = 6.58, df = 90, $P < 0.0001$), but not at 22°C vs. 16°C (t ratio = 1.45, df = 90, $P = 0.15$) (Table 2). Lake-of-origin was not a significant predictor of righting time overall (Fig. 2c), but there were significant differences in righting time among lakes ($\chi^2 = 12.67$, df = 2, $P = 0.0018$) as well as a significant

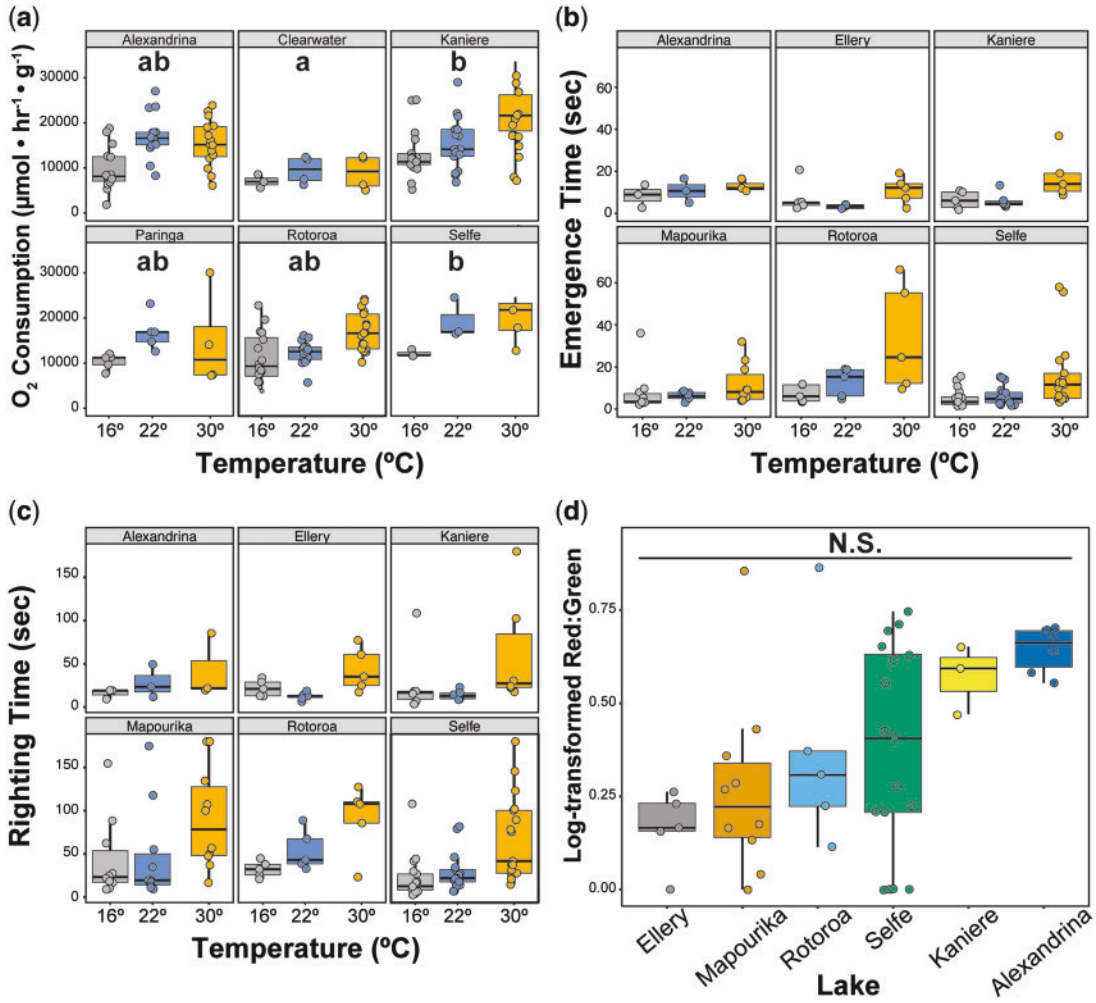

Fig. 2 Mitochondrial phenotypic variation across New Zealand lake populations of *P. antipodarum*. (**a**) Oxygen consumption/hour/gram measured at 16°C, 22°C, and 30°C in snails from six New Zealand lakes. There was a significant effect of mass, temperature, and lake-of-origin interaction on O_2 consumption (Table 2a) . Letters indicate *post hoc* statistical groupings. (**b**) Time to emergence following a startling stimulus was measured at 16°C, 22°C, and 30°C in snails from six New Zealand lakes. (**c**) Time required for righting after being flipped was measured at 16°C, 22°C, and 30°C in snails from six New Zealand lakes. (**d**) Log-transformed ratios of red: green fluorescence following treatment with JC-1 were measured using flow cytometry.

temperature × lake-of-origin interaction ($\chi^2 = 12.99$, df = 4, $P = 0.0113$) in females (Supplementary Table S1, Model S). The two behavioral responses were negatively correlated with each other at 16°C ($r^2 = 0.56$, $P < 0.0001$), 22°C ($r^2 = 0.34$, $P < 0.0001$), and 30°C ($r^2 = 0.19$, $P < 0.0023$). Together these data support a role for lake-of-origin in determining behavioral responses, with heat stress generally resulting in longer righting times and longer times to emerge from the shell after a startling response.

We measured mitochondrial membrane potential in freshly isolated mitochondrial fractions in the 46 field-collected snails that were used for behavioral trials. Mitochondrial membrane potential was not affected by lake-of-origin (Fig. 2d) and was not correlated with either of the behavioral responses ($P > 0.30$ for all comparisons). Thus, in contrast to the organismal performance traits that we expect to be related to mitochondrial function, mitochondrial membrane potential was similar across lakes for field-collected *P. antipodarum*.

Effects of reproductive mode on mitochondria-related performance traits

Whole-snail oxygen consumption rates did not differ between sexual and asexual snails (Fig. 3a), even when we restricted our analyses to snails from Lake Alexandrina (Supplementary Table S1, Model E), the one lake in the first experiment from which we sampled both sexual and asexual individuals. There was also no significant effect of reproductive mode on emergence time (Fig. 3b), even when we limited

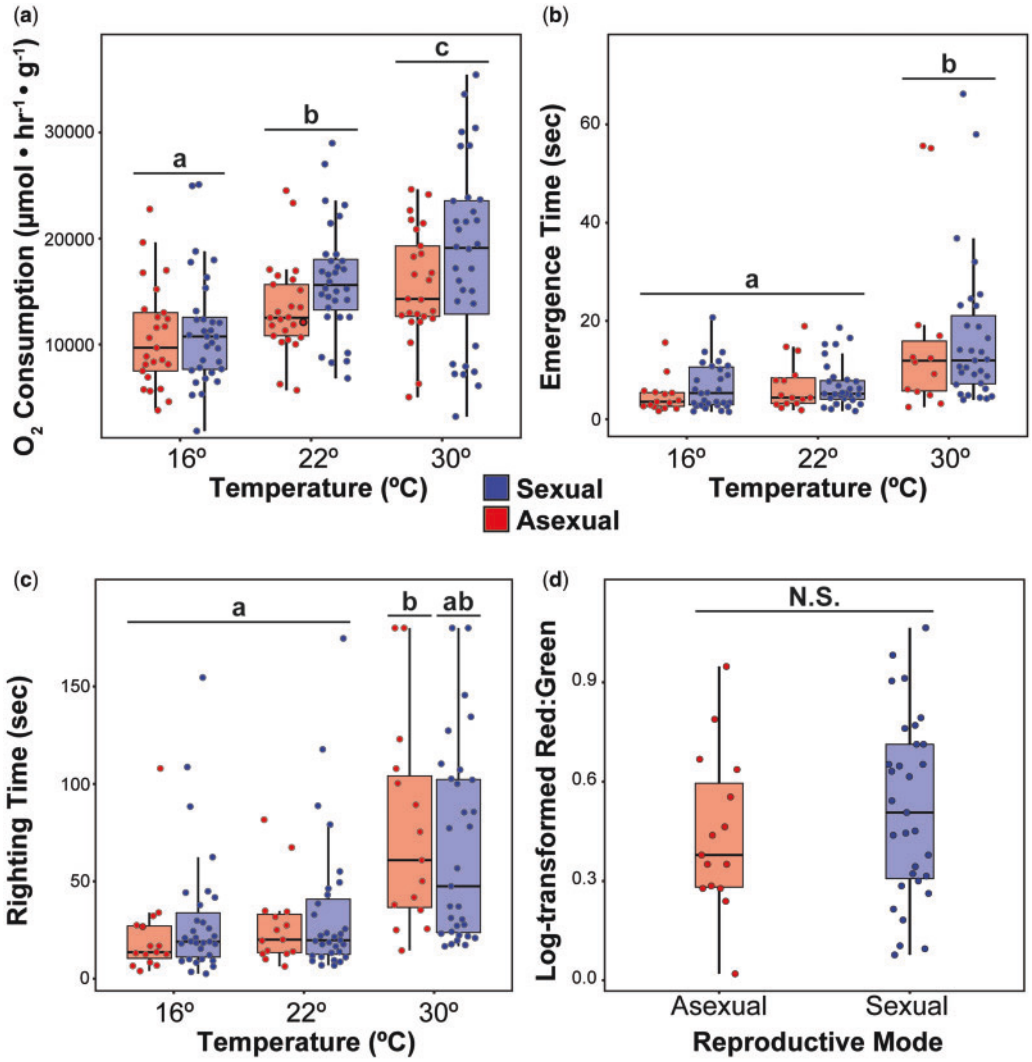

Fig. 3 Mitochondrial phenotypic variation across reproductive mode among field-collected *P. antipodarum*. Lower-case letters indicating statistical groups identified by pairwise comparisons. (**a**) Oxygen consumption per hour per gram assayed at 16°C, 22°C, and 30°C. (**b**) Time to emergence following a startling stimulus after incubation at 16°C, 22°C, and 30°C. Two outliers are not shown for the sake of display. (**c**) Righting time following being incubated at 16°C, 22°C, and 30°C, and subsequently flipped over. (**d**) Log-transformed ratios of red: green following treatment with JC-1 are depicted in sexual vs. asexual individuals.

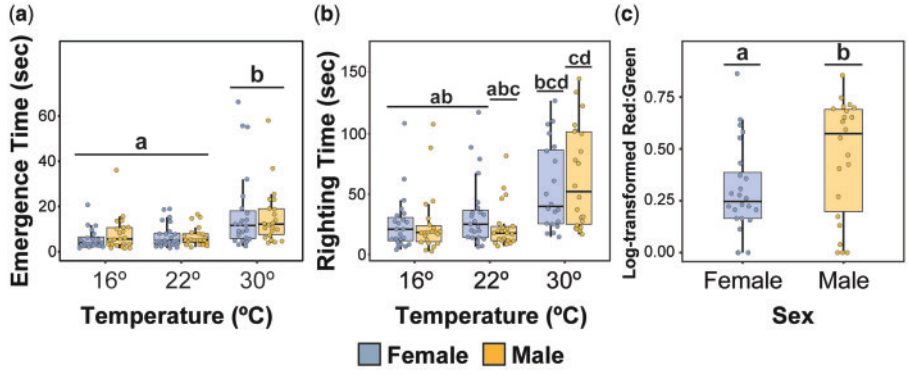

Fig. 4 Mitochondrial phenotypic variation across sexes among field-collected *P. antipodarum*. Lower-case letters indicating statistical groups identified by pairwise comparisons. (**a**) Time to emergence following a startling stimulus. (**b**) Righting time after being flipped, (**c**) Log-transformed ratios of red: green following treatment with JC-1.

our analyses to Lake Selfe, the only lake in the second experiment with adequate sample sizes to perform this comparison (Supplementary Table S1, Model F). However, reproductive mode ($\chi^2 = 4.48$, df = 1, $P = 0.0343$) and the reproductive mode × sex interaction ($\chi^2 = 12.67$, df = 2, $P = 0.0107$, Fig. 4b) were significant predictors of righting time (Table 2). Asexuals righted themselves faster than sexuals at 16°C and 22°C, but righted themselves more slowly than sexuals at 30° (Fig. 3c). Sexual males also tended to right themselves faster than sexual females, likely contributing to the significant reproductive mode × sex interaction. Reproductive mode did not affect mitochondrial membrane potential (Fig. 3d), even when we limited our analyses to Lake Selfe or to females within Lakes Mapourika and Selfe where there was adequate sample size to do this comparison (Supplementary Table S1). These data provide some evidence from a relatively small set of snails that reproductive mode and sex influence some aspects of organismal performance across temperatures, while mitochondrial membrane potential and other performance traits do not differ between asexual and sexual snails. However, low levels of within-lake sampling of asexuals and sexuals preclude a rigorous test of the effects of reproductive mode on these traits, particularly if the effects of reproductive mode vary across lakes.

Effects of sex on mitochondria-related performance traits

Emergence time was not significantly different between males and females (Fig. 4a), even when we limited our analyses to Lake Selfe (Supplementary Table S1, Model f). However, righting time was affected by an interaction between reproductive mode and sex (see above) (Fig. 4b). In this same set of field-collected snails, mitochondrial membrane potential was significantly higher in males relative to females (ANOVA $F_{1,44} = 6.84$, $P = 0.0122$, Fig. 4c). This difference between males and females was evident even when we limited our analysis to asexuals (ANOVA $F_{1,6} = 12.09$, $P = 0.0132$, Supplementary Table S1, Model K) or to Lake Selfe, which featured full replication of reproductive mode and sex (ANOVA $F_{1,15} = 6.58$, $P = 0.0216$, Model H). While sexual males also had higher mitochondrial membrane potentials than sexual females, the difference was not statistically significant (Supplementary Table S1, Model U). In sum, sex had significant effects on both mitochondrial membrane potential and righting time in *P. antipodarum*, with some evidence that the effects of sex vary in magnitude across reproductive modes.

Discussion

Lake-specific phenotypes and local adaptation for resistance to infection by the trematode parasite *Atriophallophorus winterbourne* (Lively and Jokela 1996; Bankers et al. 2017; Blasco-Costa et al. 2019), for life history traits such as growth rate and size (Larkin et al. 2016), and in the response to nutrient limitation (Krist et al. 2014) have been previously documented in *P. antipodarum*. Here, we report the first evidence of lake-structured variation for metabolic and behavioral performance traits, as well as sex-specific mitochondrial function in *P. antipodarum*, a species that is also known to have marked population structure for mitochondrial genetic variation (Neiman and Lively 2004; Paczesniak et al. 2013). A remaining question is whether the mitochondrial genetic and phenotypic variation among lakes is neutral or, like other traits studied in this snail, the result of local adaptation.

While the lack of recombination generated by asexuality should reduce the efficacy of natural selection in both nuclear (Fisher 1930; Muller 1964; Hill and Robertson 1966; Kondrashov 1993) and mitochondrial genomes (Gabriel et al. 1993; Neiman and Taylor 2009), stable transmission of mito-nuclear genotypes may also facilitate rapid mito-nuclear co-adaptation and local adaptation (Neiman and Linksvayer 2006). Surveys of mitochondrial genomes of asexual lineages (Paland and Lynch 2006; Johnson and Howard 2007; Henry et al. 2012), including in *P. antipodarum* (Osada and Akashi 2012; Larkin et al. 2016), have revealed elevated rates of putatively harmful mutations in mitochondrial genomes compared to sexual lineages. Absent nuclear compensation for mitochondrial function, we predicted that mitochondrial genomes carried by asexual lineages would, therefore, be associated with reduced mitochondrial function. However, we did not detect any global decrease in mitochondrial, organismal, or behavioral performance in asexual vs. sexual lineages in this geographically diverse sample. We conclude that either the decline in mitochondrial performance in asexual lineages was only detectable among closely related sexual and asexual pairs within the same lakes, which we were not able to adequately test for in this sample, or that asexual lineages exhibit either genetic or physiological compensation of the observed increased mutation load in their mitochondrial genomes. This compensation could be through mechanisms of physiological homeostasis (Matoo et al. 2019), clonal selection favoring lineages with mito-nuclear genotypes in which mutations in the nuclear genome compensate for the deleterious

mutations in the mitochondrial genome, or more complex compensatory mechanisms that involve signaling between the mitochondria and regulatory mechanisms encoded in the nuclear genome (Jastroch et al. 2010).

Polyploidy may also be a potentially important source for compensation of deleterious mutational effects in asexual snails, as sexual *P. antipodarum* are all diploid and asexual *P. antipodarum* are all polyploid (Jokela et al. 1997). Extra genome copies in the nuclear genome may allow for mutational masking that facilitates broader traversal of the adaptive landscape (Otto 2007). Elevated genome size is also positively correlated with larger cell size (Beaulieu et al. 2008), which likely alters the signaling, energetic, and stoichiometric landscape of the cell (Otto 2007). Moreover, the expected larger cell sizes of polyploids vs. diploids might allow for more and larger mitochondria, which in turn give rise to opportunities for compensatory mechanisms such as increasing OXPHOS capacity in the cell (Sharbrough et al. 2017c). Combined with the strong lake effect observed here, it is clear that extensive within-lake sampling combined with integrative approaches that span levels of biological organization will be required to elucidate the mechanisms of mitochondrial compensation in asexual lineages of *P. antipodarum*.

Our snails were collected from wild populations, meaning that observed differences in traits among lakes could result from genetic or environmental differences between these lakes. *Potamopyrgus antipodarum* does have significant population genetic structure (Neiman and Lively 2004; Paczesniak et al. 2013; Bankers et al. 2017), in which lakes and other geographic features of New Zealand (e.g., the Southern Alps Neiman and Lively 2004) act as major barriers to gene flow among *P. antipodarum* populations. Previous work investigating mitochondrial function in lab-cultured asexual lineages of *P. antipodarum* has identified heritable variation for mitochondrial, metabolic, and behavioral phenotypes (Sharbrough et al. 2017b). However, because the snails used in this study were born and developed to reproductive maturity in the wild before being subsequently acclimated to common laboratory conditions, it is possible that the lake effects observed here might also be the result of developmental phenotypic plasticity for metabolic rate. One tractable angle for investigating the contributions of genetic vs. environmental variation in mito-nuclear genotype to variation in mitochondrial performance can take advantage of extensive mito-nuclear discordance observed in asexual lineages of *P. antipodarum* (Paczesniak et al. 2013). Because asexual lineages stably transmit mito-nuclear genotypes across generations, asexual lineages that have reciprocal combinations of mitochondrial haplotypes with different nuclear genotypes can be used to test whether mito-nuclear epistatic variation contributes to mitochondrial function and ultimately, organismal fitness.

Maternal transmission of mitochondrial genomes has two primary consequences for selection on the mitochondrial genome in sexual taxa. First, genes in the mitochondrial genome of sexually reproducing lineages experience a reduction in the effective population size relative to nuclear genes due to both the absence of sexual recombination and uniparental inheritance. Second, mutations in the mitochondrial genome only experience selection in females (Frank and Hurst 1996; Gemmell et al. 2004). This latter phenomenon 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 mitochondrial mutations with sex-specific fitness effects (but see Innocenti et al. 2011; Patel et al. 2016; Camus and Dowling 2018) 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, and/or nuclear-encoded restorers of male function Delph et al. 2007; Dowling et al. 2007). Asexually produced males, as are occasionally produced in *P. antipodarum* (Neiman et al. 2012), represent worst-case scenarios for male-specific mitochondrial performance because their nuclear and mitochondrial genomes have been "trapped" in females for generations but are now being expressed in a male context. Because selection against any male-harming mutation, regardless of genomic compartment, is therefore ineffective in asexual lineages, asexual males are expected to exhibit particularly poor performance. Asexual male *P. antipodarum* produce a much higher frequency of abnormal sperm compared to sexual males (Jalinsky et al. 2020), indicating that degeneration of the molecular machinery responsible for male-specific function is a symptom of asexuality. Because mitochondrial membrane potential is established by OXPHOS complexes that are comprised both nuclear and mitochondrial gene products, we predicted that males would exhibit reduced mitochondrial membrane potentials if male-harming mutations have accumulated in asexual *P. antipodarum* mitochondrial genomes. In contrast to this expectation, the only strong effect of sex that we observed was that male *P. antipodarum* had higher mitochondrial membrane potential than their female

counterparts. Either male *P. antipodarum* do not suffer from sex-specific mutational effects that decrease their ability to generate mitochondrial membrane potential, or this high membrane potential may have deleterious effects that we did not measure. One of the most intriguing findings presented here was that the pattern of elevated mitochondrial membrane potential in males relative to females was stronger in asexual snails than it was in sexual snails. However, because we do not know the relationship between mitochondrial membrane potential and fitness, we cannot conclude whether this sexual dimorphism in mitochondrial membrane potential supports or refutes the presence of male-harming mitochondrial mutations in *P. antipodarum*. Nevertheless, the marked increase in mitochondrial membrane potential among asexually produced males offers a promising avenue for investigating the prevalence of evolutionary dynamics of male-harming mutations.

Conclusions

We found strong effects of temperature on oxygen consumption, righting behavior, and emergence time, lake effects on three out of four mitochondria-related performance traits, a difference among sexual and asexual individuals in one behavioral assay, and sexual dimorphism in mitochondrial membrane potential. These results suggest that the local environment and mitochondrial genetic structure may shape mitochondrial function and organismal performance in *P. antipodarum*. The strong lake effects observed demand more extensive intra-lake sampling in order to better test for the effects of reproductive mode and sex on mitochondrial and organismal performance in *P. antipodarum*. Even so, we were not able to detect a global negative effect of asexual reproduction on performance. This last finding points to physiological or genetic compensation for the accumulation of putatively deleterious mutations in mitochondrial genomes in asexual *P. antipodarum*. Together, our results establish *P. antipodarum* as a model system for evaluating the consequences of sexual reproduction for mitochondrial function and evolution and for evaluating the strength and efficacy of selection against male-harming mitochondrial mutations in sexual populations.

Acknowledgments

This work is dedicated in loving memory to J.J. Neiman-Brown, whose sparkle and curiosity give inspiration to us all. This manuscript was written in part during the Biology Department Weekly Writing Retreat at Colorado State University. Flow cytometry was performed at UI's Flow Cytometry Facility. We thank two anonymous reviewers for their wonderfully helpful reviews, and Laura Bankers, Kaitlin Hatcher, Katelyn Larkin, and Kyle McElroy for snail collections. We thank the organizers, especially Karen Burnett, and all the participants of the SICB 2020 Building Bridges from Genome to Phenome: Methods, Molecules, and Models symposium for their participation in the symposium and helpful feedback on this work.

Funding

This work was funded by the National Science Foundation [MCB – 1122176, DEB—1310825, DEB-1753695, DEB—1753851]; and the Iowa Academy of Sciences [ISF #13-10].

Data accessibility

Oxygen consumption, behavioral and mitochondrial membrane potential data, and R code have been posted to https://github.com/jsharbrough/Potamo_mt_function and FigShare (https://doi.org/10.6084/m9.figshare.11782128).

Conflict of interest statement

The authors declare no conflicts of interest.

Supplementary data

Supplementary data available at *ICB* online.

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