Title: Genetic variation for mitochondrial function in the New Zealand freshwater snail *Potamopyrgus antipodarum*

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ABSTRACT

The proteins responsible for mitochondrial function are encoded by two different genomes with distinct inheritance regimes, rendering rigorous inference of genotype–phenotype connections intractable for all but a few model systems. Asexual lineages provide a powerful means of addressing these challenges by enabling comparisons among genetically identical individuals in which nuclear and mitochondrial genomes are co-inherited. Here, we used *Potamopyrgus antipodarum*, a New Zealand freshwater snail that is an emerging model system for the evolution of sexual reproduction, adapting several methods for measuring mitochondrial function in mollusks at three distinct levels of biological organization: mitogenomic, organellar, and organismal. We applied these methods to multiple asexual lineages of *P. antipodarum* and evaluated whether there exists genetic variation (that is, the raw material for adaptive evolution) for mitochondrial genome copy number, mitochondrial membrane potential, and organismal oxygen consumption. Our analyses revealed that asexual lineages of *P. antipodarum* differed in terms of mitochondrial function at all three levels of biological organization. The discovery of lineage-level variation in terms of mitochondrial function combined with the methods developed here set the stage to study central evolutionary questions including whether mitochondrial mutation accumulation influences the maintenance of sexual reproduction in natural populations.

Keywords: Cellular respiration, oxygen consumption, mitochondrial function, asexual reproduction, mtDNA copy number, mitochondrial membrane potential

INTRODUCTION

Mitochondrial function is of critical importance to eukaryotic health (for example, Chen *et al.*, 2007, Dowling, 2014), and genetic variation for mitochondrial function has been linked to evolutionary adaptation (for example, Rawson and Burton, 2002) and disease (DiMauro and Schon, 2001). The role of genetic variation for mitochondrial function is complicated by direct interaction between nuclear encoded and mitochondrially encoded proteins, particularly with respect to oxidative phosphorylation (OXPHOS) (reviewed in Rand *et al.*, 2004). Mitonuclear cooperation is particularly important for proper enzyme function in OXPHOS complexes I, III, IV, and V because these complexes are comprised of subunits encoded by both genomes. Accordingly, discordance between mitochondrial and nuclear genomes has been demonstrated to have negative fitness and/or functional consequences in a variety of animals, including copepods (Ellison and Burton, 2006), *Drosophila* (Meiklejohn *et al.*, 2013; Pichaud *et al.*, 2013), and salamanders (Lee-Yaw *et al.*, 2014).

In sexually reproducing organisms, the maintenance of mitonuclear cooperation is further complicated by the expectation that the different mechanisms of nuclear *vs*. mitochondrial genome inheritance will differentially affect the generation, maintenance, and distribution of genetic variation. In particular, biparental inheritance and meiotic recombination in the nuclear genome will increase effective population size relative to the (typically) uniparentally inherited and non-recombinant mitochondrial genome (reviewed in Barr *et al.*, 2005; Neiman and Taylor, 2009). This logic is the basis for the expectation that mitochondrial genomes will, when compared to the nuclear genome, experience reduced efficacy of selection and suffer an increased rate of accumulation of mildly deleterious mutations (reviewed in Neiman and Taylor,

2009). This mechanism is also thought to generate selection favoring compensatory changes in nuclear-encoded mitochondrial subunits (Sloan *et al.*, 2013; Zhang and Broughton, 2013).

Asexual taxa provide a particularly interesting context in which to evaluate mitochondrial function and evolution because the absence of recombination and segregation in asexual nuclear genomes means that asexual lineages will transmit their mitochondrial genomes in complete linkage disequilibrium (LD) with the nuclear genome. The co-transmission of nuclear and mitochondrial genomes is expected to result in two non-mutually exclusive evolutionary scenarios: (1) increased LD should decrease the efficacy of selection in both genomes *via* selective interference (reviewed in Neiman and Taylor, 2009), and (2) tight linkage between nuclear and mitochondrial genomes may allow epistatic variation to become available to natural selection (reviewed in Neiman and Linksvayer, 2006). As such, the loss of sex likely has profound consequences for the evolution of mitochondrial function, most of which remain unevaluated.

Potamopyrgus antipodarum, a New Zealand freshwater snail, is very well suited for investigating mitochondrial function in the absence of sex because otherwise similar obligately sexual and obligately asexual individuals frequently coexist in natural populations. The transition from sexual ancestor to asexual descendent has occurred multiple separate times within this species, providing many so-called “natural experiments” into the consequences of asexuality (Neiman *et al.*, 2011; Paczesniak *et al.*, 2013). Surveys of patterns of molecular evolution in the mitochondrial genome of *P. antipodarum* have demonstrated that asexual lineages accumulate nonsynonymous substitutions in their mitochondrial genomes more rapidly than their sexual counterparts (Neiman *et al.*, 2010; Sharbrough *et al.*, 2016) and retain radical amino acid-changing polymorphisms longer than sexual lineages (Sharbrough *et al.*, 2016). What remains

unevaluated is whether and how these accumulated changes resulting from the loss of sex have shaped variation in mitochondrial function. Because natural selection can only operate on genetic variation that is linked to fitness, the critical first step in evaluating how asexuality impacts evolution of mitochondrial function in *P. antipodarum* is to evaluate whether asexual lineages exhibit variation in mitochondrial function. The direct transmission of nuclear and mitochondrial genetic information from mother to daughter in asexuals means that comparisons of mitochondrial function across asexual lineages raised under shared environmental conditions will provide an estimate of heritable variation for mitochondrial function in asexual lineages of *P. antipodarum*.

Here, we use a common-garden approach to test whether asexual lineages of *P. antipodarum* vary in mitochondrial function at three distinct levels of biological organization: (1) mitogenomic (mtDNA copy number), (2) organellar (mitochondrial membrane potential and electron transport), and (3) organismal (total oxygen (O_2) consumption), three traits that have all been linked to proper mitochondrial function in other taxa. First, mitochondrial genome copy number is tightly regulated and is positively correlated with cytochrome C oxidase activity (Van den Bogert *et al.*, 1993), and low copy number has been implicated in a number of human diseases (reviewed in Montier *et al*., 2009). Second, elevated mitochondrial membrane potential, generated by electron transport through OXPHOS, is positively correlated with the ratio of ATP: ADP, indicating that stronger membrane potentials reflect healthier mitochondria (Nicholls, 2004). Third, because O_2 is the final electron acceptor for the production of ATP (Chance and Williams, 1955), total O_2 consumption represents an integrated measure of the ATP production capacity of an organism. We therefore adapted well-established mitochondrial functional assays, previously employed exclusively in model organisms, to probe the number of mitochondrial

genome copies relative to the nuclear genome, estimate the strength of the electrochemical gradient, and quantify the flow of electrons through the electron transport chain (ETC) to test for genetic variation in mitochondrial function in asexual lineages of *P. antipodarum*. Because heat stress can increase inefficiencies in oxidative phosphorylation (Heise *et al.*, 2003), we also used heat treatments and aquatic respirometry to quantify and compare total O_2 consumption under heat stress across asexual *P. antipodarum* lineages. Together, these analyses revealed substantial variation for mitochondrial function across asexual lineages at all three levels of biological organization. More broadly, the assays adapted here provide a suite of useful experimental tools for investigating mitochondrial function in *P. antipodarum* and, potentially, other mollusks.

MATERIALS AND METHODS

Snail husbandry

We compared mitochondrial function across a diverse array of asexual *P. antipodarum* lineages (Neiman *et al.,* 2011; Paczesniak *et al.,* 2013; Table S1) reared under identical conditions for multiple generations, enabling us to interpret across-lineage variation in our various measures of mitochondrial function as representing genetic variation for these traits. Asexuality was established for each lineage by determining ploidy using flow cytometry (sexual *P. antipodarum* are diploid, asexuals are polyploid), as described in Neiman *et al.* (2011). Asexual lineages were chosen for functional assays to represent the range of mitochondrial diversity found in New Zealand populations (Neiman *et al.,* 2011) and to maximize our ability to compare different functional assays. Adult female snails were selected arbitrarily from lineage populations for each assay. Following standard laboratory protocols for *P. antipodarum* (e.g., Zachar and Neiman, 2013), snails were housed at 16ºC on an 18 hr light/6 hr dark schedule and fed *Spirulina* algae 3x per week.

Mitochondrial function at the genomic level

Mitochondrial genome copy number is known to affect respiratory capacity (Van den Bogart *et al.,* 1993) and to be dynamically regulated in response to various cellular environmental cues (Hori *et al.,* 2009; Matsushima *et al.,* 2010; Kelly *et al*., 2012). As such, mitochondrial genome copy number represents a potentially important phenotypic level at which selection could favor a compensatory mechanism to reduce the potentially negative consequences of mitochondrial mutation accumulation. To test whether asexual *P. antipodarum* exhibited across-lineage phenotypic variation for mitochondrial copy number, we estimated mitochondrial genome copy

number relative to a putatively single-copy nuclear gene using quantitative PCR (qPCR) in six asexual triploid lineages. A putatively single-copy gene from the *P. antipodarum* nuclear genome (*rad21*; Neiman *et al.*, unpublished) and mitochondrial *cytB* were chosen for analysis. Primer pairs were designed to generate a 264-bp *rad21* product (F: 5'–GATTCCAACAACTGATGTTTG –3', R: 5'–CAAAACTTACTCTAAATCTGC–3') and a 194-bp amplicon from *cytB* (F: 5'–TATGAATATTCAGATTTTTTAAATA–3', R: 5'–CCTTAACTCCTAATCTTGGTAC–3'). For measurement standards, each of these products was cloned from total DNA from a single *P. antipodarum* individual into the pGEM T-Easy plasmid vector (Promega Corp., Madison, WI). Linearized plasmids were diluted in the presence of carrier human genomic DNA to produce samples containing 300 – 300,000 copies of either the nuclear or mitochondrial amplicon. To evaluate genome copy numbers, total DNA was isolated from three to five individual snails from each lineage using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA), and nuclear and mitochondrial targets were amplified in triplicate in separate reactions on the same plate, together with serial dilutions of the cloned standards, using quantitative PCR on a StepOne Plus real-time thermal cycler (Applied Biosystems, Foster City, CA). We converted quantitation cycle values (Cq, the PCR cycle at which amplification products accumulated above a defined threshold) from snail samples into copy numbers using standard curves generated from the cloned standards, as in Miller *et al.* (2003), and the ratio of mitochondrial to haploid nuclear genome copies was determined for each sample; all lineages examined were triploid, established by flow cytometric data. Because the ratio of *cytB* to *rad21* fit the assumptions of normality (Shapiro-Wilks $W = 0.956$, $p = 0.228$) and variances between lineages were not significantly different (Levene's $F = 0.632$, $p = 0.677$), we compared inferred

mitochondrial genome copy number across lineages using a one-way ANOVA and pairwise t-tests as implemented in the R v 3.2.4 (R Core Team 2016).

Mitochondrial function at the organellar level

JC-1 assay

Mitochondrial membrane potential determines the strength of the electrochemical gradient mitochondria use to phosphorylate ADP to ATP. Accordingly, the amplitude of membrane potential is positively correlated with the ratio of ATP to ADP in the cell (Nicholls, 2004). Variation in this mitochondrial membrane potential has been linked to cellular aging (Nicholls, 2004) and survival (Callegari *et al.*, 2011), making mitochondrial membrane potential a useful measure of mitochondrial performance. The JC-1 assay (Garner and Thomas, 1999) measures the strength of the electrochemical gradient in mitochondrial isolates using a fluorescent dye, JC-1. With UV illumination, JC-1 will fluoresce green when dispersed and red when aggregated. In the absence of an electrochemical gradient, JC-1 will diffuse randomly across membranes and will aggregate in proportion to its concentration, such that under our experimental conditions, the ratio of red: green fluorescence should be approximately 1. If isolated mitochondria establish and maintain electrochemical gradients, JC-1, a small, positively charged molecule, will diffuse down the gradient and accumulate inside mitochondrial matrices, yielding an elevated ratio of red: green. A relatively high ratio of red: green is indicative of increased mitochondrial membrane potential and thereby suggests increased mitochondrial function (Garner and Thomas, 1999). We used the uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) to abolish the electrochemical gradient across the mitochondrial inner membrane in replicate subsamples,

allowing us to control for background levels of fluorescence of JC-1 and of mitochondrial membranes unrelated to mitochondrial function.

To test for genetic variation in mitochondrial function in *P. antipodarum* in terms of mitochondrial membrane potential, we assayed 8-10 individual adult female snails from six distinct asexual lineages representing a diverse subset of the natural mitochondrial haplotype diversity found in New Zealand (Neiman *et al.*, 2011; Paczesniak *et al.*, 2013; Table S1). For each snail, we first removed its shell and briefly washed the collected tissues by centrifugation at 600 x g in extraction buffer (10.0 mM HEPES (pH 7.5), 0.2 M mannitol, 70.0 mM sucrose, 1.0 mM EGTA). We rapidly homogenized these tissues on ice in extraction buffer containing 2 mg/ml fatty acid-free bovine serum albumin (fafBSA) using a micropestle and then centrifuged the homogenate at 4ºC for 5 minutes at 600 x g. The supernatant was recovered and held on ice separately while the pellet was re-homogenized and centrifuged again, as above. The pooled mitochondrial-enriched supernatant was centrifuged at 12,000 x g for 10 minutes and the pellet resuspended in buffer containing 10.0 mM HEPES (pH 7.5), 0.25 M sucrose, 1.0 mM ATP, 0.08 mM ADP, 5.0 mM sodium succinate, 2.0 mM K_2HPO_4, and 1mM DTT. We divided each sample into three subsamples of 30 µl and added 500 µl assay buffer containing 20.0 mM MOPS (pH 7.5), 110.0 mM KCl, 10mM ATP, 10.0 mM $MgCl_2$, 10.0 mM sodium succinate, and 1.0 mM EGTA. The first subsample was incubated with buffer alone, to monitor background fluorescence, the second subsample was incubated with 2 µM JC-1, and the third with 2 µM JC-1 and 30 µM CCCP. All three subsamples were incubated in the dark at 37ºC for 20 minutes, after which the ratio of red: green fluorescence was determined using flow cytometry (Becton Dickinson FacsCalibur, Franklin Lakes, NJ). Ungated data were collected from several hundred to several thousand mitochondrial particles per sample, on forward scatter (FSC) and side scatter

(SSC), FL1 (green fluorescence, log scale), and FL2 (red fluorescence, log scale). We plotted FL1 vs. FL2 for each sample, after gating out debris in FlowJo v 10.0.8 (FlowJo, Ashland, OR) and derived the ratio of red to green for each particle. The median ratio for each sample run was used for all comparisons.

Because red: green fluorescence ratios were not normally distributed (Shapiro-Wilks W = 0.858, $p = 7.131 \times 10^{-6}$), we used a log transformation of the data to meet the normality assumption of parametric analyses (Shapiro-Wilks W = 0.969, p = 0.15). We also found that ratios of red: green across lineages did not have equal variances (Levene's F = 3.093, p = 0.016), so we used a one-way ANOVA with a White adjustment for unequal variances (McKinnon and White, 1985) and lineage as a random factor to determine whether lineage affected the dependent variable of red: green ratio. We performed pairwise comparisons between lineages using Welch's t-tests (to reflect unequal variances) and the Holm procedure for Bonferroni correction (Holm 1979) using R v 3.2.4.

MTT assay

Electron flow through the electron transport chain (ETC) provides the energy necessary to establish a proton gradient, such that increased flow should produce a corresponding increase in mitochondrial membrane potential. When isolated mitochondria are incubated with the compound MTT (3-(4,5-dimethylthizol-2-yl) diphenyltetrazolium bromide), MTT accepts electrons from the ETC, forming a purple formazan product that is proportional to electron flow, and is the basis of a colorimetric assay of electron transport (Liu *et al.*, 1997). We performed the MTT assay by resuspending mitochondrial pellets pooled from 3-4 snails (obtained as described above) in 100 µl buffer (125 mM KCl, 2 mM K_2HPO_4, 1 mM $MgCl_2$, and 20 mM HEPES,

adjusted to pH 7.4 with KOH) with 6 mM succinate as an energy substrate. We then added these resuspended mitochondria to wells in 96-well plates. The MTT reaction was initiated by adding 10 µl of 2.5 mg/ml MTT to each well, and the plate was incubated for 2 hours at 37°C to allow electrons from the ETC to reduce MTT. A 20% SDS 50% dimethylformamide solubilization solution was applied to each well and the plate incubated overnight, after which the reduced formazan product was measured as A_{570}, in a XL-800 microplate reader (Bio-Tek Instruments, Winooski, VT). We determined background absorbance from duplicate mitochondrial samples incubated without an energy substrate and subtracted this background value from all readings. Phosphate-buffered saline was used as a negative control and 0.1 mM dithiothreitol as a positive control for MTT reduction. A fraction of each original mitochondrial sample was lysed in SDS and then used in a bicinchoninic acid assay (Smith *et al.*, 1985) to determine protein concentration. MTT reduction was expressed as A_{570}/ug protein.

While MTT values did not fit the assumptions of a normal distribution (Shapiro-Wilks $W = 0.840$, $p = 2.436 \times 10^{-5}$), these values did meet the assumptions of normality following a log transformation (Shapiro-Wilks $W = 0.961$, $p = 0.140$). Log-transformed MTT values did not display unequal variances (Levene's $F = 0.964$, $p = 0.452$), allowing us to compare log-transformed MTT values using a one-way ANOVA with lineage as a random factor to test whether different asexual lineages exhibited different levels of electron flow through the ETC (as implemented in R v 3.2.4).

Mitochondrial function at the organismal level

Ectotherms respire at higher rates at elevated temperatures, such that O_2 becomes limiting as temperature increases (Abele *et al.*, 2007). Accordingly, heat stress can reveal inefficiencies in

ATP production (Heise *et al.*, 2003). By this logic, we hypothesized that measuring O_2 consumption under heat stress would increase our ability to detect existing genetic variation for mitochondrial function. There is some evidence from earlier studies that *P. antipodarum* are susceptible to heat stress: at elevated temperature, snails exhibit decreased fecundity (Dybdahl and Kane, 2005) and tend to consume more O_2 (Hudson, 1983).

To further define the range of heat stress likely to alter mitochondrial function in *P. antipodarum*, we used a behavioral assay that measures the amount of time that a snail takes to right itself when placed ventral side-up and compared righting ability in 13 asexual lineages (N = 10 individuals per lineage) across three temperature treatments (16ºC, 22ºC, 30ºC). Righting ability is a commonly used method to gauge levels of snail stress (for example, Orr *et al.*, 2007). For example, snails exposed to hypoxic conditions exhibit increased righting time (and elevated HSP70 gene expression) compared to unexposed snails (Fei *et al.*, 2008). We began by incubating adult female *P. antipodarum* in carbon-filtered tap water (that is, the water in which the snails are housed) at the test temperature for 1 hour. Next, we placed snails ventral side-up in a petri dish and measured the number of seconds elapsed until the snail righted itself, up to 180 seconds. We compared righting time across temperatures and lineages with a Kruskal-Wallis test (as implemented in R v 3.2.4) and found that both temperature (Kruskal-Wallis, $\chi^2 = 14.218$, df = 2, $p = 0.00082$) and lineage (Kruskal-Wallis, $\chi^2 = 122.64$, df = 12, $p < 2.2 \times 10^{-16}$) significantly affected righting time. Righting time was ~24% faster at 22ºC (mean (+/- SD) = 48.82 (+/- 56.09) seconds) than at 16ºC (mean (+/- SD) = 62.50 (+/- 63.26) seconds), but this difference was not significant (Mann-Whitney, $U = 9244.5$, $p = 0.0754$). Conversely, righting took ~ 37% longer at 30ºC (mean (+/- SD) = 83.02 (+/- 73.74) seconds) than at 16ºC (Mann-Whitney U = 8192, $p = 0.0254$) and ~85% longer at 30ºC than at 22ºC (Mann-Whitney, $U = 5996.5$, $p =$

0.00014, Figure S1). These results are consistent with other righting time assays from other snail species in that some amount of stress decreases righting time (for example, presence of predators, Orr *et al.*, 2007), and some stimuli increase righting time (for example, hypoxia, Fei *et al.*, 2008), suggesting that our observed responses indicate stress in *P. antipodarum* exposed to elevated temperature (here, 30ºC).

After determining that snails do appear to be stressed by elevated temperatures, we tested whether there exists genetic variation for O_2 consumption under heat stress in *P. antipodarum* by performing closed-system aquatic respirometry on seven asexual lineages of *P. antipodarum* (N = 10 per lineage) at three different incubation temperatures (16ºC, 22ºC, 30ºC) using a Strathkelvin Instruments RC200a respiration chamber, 892 Oxygen Meter, and a 1302 Clark-type oxygen electrode (Strathkelvin Instruments, Motherwell, UK). We calibrated the electrode daily using the solubility of O_2 at each respective temperature (16ºC – 309.0 µmol/liter, 22ºC – 279.0 µmol/liter, 30ºC – 236.0 µmol/liter). A high calibration point was obtained by stirring carbon-filtered water vigorously for 30 minutes prior to calibration, while we used a 2% sodium sulfite solution as a low calibration standard. We incubated each snail at the prescribed temperature for one hour prior to measurement, placed snails into the cell chamber, and obtained O_2 concentration readings for each snail every second for 1 hour. We maintained constant temperature in the respiration chamber by pumping temperature-controlled water into the cell chamber's water jacket. Upon completion of the 1-hour test period, we blotted each snail dry and measured its wet mass on a Denver Instruments Cubis Analytical Balance (Denver Instrument, Bohemia, NY).

Snail wet mass was significantly and positively correlated with O_2 consumption (Spearman's rho = 0.17, p = 0.016, Figure S2). Because there is significant variation for snail

wet mass between asexual lineages (Kruskal-Wallis, $\chi^2 = 76.82$, df = 6, $p < 0.0001$), we calculated the residuals of wet mass *vs*. O_2 consumption using a linear model. Square-root-transformed O_2 consumption residuals were not significantly different from a normal distribution (Shapiro-Wilks, $W = 0.9894$, $p = 0.14$), allowing us to implement a linear regression model to test whether temperature and/or lineage affected O_2 consumption. We then used a two-way ANOVA to address whether the fixed factor of temperature, the random factor of lineage, and the interaction between temperature and lineage affected the dependent variable of wet-mass-corrected O_2 consumption.

Comparison of mitochondrial functional assays

Because each of the three assays that we developed and employed is designed to reflect some aspect of mitochondrial function, we should in principle expect some correlations between different assays if the assays in question actually provide meaningful measures of mitochondrial function. In particular, mitochondrial genome copy number measures overall system capacity for ATP production, the JC-1 assay measures the strength of the electrochemical gradient, the MTT assay measures electron flow though the OXPHOS pathway, and O_2 consumption under heat stress measures the overall electron acceptor turnover in conditions when ATP is in demand. To test for associations between different mitochondrial functional assays, we performed all 15 possible pairwise comparisons of mitochondrial functional assays by comparing the mean trait value per lineage across assays using Spearman's rank correlation (as implemented by the *Hmisc* package for R v 3.2.4), correcting for multiple comparisons using the Holm procedure. For these analyses, we included two additional sample populations that were not used in any of the tests for genetic variation: one field-collected sample from a lake with a high frequency of sexual

individuals (“KnSF12”) and an inbred sexual lineage that has been maintained in the lab for >20 generations (“Y2”, Table S1).

RESULTS

Mitochondrial function at the genomic level

To test whether *P. antipodarum* exhibits heritable variation for mtDNA copy number, we compared qPCR amplification of the mitochondrially encoded *cytB* locus to amplification of a putatively single copy nuclear gene, *rad21*, in six asexual lineages of *P. antipodarum* reared in a common-garden setting (N = 3-8 per lineage). The mean (+/- SD) number of *cytb* copies to the number of *rad21* copies was 13.715 (+/- 3.571), though we also found significant differences in this ratio across asexual lineages (one-way ANOVA, $F_{5, 25} = 2.72$, $p < 0.0428$, Figure 1), indicating that lineages differ in mitochondrial genome copy number. We performed pairwise t-tests of copy number ratio among each pair of lineages and found that a single lineage (Gr5; mean copy number ratio = 9.423, SD = 1.483) with very low mitochondrial copy number appeared to be driving the significant among-lineage variance. To test if the lack of differences amongst the remaining lineages could be an artifact of low statistical power, we performed power analyses using the *pwr* package in R v 3.2.4 for each of the 28 pairwise comparisons. We found mean power (+/- SD) = 0.402 (+/- 0.398), indicating both that power was low to moderate but also that power varied substantially across comparisons. For all comparisons involving the Gr5 lineage, mean power (+/- SD) = 0.881 (+/- 0.224), while for all comparisons not involving Gr5, mean power (+/- SD) was 0.162 (+/- 0.183). This result is likely driven by the low mitochondrial genome copy number of Gr5 relative to the other lineages and suggests that sample size was likely too small to detect the more minor differences in copy number, if any, that might exist between other asexual lineages.

Mitochondrial function at the organellar level

JC-1

Membrane potentials in mitochondria isolated from six asexual lineages of *P. antipodarum* were compared using the JC-1 assay, in which the ratio of red to green fluorescence is indicative of membrane potential. We found significant differences in the log-transformed ratios of red: green across asexual lineages (Welch's one-way ANOVA, $F_{5, 52} = 6.628$, $p = 7.671 \times 10^{-5}$, Figure 2a). Pairwise *posthoc* comparisons revealed three significantly different groups ($p < 0.05$ after correcting for multiple comparisons) amongst the six lineages (Figure 2a). Unlike mitochondrial genome copy number, this variation in mitochondrial membrane potential does not appear to be driven by a single lineage. Because CCCP-treated samples did not vary across lineages (one-way ANOVA, $F_{5,52} = 1.367$, $p = 0.252$), the variation observed for red: green fluorescence across lineages likely reflects variation in mitochondrial membrane potential. In particular, these data suggest that under the current rearing and assay conditions, the Gr5 lineage appears to have the strongest mitochondrial membrane potential (mean (+/- SD) = 3.859 (+/- 1.546)) and the DenA lineage (mean (+/- SD) = 1.775 (+/- 0.468)) appears to have the weakest mitochondrial membrane potential.

MTT

MTT reduction by isolated mitochondria is due to acceptance of electrons from the electron transport chain of the inner membrane, meaning that the extent of MTT reduction should reflect electron flow through the OXPHOS pathway. In general, the outcome of the MTT assay would be expected to be correlated with the strength of the proton gradient measured in the JC-1 assay, although that gradient can also be affected by the presence of uncoupling proteins, which reduce

the gradient by letting protons leak back into the matrix, dissipating energy as heat. Additionally, MTT does not accept electrons from all the reactions in the electron transport chain, so that some complex-specific differences in function may not be represented. Despite clear evidence for across-lineage variation in JC-1, we did not detect any differences amongst asexual lineages in MTT reduction (one-way ANOVA, $F_{5, 38} = 0.751$, $p = 0.591$, Figure 2b), even though we had relatively high power (=0.92, using the pwr.anova.test function implemented in the *pwr* package in R v 3.2.4).

Mitochondrial function at the organismal level

Organismal O_2 consumption under stressful conditions is expected to reflect electron acceptor turnover under maximal ATP production (Abele *et al.*, 2007). To test for genetic variation in O_2 consumption in heat-stressed *P. antipodarum*, we performed closed-system aquatic respirometry for seven asexual lineages (N = 10 per lineage) at 16ºC, 22ºC, and 30ºC. We found that elevated temperatures significantly affected mass-corrected O_2 consumption residuals (two-way ANOVA, $F_{1, 191} = 79.118$, $p = 4.44 \times 10^{-16}$) and that there was a significant interaction between lineage and temperature (Figure 3, two-way ANOVA, $F_{1, 191} = 3.919$, $p = 0.00102$). Using a one-way ANOVA within each temperature treatment, we found that lineage had a significant effect on O_2 consumption at 22ºC (one-way ANOVA, $F_{6, 58} = 3.002$, $p = 0.0127$) and at 30ºC (one-way ANOVA, $F_{6, 62} = 3.823$, $p = 0.00263$), but not at 16ºC (one-way ANOVA, $F_{6, 64} = 1.892$, $p = 0.096$). In particular, lineages responded differently to elevated temperature, with some lineages (for example, Ta10) exhibiting relatively high O_2 consumption at high temperatures and others maintaining similar levels of O_2 consumption (for example, Gn5) across temperatures. This

result demonstrates that genetically distinct lineages of *P. antipodarum* consume different amounts of O_2 in response to heat stress.

Comparison of mitochondrial functional assays

To determine the extent to which our different measures of mitochondrial function are associated, we performed Spearman's rank correlation for each of the 15 possible pairwise comparisons of the assays for mitochondrial genome copy number, log-transformed JC-1 red: green ratios, log-transformed MTT values, and square-root-transformed O_2 consumption at each temperature. None of the correlations remained significant after the Holm correction for multiple comparisons. The absence of significant relationships between assay results is not surprising in light of the fact that we had power of ~ 0.35 – 0.65 (using the pwr.r.test function implemented in the *pwr* package in R v 3.2.4) to detect a relationship between two variables at the $p < 0.003$ level, the alpha required by the Holm procedure. Power notwithstanding, there were some interesting results that deserve further scrutiny. First, neither mitochondrial genome copy number nor O_2 consumption values for 16º C or 30º C were correlated with any other phenotypic measure (Figure 4). Second, while the MTT assay requires pooling of mitochondria from 3-4 individual snails per measurement and is thus somewhat less sensitive than JC-1, which only requires mitochondria from one snail, we did find that MTT reduction was positively correlated with JC-1 measures of the proton gradient in the six asexual and two sexual *P. antipodarum* lineages examined (Spearman's rho = 0.81, $p = 0.0149$, Figure 4f). There was a trend towards a negative correlation between mean log-transformed JC-1 values and O_2 consumption at 22ºC for the six asexual lineages (Spearman's rho = -0.77, $p = 0.0724$, Figure 4k), and mean log-transformed MTT values were negatively correlated with O_2 consumption at 22ºC for the six asexual lineages

(Spearman's rho = -0.89, p = 0.0188, Figure 4k). Together, these tentative relationships between the MTT assay (reflecting OXPHOS electron flow) and O_2 consumption and between the MTT assay and the JC-1 assay (reflecting mitochondrial membrane potential) suggest that high mitochondrial membrane potential is expected to result in low O_2 consumption under O_2-limiting conditions in *P. antipodarum.* These results also suggest that genome copy number, JC-1, and organismal O_2 consumption provide orthogonal lines of inquiry into mitochondrial function.

DISCUSSION

Genetic variation for mt function at three different levels of biological organization

We used obligately asexual lineages of *Potamopyrgus antipodarum*, a freshwater New Zealand snail, to test for genetic variation in mitochondrial function in a common-garden setting. We found significant levels of variation at all three levels of biological organization that we assayed: mitochondrial genome copy number, mitochondrial membrane potential, and variation in O_2 consumption in response to heat stress. These results demonstrate that substantial levels of variation for mitochondrial function exist in asexual lineages of this species and suggest that mitochondrial function at the organellar level is related to mitochondrial function at the organismal level. Importantly, the methods described here can be easily adapted to other non-model organisms, especially mollusks, providing a new means of quantifying genotype-phenotype relationships.

The extent to which this variation in mitochondrial functional phenotypes contributes to fitness in *P. antipodarum* remains to be directly evaluated. Even so, the substantial across-lineage variation that we discovered provides functional evidence of high levels of asexual phenotypic diversity in *P. antipodarum*, consistent with previous reports that asexual *P. antipodarum* harbor substantial genetic (Jokela *et al.*, 2003; Paczesniak *et al.*, 2013) and phenotypic (Kistner and Dybdahl, 2013) diversity. The multiple separate transitions to asexuality in *P. antipodarum* (Neiman *et al.*, 2011; Paczesniak *et al.*, 2013) may help explain the substantial levels of variation found here, as asexual lineages represent “snapshots” of local sexual population diversity. Thus, variation within asexual lineages should be a subset of the variation found in source sexual populations (for example, Jokela *et al.*, 1997). The mitochondrial phenotypes we observed in one lineage, Gr5, were particularly distinct: snails from this lineage

exhibited relatively low mitochondrial copy number, high mitochondrial membrane potential and electron flow, and low O_2 consumption at 22ºC. Together, these phenotypic values suggest that the Gr5 lineage exhibits relatively high mitochondrial function, indicating that genetic dissection of its mitochondrial haplotype may prove illuminating. Further comparisons with other *P. antipodarum* lineages and in other conditions will provide substantial insight into the relative fitness of this particular mitonuclear combination. Future studies should also focus on a particular mitochondrial haplotype that appears to be especially common amongst asexual *P. antipodarum* (Neiman *et al.,* 2011; Paczesniak *et al.,* 2013). Paczesniak *et al.* (2013) showed that this haplotype is often found in divergent nuclear backgrounds, consistent with a scenario where this haplotype is spreading into new populations and lineages. Evaluating mitochondrial function of the common mitochondrial haplotype in *P. antipodarum* with divergent vs. similar nuclear backgrounds and in various biologically relevant conditions would shed light onto intraspecific mitonuclear coevolution in the context of asexuality and on whether selection is effective at favoring epistatic combinations in the absence of sex.

That the phenotypic variation for mitochondrial function shown here resides in traits that have been demonstrated to influence fitness in other organisms (Chen *et al.,* 2007; Dowling, 2014) means that this variation could have major implications for asexual lineage success. In particular, asexual *P. antipodarum* are known to harbor mitochondrial genomes with high mutational loads relative to sexual lineages (Neiman *et al.,* 2010; Sharbrough *et al.,* 2016), suggesting that accelerated mutation accumulation in asexual lineages likely affects mitochondrial function. Whether the mutations present in asexual mitochondrial genomes negatively impact fitness and/or function remains an unanswered question of great interest, and comparing mitochondrial function in *P. antipodarum* across reproductive modes will provide

critical tests of the hypothesis that mitochondrial mutation accumulation in asexuals is deleterious.

Relationship between mitochondrial functional assays

All else being equal, stronger membrane potentials, greater electron flow, lower O_2 consumption, and higher mitochondrial genome copy number should all positively correlate with overall mitochondrial function. Because all else is almost certainly not equal (for example, variation in temperature/resources in native range, phylogenetic constraint, *etc*.), it is possible that high mitochondrial performance in common-garden conditions does not reflect mitochondrial performance in nature. Despite this caveat, the tools developed here will provide an important starting point by which to interrogate mitochondrial function in non-model systems.

Although the number of asexual lineages included in the present study was relatively small, we did observe some interesting relationships between mitochondrial genome copy number, electron flux through the ETC, mitochondrial membrane potential, and organismal O_2 consumption (Figure 4). In particular, we found an inverse relationship between O_2 consumption at 22ºC and electron flow through the ETC and a positive relationship between electron flow through the ETC and mitochondrial membrane potential. The latter relationship is not particularly surprising in light of the fact that as electrons pass through the ETC, we expect a corresponding increase in mitochondrial membrane potential as H+ ions are released into the intermembrane space. The inverse relationship between decreased electron flux and elevated O_2 consumption is more difficult to understand because O_2 consumption is expected to increase with electron flux. Importantly, correlations between organismal-level O_2 consumption and organelle-level electron flux are difficult to disentangle because many layers of respiratory regulation can

contribute to increased organismal O_2 consumption beyond ETC inefficiencies (Brand and Nicholls, 2011). Furthermore, electrons leaked back across the inner membrane though uncoupling proteins contribute to electron flux but not O_2 consumption, and elevated temperatures in a marine mollusk have been shown to increase electron leakage of this type, as well as O_2 consumption in the formation of reactive oxygen species (Abele *et al.*, 2002), such that connecting organismal O_2 consumption to organellar electron flux is a non-trivial exercise. Understanding the relationship between organellar and organismal variation in mitochondrial function would provide helpful context for interpreting functional variation in *P. antipodarum*, and future efforts towards examining these relationships in more detail are necessary.

Implications for the loss of sex

One of the primary hypotheses put forth as an explanation for the prevalence of sexual reproduction in eukaryotes is the accumulation of slightly deleterious mutations in asexual lineages, eventually causing mutational meltdown and lineage extinction (Neiman and Taylor, 2009). Empirical evidence in support of this hypothesis remains largely indirect: (1) the relatively "twiggy" distribution of asexual taxa on the eukaryotic phylogeny compared to sexual taxa (Williams, 1975), and (2) the accumulation of putatively deleterious mutations in nuclear (Tucker *et al.*, 2013; Hollister *et al.*, 2015) and organelle genomes of asexual lineages (Neiman *et al.*, 2010; Henry *et al.*, 2012; Sharbrough *et al.*, 2016). What remains unresolved is whether the mutations being accumulated by asexual lineages actually decrease function and/or fitness. Establishing the presence of genetic variation for mitochondrial function represents the first step in determining whether mitochondrial mutation accumulation in the absence of sex causes such a decline. More specifically, if mitochondrial haplotype is not linked to mitochondrial phenotype,

mutation accumulation in mitochondrial genomes in asexual lineages would not cause decreases in function, such that sexual reproduction must be associated with other benefits to persist in the face of asexual competitors. Here, we show that snails with different mitochondrial genetic backgrounds have different mitochondrial phenotypes in a common-garden environment, suggesting that mutations present in certain lineages have some impact on function, thereby setting the stage for direct comparisons between mitochondrial function in sexual and asexual *P. antipodarum*.

One alternative possibility to decreased mitochondrial function in asexual lineages is that asexuals have access to genetic variation that sexuals do not. This scenario is not wholly unexpected: because the nuclear and mitochondrial genomes are transmitted in complete LD in asexuals, advantageous combinations of mutations (that is, epistatic variation) can become visible to selection (Neiman and Linksvayer, 2006). By contrast, in sexual lineages, the mitochondrial genome is swapped across diverse nuclear backgrounds each generation, such that selection cannot act effectively on phenotypes linked to epistatic mitonuclear interactions. The implications are that interacting nuclear and mitochondrial subunits may experience particularly tight and effective coevolution. Such a phenomenon might exhibit a very similar pattern of molecular evolution to that of accelerated deleterious mutation accumulation, meaning that testing the functional consequences of asexual mutation accumulation is critical to determining whether the absence of sex inevitably leads to mutation meltdown and lineage extinction.

DATA ARCHIVING

All data will be made available on Dryad upon acceptance.

CONFLICT OF INTEREST

The authors declare no conflict of interest for the work described here.

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TITLES AND LEGENDS TO FIGURES

Figure 1. MtDNA copy number variation in six asexual lineages of *P. antipodarum*. Box-and-whisker plot depicting quantitative PCR estimates (rank-ordered by median) of *cytB* copy number relative to a putatively single-copy nuclear gene, *rad21*. MtDNA copy number was significantly different across lineages (one-way ANOVA, $F = 2.72$, df = 5, $p = 0.043$), which appears to be driven by lineage Gr5. Boxes represent Inner Quartile Ranges (IQR) with error bars extending 1.5x beyond IQRs. Data points falling outside error bars are denoted by open circles. Shared lowercase letters indicate $p > 0.05$ for pairwise t-tests corrected for multiple comparisons using the Holm procedure for multiple comparisons. N = 10 individuals for all lineages.

Figure 2. Organellar function of mitochondrial fractions isolated from six lineages of *P. antipodarum*. a) Estimate of mitochondrial membrane potential using the ratio of red to green JC-1 dye fluorescence from individual snails (N = 10 individuals for all lineages). Lineages were rank-ordered by median with boxes representing Inner Quartile Ranges (IQR); error bars extend 1.5x beyond IQRs. Data points falling outside error bars are denoted by open circles. Shared lowercase letters indicate $p > 0.05$ for pairwise Welch's T-tests, corrected using the Holm procedure for multiple comparisons. b) Estimate of electron flux through OXPHOS pathway using the colorimetric MTT reduction assay from pooled mitochondrial extractions (3 snails pooled per replicate, 3 – 6 replicates per lineage). Lineages were rank-ordered by median value with boxes representing IQRs and error bars extending 1.5x beyond IQRs. Data points falling outside error bars are denoted by open circles. Lineages did not appear to differ in MTT reduction.

Figure 3. Interaction plot between O_2 consumption residuals, temperature, and snail lineage. Lines indicate best-fit linear regression of O_2 consumption across temperature pairs (for example, 16ºC – 22ºC) for seven asexual lineages of *P. antipodarum.* O_2 consumption was measured for 10 individual snails at each temperature for each lineage. O_2 consumption is affected by temperature as well as by the interaction between temperature and lineage. Within temperatures, lineage had a significant effect on O_2 consumption at 22ºC (one-way ANOVA, $F_{6, 58} = 3.002$, $p = 0.0127$) and at 30ºC (one-way ANOVA, $F_{6, 62} = 3.823$, $p = 0.00263$).

Figure 4. Comparisons of mitochondrial functional assays. Spearman's rank correlations for all pairwise comparisons of mitochondrial functional assays. Best-fit linear regression lines (black) are only shown for those comparisons that were significant at the $p < 0.05$ level. a) mtDNA copy number vs. JC-1 (Spearman's rho = -0.1, $p = 0.823$). b) mtDNA copy number vs. MTT reduction (Spearman's rho = -0.33, $p = 0.42$). c) mtDNA copy number vs. O_2 consumption at 16ºC (Spearman's rho = 0.03, $p = 0.957$). d) mtDNA copy number vs. O_2 consumption at 22ºC (Spearman's rho = 0.03, $p = 0.957$). e) mtDNA copy number vs. O_2 consumption at 30ºC (Spearman's rho = 0.43, $p = 0.396$). f) JC-1 vs. MTT reduction (Spearman's rho = 0.81, $p = 0.0149$). g) JC-1 vs. O_2 consumption at 16ºC (Spearman's rho = -0.43, $p = 0.397$). h) JC-1 vs. O_2 consumption at 22ºC (Spearman's rho = -0.77, $p = 0.0724$). i) JC-1 assay vs. O_2 consumption at 30ºC (Spearman's rho = 0.2, $p = 0.704$). j) MTT reduction vs. O_2 consumption at 16ºC (Spearman's rho = 0.14, $p = 0.787$). k) MTT reduction vs. O_2 consumption at 22ºC (Spearman's rho = -0.89, $p = 0.0188$). l) MTT reduction vs. O_2 consumption at 30ºC (Spearman's rho = -0.09, $p = 0.872$). m) O_2 consumption at 16ºC vs. O_2 consumption at 22ºC (Spearman's rho = -0.36, $p =$

0.432). n) O_2 consumption at 16ºC vs. O_2 consumption at 30ºC (Spearman's rho = -0.14, *p* = 0.76). o) O_2 consumption at 22ºC vs. O_2 consumption at 30ºC (Spearman's rho = -0.04, *p* = 0.939).

SUPPLEMENTARY MATERIAL TITLES TO FIGURES AND LEGENDS

Figure S1. Variation in righting time in response to heat stress in *P. antipodarum*. Ten asexual lineages (N = 10 snails per lineage) were incubated at either 16ºC, 22ºC, or 30ºC for 1 hour prior to assaying behavioral responses to elevated temperature. Temperature (Kruskal-Wallis, $\chi^2 = 14.218$, df = 2, $p = 0.00082$) and lineage (Kruskal-Wallis, $\chi^2 = 122.64$, df = 12, $p < 2.2 \times 10^{-16}$) both significantly affected righting time, indicating that elevated temperature can be used to induce stress in *P. antipodarum*.

Figure S2. Relationship between snail wet mass and oxygen consumption in *P. antipodarum*. There was a significant positive correlation between snail wet mass and O_2 consumption (Spearman's rho = 0.2, $p = 0.0044$).

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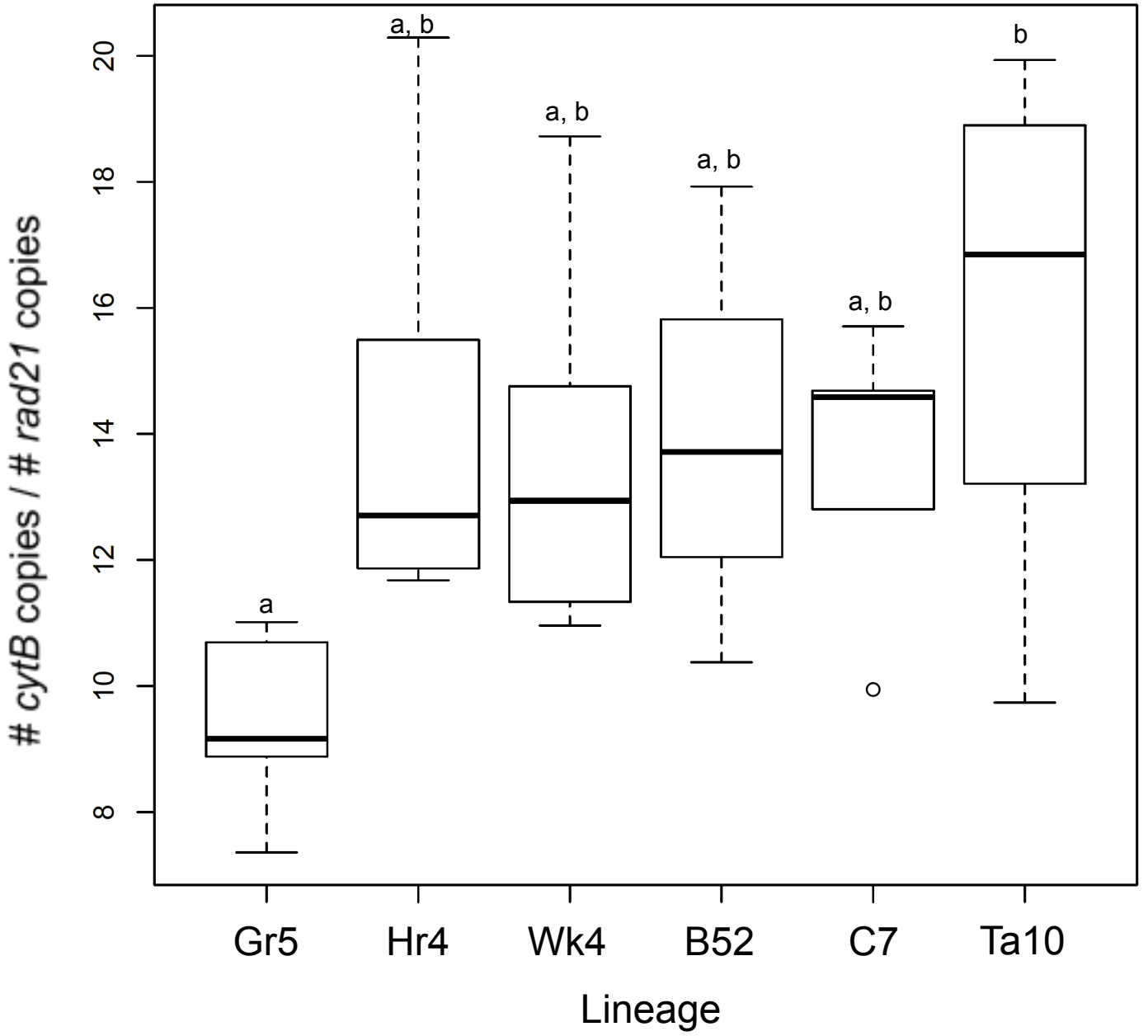
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14
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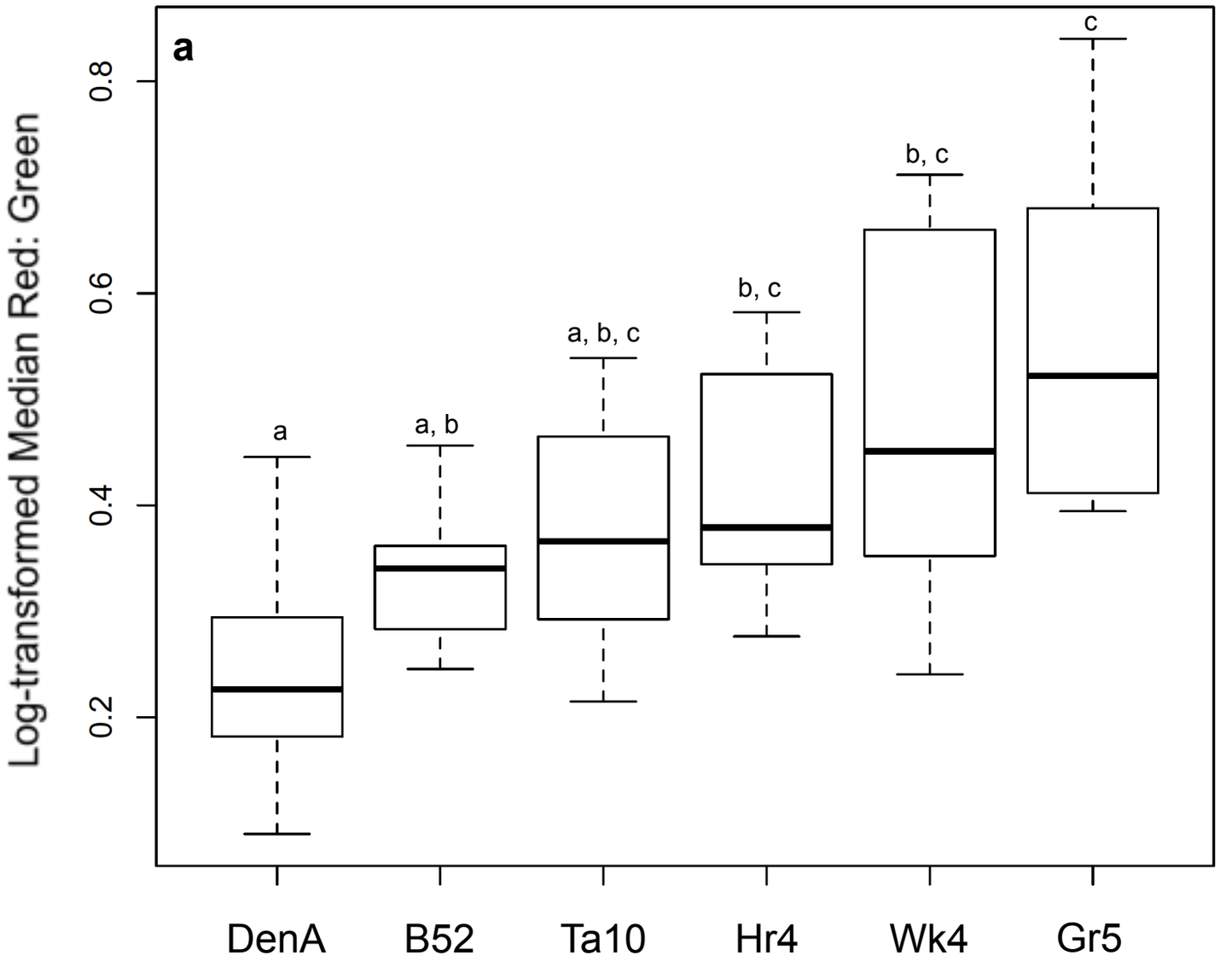
Lineage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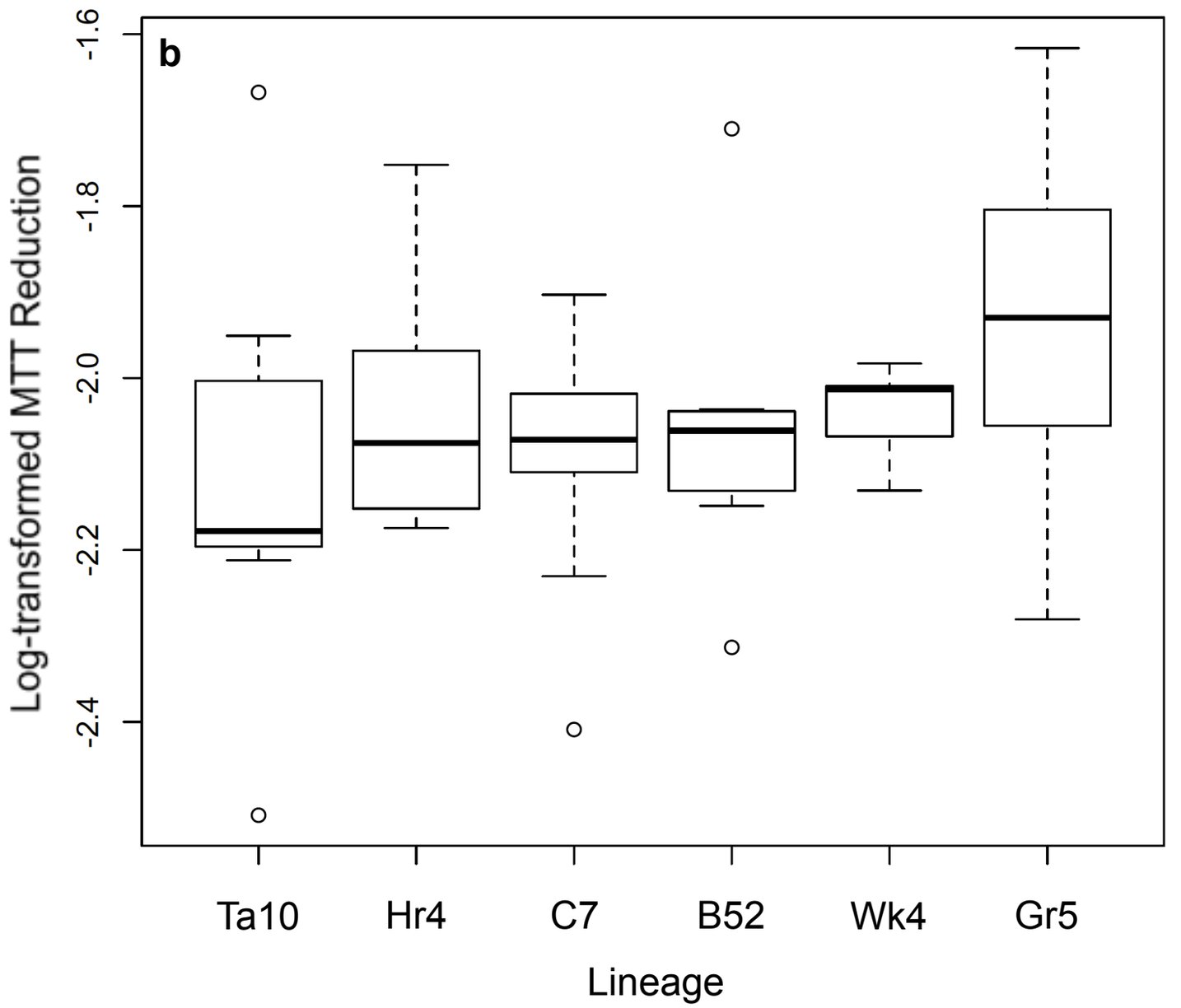
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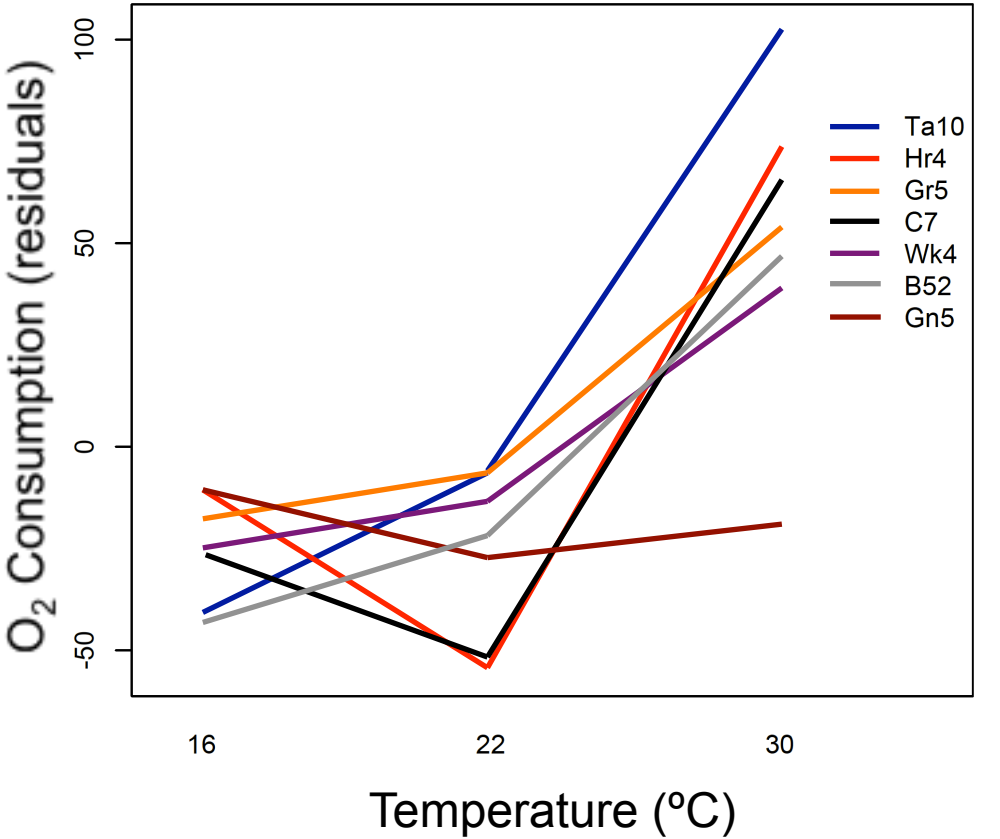
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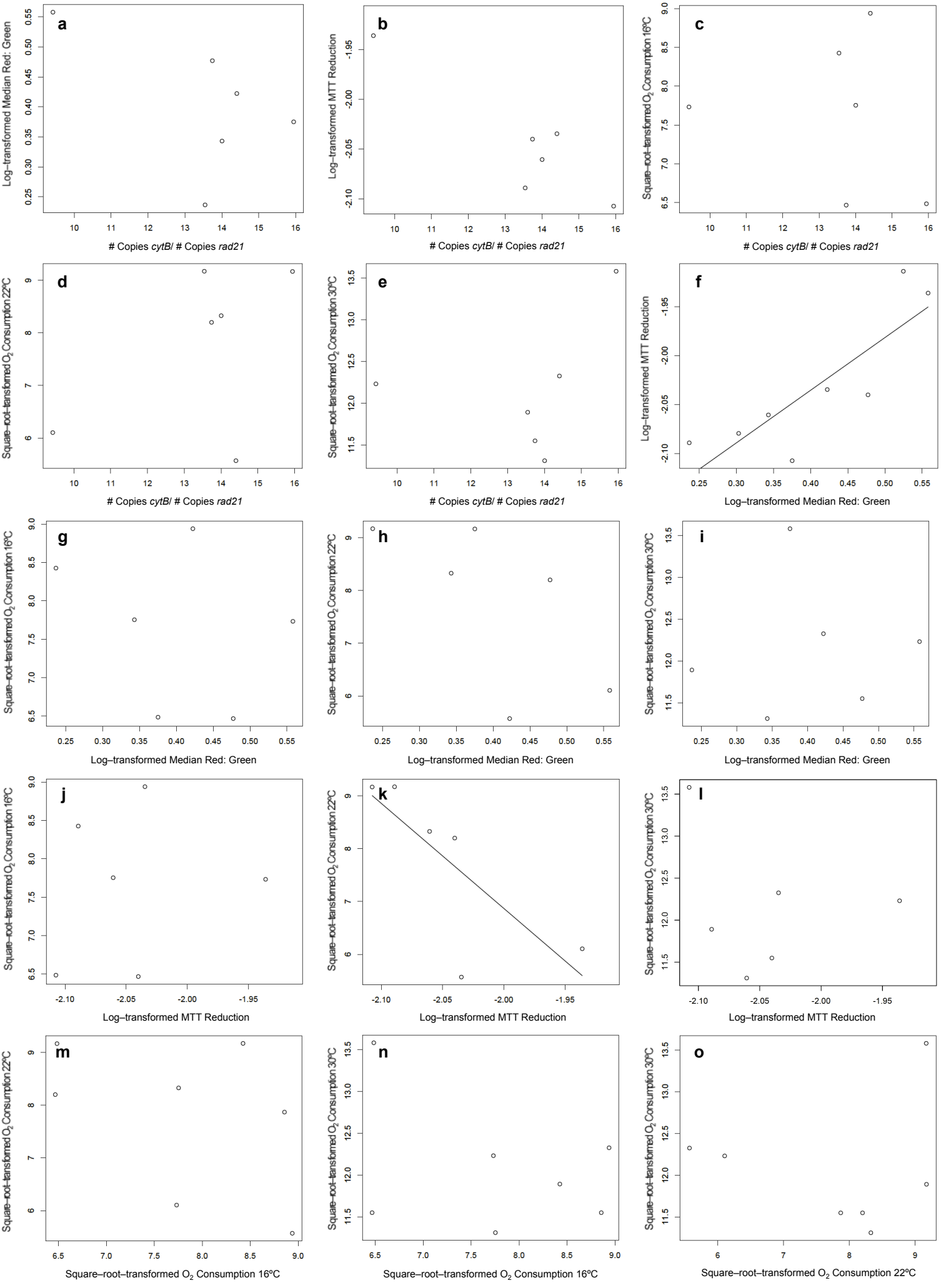
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