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 organisms provide a powerful means for addressing these challenges because offspring produced without recombination inherit both nuclear and mitochondrial genomes from a single parent. As such, these offspring inherit mitonuclear genotypes that are identical to the mitonuclear genotypes of their parents and siblings and different from those of other asexual lineages. Here, we compared mitochondrial function across distinct asexual lineages of *Potamopyrgus antipodarum*, a New Zealand freshwater snail model for understanding the evolutionary consequences of asexuality. Our analyses revealed substantial phenotypic variation across asexual lineages at three levels of biological organization: mitogenomic, organellar, and organismal. These data demonstrate that different asexual lineages have different mitochondrial function phenotypes and that there exists heritable variation (that is, the raw material for evolution) for mitochondrial function in *P. antipodarum*. The discovery of this variation combined with the methods developed here sets the stage to use *P. antipodarum* to study central evolutionary questions involving mitochondrial function, 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). Successful mitonuclear interaction is particularly important for proper enzyme function in OXPHOS complexes I, III, IV, and V because these complexes are composed 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), seed beetles (Dowling *et al.*, 2007), and salamanders (Lee-Yaw *et al.*, 2014).

In sexually reproducing organisms, the maintenance of mitonuclear compatibility is further complicated by the expectation that the different mechanisms of nuclear *vs.* mitochondrial genome (mtDNA) inheritance will differentially affect the generation, maintenance, and distribution of genetic variation. In particular, biparental inheritance and meiotic recombination in the nuclear genome should increase effective population size relative to the (typically) uniparentally inherited and non-recombinant mtDNA (reviewed in Barr *et al.*, 2005; Neiman and Taylor, 2009). This logic is the basis for the expectation that mtDNA 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 asexually inherited nuclear genomes means that asexual lineages will transmit their mtDNA in complete linkage disequilibrium (LD) with the nuclear genome. To date, inbred sexual lineages have been the primary genetic tool used to investigate genotype-phenotype connections relating to mitochondrial function (for example, Ellison and Burton, 2006; Montooth *et al*., 2010; Latorre-Pellicer *et al*., 2016). While these studies are powerful, the inferences that they generate are in part limited by the fact that inbreeding can introduce other off-target effects (for example, inbreeding depression, purging of harmful recessive mutations). Alternatively, full-factorial crossing of mtDNA onto various nuclear backgrounds (Willett and Burton, 2003; Dowling *et al*., 2007), perhaps represents the best of both worlds, but it is not tenable for species with long generation times. These challenges can be circumvented in asexual lineages, in which mitonuclear LD can be used as a relatively straightforward means of exploring genotype-phenotype connections in natural populations. This asexual-focused approach also has the substantial additional benefit of providing information relevant to understanding how sexual reproduction (and its absence) influences mitochondrial function.

Potamopyrgus antipodarum, a New Zealand freshwater snail, is very well suited for investigating mitochondrial function in the absence of sex because obligately sexual and obligately asexual individuals frequently coexist in natural populations (Lively 1987). Multiple separate transitions from sexual ancestor to asexual descendent have occurred within this species, providing many so-called “natural experiments” into the consequences of asexuality (Neiman *et*

96 *al.*, 2011; Paczesniak *et al.*, 2013). Asexuality in *P. antipodarum* appears to occur via apomictic
97 parthenogenesis (Phillips and Lambert, 1989), meaning that asexually produced offspring inherit
98 both their nuclear and mtDNA from a single parent and that the nuclear genome is transmitted
99 without recombination. The implications are that *P. antipodarum* individuals descended from the
100 same mother (what we term "asexual lineages") should share the same mitonuclear genotype,
101 barring *de novo* mutations.
102 Here, we use a common-garden approach, which isolates genetic (*vs.* environmental)
103 effects on phenotypic variation, to test whether distinct asexual lineages of *P. antipodarum* (our
104 proxy for mitonuclear genotype) vary in mitochondrial function at three distinct levels of
105 biological organization: (1) mitogenomic (mtDNA copy number), (2) organellar (mitochondrial
106 membrane potential and electron transport), and (3) organismal (total oxygen (O_2) consumption).
107 All three of these traits have been linked to mitochondrial performance in other taxa. In
108 particular, mtDNA copy number is thought to affect mitochondrial function (Van den Bogert *et*
109 *al.*, 1993; Taanman *et al.*, 1997; Moraes, 2001; Salminen *et al.*, 2017) and to be dynamically
110 regulated in response to various cellular environmental cues (Hori *et al.*, 2009; Matsushima *et al.*,
111 2010; Kelly *et al.*, 2012). This regulation is also thought to be tuned, at least in part, as a
112 response to the energy demands of a cell (Moraes, 2001). Elevated mtDNA copy number has
113 even been shown to compensate for deletions in mtDNA (Bai and Wong, 2005), but whether
114 copy number elevation represents a general compensatory mechanism for mitochondrial
115 mutation accumulation remains unclear (Montier *et al.*, 2009). Second, mitochondrial membrane
116 potential, generated by electron transport, determines the strength of the electrochemical gradient
117 mitochondria use to phosphorylate ADP to ATP (Nicholls, 2004). Variation in mitochondrial
118 membrane potential has been linked to cellular aging (Nicholls, 2004) and longevity (Callegari *et*

119 *al.*, 2011). The JC-1 assay measures the strength of the electrochemical gradient in mitochondrial
120 isolates using JC-1, a small positively charged molecule that fluoresces green when dispersed
121 and red when aggregated under ultraviolet (UV) illumination (Garner and Thomas, 1999).
122 Gradient strength can then be estimated from the ratio of red aggregate fluorescence to green
123 fluorescence of the dye monomer. Third, electron flow through the electron transport chain
124 (ETC) provides the energy necessary to establish a proton gradient (Brand and Murphy, 1987),
125 such that increased electron flow should produce a corresponding increase in mitochondrial
126 membrane potential. When isolated mitochondria are incubated with the compound MTT (3-
127 (4,5-dimethylthizol-2-yl) diphenyltetrazolium bromide), MTT accepts electrons from the ETC,
128 forming a purple formazan product, the quantity of which positively correlates with electron flow
129 (Liu *et al.*, 1997). Finally, because O_2 is the final electron acceptor for the production of ATP
130 (Chance and Williams, 1955), total O_2 consumption represents an integrated measure of the ATP
131 production capacity of an organism. Aquatic respirometry using a Clark-type electrode can
132 detect changes in O_2 concentrations over time, allowing us to evaluate the rate at which whole
133 organisms produce ATP. Because ectotherms respire at higher rates at elevated temperatures,
134 heat stress can reveal inefficiencies in ATP production (Heise *et al.*, 2003; Abele *et al.*, 2007),
135 allowing us to also use aquatic respirometry to compare ATP production in ambient *vs.* stress-
136 inducing temperatures.

137 We adapted these well-established mitochondrial functional assays, to date employed
138 exclusively in model organisms, to probe the number of mtDNA copies relative to the nuclear
139 genome, estimate the strength of the electrochemical gradient, quantify the flow of electrons
140 through the electron transport chain (ETC), and to quantify and compare total organismal O_2
141 consumption under heat stress across asexual *P. antipodarum* lineages. Together, these analyses

142 revealed substantial variation for mitochondrial function across distinct asexual lineages at all
143 three levels of biological organization. More broadly, the assays adapted here provide a suite of
144 useful experimental tools for investigating mitochondrial function in *P. antipodarum* and,
145 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. Asexual lineages were chosen for functional assays to represent the range of mitochondrial genetic diversity found in New Zealand populations (as demonstrated by Neiman and Lively, 2004; Neiman *et al.*, 2011; Paczesniak *et al.*, 2013) and to maximize our ability to compare across functional assays. Because there is notably high genetic diversity within asexual assemblages (Jokela *et al.*, 2003; Paczesniak *et al.*, 2013) and marked across-lake genetic structure (Neiman and Lively, 2004; Paczesniak *et al.*, 2013) in New Zealand *P. antipodarum*, and because nearly all of the lineages were from different lakes (Table S1), we can confidently 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). We did not determine ploidy level for field-collected snails, which is why we did not use these individuals in lineage-level comparisons of mitochondrial performance. Adult female snails were selected arbitrarily from lineage populations or field collections for each assay. Following standard laboratory protocols for *P. antipodarum* (for example, 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

To test whether asexual *P. antipodarum* exhibited across-lineage phenotypic variation for mitochondrial copy number, we used quantitative PCR (qPCR) to estimate mtDNA copy number relative to a putatively single-copy nuclear gene in six asexual triploid lineages. To identify a suitable nuclear-encoded gene to use as a control, we performed an all-by-all BLAST search of the transcriptomes available at http://bioweb.biology.uiowa.edu/neiman/blastsearch.php (Wilton *et al.*, 2013) to identify assembled transcripts that hit themselves and only themselves. We randomly selected 30 transcripts satisfying that criterion, designed primers, amplified sequences *via* PCR, and sequenced each transcript on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Because *rad21* was the most consistent performer in PCR amplification experiments, we used our sequencing information to design new internal primers designed to produce a 264-bp product (F: 5'–GATTCCAACAACTGATGTTTG –3', R: 5'–CAAAACTTACTCTAAATCTGC–3') for use as a nuclear genome standard in qPCR experiments. We then designed primers to produce a 194-bp amplicon from *cytB* (F: 5'–TATGAATATTCAGATTTTTTAAATA–3', R: 5'– CCTTAACTCCTAATCTTGGTAC–3'), our mitochondrial standard. 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 mtDNA copy numbers, total DNA was isolated from three to five individual snails from each of the six lineages using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA). We used this DNA to amplify nuclear and mitochondrial targets 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). We then used this information to determine the ratio of mitochondrial to haploid nuclear genome copies for each sample. Finally, we compared inferred mtDNA copy number across lineages using a one-way ANOVA and pairwise t-tests, ensuring that mtDNA copy numbers were normally distributed (Shapiro-Wilks $W = 0.96$, $p = 0.23$) and that variances between lineages were not significantly different (Levene's $F = 0.63$, $p = 0.68$). All statistical tests were performed in R v 3.2.4 (R Core Team, 2012), and all plots were produced using the *car* R package (Fox and Weisberg, 2011).

Mitochondrial function at the organellar level

Except where noted, all reagents were obtained from Sigma-Aldrich, St. Louis, MO.

JC-1 assay

To test for genetic variation in mitochondrial function in *P. antipodarum* in terms of mitochondrial membrane potential, we assayed mitochondrial membrane potentials using the JC-1 dye assay in 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). 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.

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 (Calbiochem, San Diego, CA), 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 subsample after gating out debris in FlowJo v 10.0.8 (FlowJo, Ashland, OR), and derived the ratio of red to green for each particle. Unstained subsamples showed very low background autofluorescence that did not vary across lineages. Stained subsamples uncoupled by CCCP had low red:green ratios representative of dissipated charge gradients, while subsamples without uncoupler contained an additional fraction

of mitochondrial particles with higher ratios, representing coupled mitochondria. We used one-way ANOVA with lineage as a random factor to compare the median red: green ratio of particles in this final fraction across lineages following log transformation of red: green fluorescence ratios (Shapiro-Wilks $W = 0.858$, $p = 7.131 \times 10^{-6}$ prior to log transformation; Shapiro-Wilks $W = 0.969$, $p = 0.15$ following log transformation). Our ANOVA approach required a White adjustment (MacKinnon and White, 1985) because ratios of red: green fluorescence exhibited unequal variances across lineages, (Levene's $F = 3.093$, $p = 0.016$).

MTT assay

We next tested whether there exists genetic variation for electron flux through the ETC in *P. antipodarum* by comparing MTT reduction across asexual lineages. We resuspended 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. We then incubated the plate for 2 hours at 37°C to allow electrons from the ETC to reduce MTT. Next, a 20% SDS 50% dimethylformamide solubilization solution was applied to each well and the plate was 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. We performed a log transformation of the raw MTT values (Shapiro-Wilks $W = 0.840$, $p = 2.436 \times 10^{-5}$) to meet the assumptions of normality (Shapiro-Wilks $W = 0.961$, $p = 0.140$) and test for unequal variances (Levene's $F = 0.964$, $p = 0.452$). We then compared 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.

Mitochondrial function at the organismal level

To test for variation in O_2 consumption in response to heat stress, we first needed to establish the range of heat stress likely to alter mitochondrial function in *P. antipodarum.* We accomplished this goal by using a behavioral assay that measures the amount of time that a snail takes to right itself when placed ventral side-up to compare 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.

After determining that snails do appear to be stressed by elevated temperatures (see Results, Figure S1), we tested whether there exists genetic variation for O_2 consumption under

heat stress by performing closed-system aquatic respirometry on seven asexual lineages of *P. antipodarum* (N = 10 per lineage) at the same three incubation temperatures used for the righting assay (16ºC, 22ºC, 30ºC) with a Strathkelvin Instruments RC200a respiration chamber, a 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). After correcting for snail wet mass, 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 mass-corrected O_2 consumption.

Comparison of mitochondrial functional assays

Our mitochondrial function assays were aimed at different elements of mitochondrial performance and different levels of biological organization; whether and to what extent these assays are measuring related *vs*. orthogonal determinants of mitochondrial function remains unclear, especially as newly applied to *P. antipodarum*. We addressed this question by

305 performing all 15 possible pairwise comparisons of mitochondrial functional assays (that is,
306 mtDNA copy number, mitochondrial membrane potential, electron flux, O_2 consumption at 16ºC,
307 O_2 consumption at 22ºC, and O_2 consumption at 30ºC), comparing the mean trait value per
308 lineage across assays using Spearman's rank correlation (as implemented by the *Hmisc* R
309 package [Harrell, 2008]) and correcting for multiple comparisons using the Holm procedure
310 (Holm 1979). For these analyses, we included two additional sample populations that were not
311 used in any of the tests for across-lineage variation: one field-collected sample from a lake with a
312 high frequency of sexual individuals ("KnSF12") and an inbred diploid sexual lineage that has
313 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 13.72 (+/- 3.57), though we also found significant differences in this ratio across asexual lineages (one-way ANOVA, $F_{5, 25} = 2.72$, $p = 0.043$, Figure 1), indicating that lineages differ in mtDNA copy number. We performed pairwise t-tests of mtDNA copy number ratio among each pair of lineages, which revealed that a single lineage (Gr5; mean copy number ratio [+/- SD] = 9.42 [+/- 1.48]) with very low copy number appeared to be driving the significant among-lineage variance. This conclusion is supported by the fact that when Gr5 is excluded, other asexual lineages exhibit no differences in copy number (one-way ANOVA, $F_{4, 21} = 0.55$, $p = 0.70$).

Mitochondrial function at the organellar level

JC-1

We compared membrane potential among mitochondria isolated from six asexual lineages of *P. antipodarum* using the JC-1 assay, in which the ratio of red to green fluorescence indicates the relative strength of the proton gradient. Our comparisons revealed significant differences in 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). We next performed *posthoc* pairwise comparisons between lineages using Welch's t-tests (to reflect unequal variances) and the Holm procedure for

337 Bonferroni correction (Holm 1979), which revealed three significantly different groups ($p < 0.05$
338 after correcting for multiple comparisons) amongst the six lineages (Figure 2a). Unlike mtDNA
339 copy number, this variation in mitochondrial membrane potential does not appear to be driven by
340 a single lineage. Because CCCP-uncoupled samples did not vary across lineages (one-way
341 ANOVA, $F_{5, 52} = 1.37$, $p = 0.25$), the variation observed for red: green fluorescence across
342 lineages likely reflects true variation in mitochondrial membrane potential. In particular, these
343 data suggest that under the current rearing and assay conditions, the Gr5 lineage appears to have
344 the strongest mitochondrial membrane potential (mean [+/- SD] = 3.86 [+/- 1.55]) and the DenA
345 lineage (mean [+/- SD] = 1.78 [+/- 0.47]) appears to have the weakest mitochondrial membrane
346 potential.
347
348 *MTT*
349 We next compared electron flux through the ETC using the colorimetry-based MTT assay. We
350 did not detect any differences amongst asexual lineages in MTT reduction (one-way ANOVA, $F_{5,}$
351 $_{38} = 0.75$, $p = 0.59$, Figure 2b) despite relatively high statistical power for this comparison (= 0.92,
352 as estimated by the *pwr* package in R [Champely, 2012]). This result therefore indicates that *P.*
353 *antipodarum* lacks substantial genetic variation for electron flux though the ETC, at least under
354 the benign environmental conditions in which we performed this assay.
355
356 **Mitochondrial function at the organismal level**
357 Organismal O_2 consumption under stressful conditions is expected to reflect electron acceptor
358 turnover under maximal ATP production (Abele *et al.*, 2007), which is the logical basis for our
359 application of heat stress to detect genetic variation in O_2 consumption. We began by comparing

360 righting time across ambient (16ºC) and elevated (22ºC, 30ºC) temperatures and lineages with a
361 Kruskal-Wallis test, and found that both factors significantly affected righting time (temperature:
362 $\chi^2 = 14.218$, df = 2, $p = 0.00082$; lineage: $\chi^2 = 122.64$, df = 12, $p < 2.2 \times 10^{-16}$). *Post-hoc* Mann-
363 Whitney U tests revealed that righting took ~ 37% longer at 30ºC (mean [+/- SD] = 83.02 [+/-
364 73.74] seconds) than at 16ºC (Mann-Whitney U = 8192, $p = 0.025$) and ~85% longer at 30ºC
365 than at 22ºC (Mann-Whitney, $U = 5996.5$, $p = 0.00014$, Figure S1). While righting time was
366 ~24% faster at 22ºC (mean [+/- SD] = 48.82 [+/- 56.09] seconds) than at 16ºC (mean [+/- SD] =
367 62.50 [+/- 63.26] seconds), this difference was not significant (Mann-Whitney, $U = 9244.5$, $p =$
368 0.075). These results are consistent with other righting time assays from other snail species in
369 that some amount of stress decreases righting time (for example, presence of predators, Orr *et al.*,
370 2007), and some stimuli increase righting time (for example, hypoxia, Fei *et al.*, 2008),
371 suggesting that our observed responses indicate stress in *P. antipodarum* exposed to elevated
372 temperature (here, 30ºC).

373 To test for genetic variation in O_2 consumption in heat-stressed *P. antipodarum*, we
374 performed closed-system aquatic respirometry for seven asexual lineages (N = 10 per lineage) at
375 16ºC, 22ºC, and 30ºC. Snail wet mass was significantly and positively correlated with O_2
376 consumption (Spearman's rho = 0.19, $p = 0.0086$, Figure S2). Because there is significant
377 variation for snail wet mass across asexual lineages (Kruskal-Wallis, $\chi^2 = 75.09$, df = 6, $p <$
378 0.00010), we calculated the residuals of wet mass *vs.* O_2 consumption using a linear model. Cube
379 root-transformed, mass-corrected O_2 consumption residuals were not significantly different from
380 a normal distribution (Shapiro-Wilks $W = 0.99$, $p = 0.060$), allowing us to implement a linear
381 regression model to test whether temperature and/or lineage affected O_2 consumption. We found
382 that elevated temperatures significantly affected mass-corrected O_2 consumption (two-way

ANOVA, $F_{2, 175} = 46.22$, $p = 2.2 \times 10^{-16}$) and that there was a significant interaction between lineage and temperature (Figure 3, two-way ANOVA, $F_{6, 175} = 3.40$, $p = 1.7 \times 10^{-4}$). We also found a trend towards an effect of lineage on mass-corrected O_2 consumption (two-way ANOVA, $F_{6, 175} = 2.12$, $p = 0.053$). A series of one-way ANOVAs within each temperature treatment revealed that lineage had a significant effect on O_2 consumption at 22ºC (one-way ANOVA, $F_{6, 54} = 2.83$, $p = 0.018$) and at 30ºC (one-way ANOVA, $F_{6, 62} = 3.85$, $p = 0.0025$), but not at 16ºC (one-way ANOVA, $F_{6, 59} = 2.10$, $p = 0.067$). 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 (Figure 3). This result demonstrates that genetically distinct lineages of *P. antipodarum* consume different amounts of O_2 in response to elevated temperature (22ºC) and heat stress (30ºC).

Comparison of mitochondrial functional assays

To determine whether and to what extent 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 mtDNA copy number, log-transformed JC-1 red: green ratios, log-transformed MTT values, and cube root-transformed, mass-corrected O_2 consumption residuals at each of our three study temperatures. None of the correlations remained significant after the Holm correction for multiple comparisons (Figure 4). 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 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 potential trends that deserve

406 further scrutiny. First, mtDNA copy number appears to be positively correlated with O_2
407 consumption at 30ºC among the six asexual lineages assayed (Spearman's rho = 0.94, p = 0.0048,
408 Figure 4e). Second, while the MTT assay required pooling of mitochondria from 3-4 individual
409 snails per measurement and is thus somewhat less sensitive than the JC-1 assay, which only
410 requires mitochondria from one snail, we did find a trend towards a positive correlation between
411 electron flux and mitochondrial membrane potential in the six asexual and two sexual *P.*
412 *antipodarum* lineages examined (Spearman's rho = 0.81, p = 0.015, Figure 4f). Third, there
413 electron flux appeared to be negatively correlated with O_2 consumption at 22ºC for the six
414 asexual lineages (Spearman's rho = -0.89, p = 0.019, Figure 4k). These tentative relationships
415 between mtDNA, electron flux through the ETC, mitochondrial membrane potential, and
416 organismal O_2 consumption suggest that 1) the methods we employed here assay distinct yet
417 associated mitochondrial phenotypes, and 2), evaluating mitochondrial performance at multiple
418 levels of biological organization is necessary to describe adequately the phenotypic variation for
419 mitochondrial function in *P. antipodarum*.

DISCUSSION

Genetic variation for mitochondrial function in asexual lineages

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, 1) mtDNA copy number, 2) mitochondrial membrane potential, and 3) variation in O_2 consumption in response to heat stress. These results demonstrate that substantial variation for mitochondrial function exists across asexual lineages of this species.

While the extent to which the variation in mitochondrial function described here contributes to fitness in *P. antipodarum* remains to be directly evaluated, the close link between mitochondrial function and fitness in other organisms (Chen *et al.*, 2007; Dowling, 2014) suggests that phenotypic variation across mitonuclear genotypes could have major implications for asexual lineage success. In particular, asexual *P. antipodarum* are known to harbor high mtDNA mutational loads relative to sexual lineages (Neiman *et al.*, 2010; Sharbrough *et al.*, 2016), and heat stress response can affect fecundity (Dybdahl and Kane, 2005) and respiration rates (Hudson, 1983, present study) in *P. antipodarum*. These findings suggest that variation in mitochondrial function might very well confer fitness consequences, especially amongst asexual lineages that are experiencing stressful conditions.

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 (Neiman *et al.*, 2009, 2013; Kistner and Dybdahl, 2013) diversity. The multiple separate transitions to asexuality in *P. antipodarum* (Neiman *et al.*, 2011; Paczesniak *et*

443 *al.*, 2013) may help explain the substantial levels of variation found here, as asexual lineages
444 represent "snapshots" of local sexual population diversity (Dybdahl and Lively, 1995; Jokela *et*
445 *al.*, 1997).

446 The mitochondrial phenotypes we observed in one lineage, Gr5, were particularly
447 distinct: snails from this lineage exhibited relatively low mitochondrial copy number and high
448 mitochondrial membrane potential and electron flow, as well as relatively low O_2 consumption at
449 22ºC. Together, these phenotypic values suggest that the Gr5 lineage exhibits relatively high
450 mitochondrial function, indicating that genetic dissection of its mitochondrial haplotype may
451 prove illuminating. Further comparisons with other *P. antipodarum* lineages and in other
452 conditions will provide substantial insight into the relative fitness of this particular mitonuclear
453 combination. Future studies should also focus on a particular mitochondrial haplotype that
454 appears to be especially common amongst asexual *P. antipodarum* (Neiman *et al.*, 2011;
455 Paczesniak *et al.*, 2013). Paczesniak *et al.* (2013) showed that this haplotype is often found in
456 divergent nuclear backgrounds, consistent with a scenario in which this haplotype is spreading
457 into new populations and lineages. Evaluating mitochondrial function of the common
458 mitochondrial haplotype against a variety of nuclear backgrounds and in various biologically
459 relevant conditions would shed light on intraspecific mitonuclear coevolution and whether
460 asexuality contributes to decreased mitochondrial function in *P. antipodarum*, as the mutational
461 hypotheses for sex would predict.
462
463 **Relationships among mitochondrial functional assays**
464 All else being equal, stronger mitochondrial membrane potentials and greater electron flow
465 should indicate relatively high mitochondrial performance. The relationships between whole-

organismal O_2 consumption and mitochondrial performance or between mtDNA copy number and mitochondrial performance are expected to be more complex and to reflect compensatory mechanisms at the cellular and/or organismal level. Because other factors (for example, environmental conditions, local adaptation) are virtually certain to influence mitochondrial function, it is also 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 with 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 tentative relationships between mtDNA copy number, electron flux through the ETC, mitochondrial membrane potential, and organismal O_2 consumption (Figure 4). The observation that lineages with high mtDNA copy numbers consume more O_2 at elevated temperatures than lineages with lower mtDNA copy numbers may reflect saturation of OXPHOS pathways during heat stress in lineages with low mtDNA copy numbers. Compensation for reduced mitochondrial function by increasing mtDNA copy number has been observed in human tissues carrying a variety of small deletion mutations (Bai and Wong, 2005), although the relationship between mtDNA copy number and respiratory capacity remains complex (Moraes, 2001; Montier e*t al*., 2009). The positive relationship between electron flux and mitochondrial membrane potential is not particularly surprising in light of the fact that as electrons pass through the ETC, a corresponding increase in mitochondrial membrane potential is expected as H+ ions are released into the intermembrane space (Chen, 1988). The association between relatively low electron flux and high O_2 consumption is more difficult to understand because O_2 consumption is expected to increase with electron flux (Jastroch *et al*., 2010). In

489 general, relationships between organismal-level O_2 consumption and organelle-level electron
490 flux are difficult to disentangle because many layers of respiratory regulation can contribute to
491 increased organismal O_2 consumption beyond ETC inefficiencies (Brand and Nicholls, 2011). A
492 particularly relevant example is provided by combined observations that electrons leaking back
493 across the inner membrane through uncoupling proteins contribute to electron flux but not O_2
494 consumption (reviewed in Jastroch *et al*., 2010) and that elevated temperatures in a marine
495 mollusk have been shown to increase electron leakage of this type (Abele *et al*., 2002), such that
496 connecting organismal O_2 consumption to organellar electron flux is a non-trivial exercise.
497 Understanding the relationship between organellar and organismal variation in mitochondrial
498 function will provide helpful context for interpreting functional variation in *P. antipodarum*, and
499 future efforts towards examining these relationships in more detail are necessary. More broadly,
500 the methods described here can be easily adapted to other non-model organisms, especially
501 mollusks, providing a new means of quantifying the genotype-phenotype relationships of
502 mitonuclear interactions.

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 qPCR estimates (rank ordered by median) of *cytB* copy number relative to a putatively single-copy nuclear gene, *rad21*. Boxes represent Inner Quartile Ranges (IQR), with error bars extending 1.5x beyond IQRs. Data points falling outside whiskers 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 IQRs; 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 depicting relationship 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.

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, $p = 0.82$). 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 = -0.14, $p = 0.76$). d) mtDNA copy number vs. O_2 consumption at 22ºC (Spearman's rho = 0.2, $p = 0.70$). e) mtDNA copy number vs. O_2 consumption at 30ºC (Spearman's rho = 0.94, $p = 0.0048$). f) JC-1 vs. MTT reduction (Spearman's rho = 0.81, $p = 0.015$). g) JC-1 vs. O_2 consumption at 16ºC (Spearman's rho = 0.0, $p = 1.0$). h) JC-1 vs. O_2 consumption at 22ºC (Spearman's rho = -0.71, $p = 0.11$). i) JC-1 assay vs. O_2 consumption at 30ºC (Spearman's rho = -0.09, $p = 0.87$). j) MTT reduction vs. O_2 consumption at 16ºC (Spearman's rho = 0.5, $p = 0.25$). k) MTT reduction vs. O_2 consumption at 22ºC (Spearman's rho = -0.89, $p = 0.019$). l) MTT reduction vs. O_2 consumption at 30ºC (Spearman's rho = -0.43, $p = 0.40$). m) O_2 consumption at 16ºC vs. O_2 consumption at 22ºC (Spearman's rho = -0.39, $p = 0.38$). 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 = 0.32, $p = 0.48$).

SUPPLEMENTARY MATERIAL TITLES TO FIGURES AND LEGENDS

Figure S1. Interaction plot depicting relationship between temperature, lineage, and righting time in *P. antipodarum*. Lines represent best-fit lines between righting times for a given lineage across 16ºC – 22ºC and 22ºC – 30ºC. Red lines represent asexual lineages; blue lines represent sexual lineages; black lines represent "mixed" field-collected populations. Snails righted themselves significantly more slowly at 30ºC than 16ºC (Mann-Whitney U = 8192, p = 0.025) and 22ºC (Mann-Whitney, U = 5996.5, p = 0.00014), indicating that 30ºC water temperatures represent a significant source of heat stress.

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 = 0.19, p = 0.0086).

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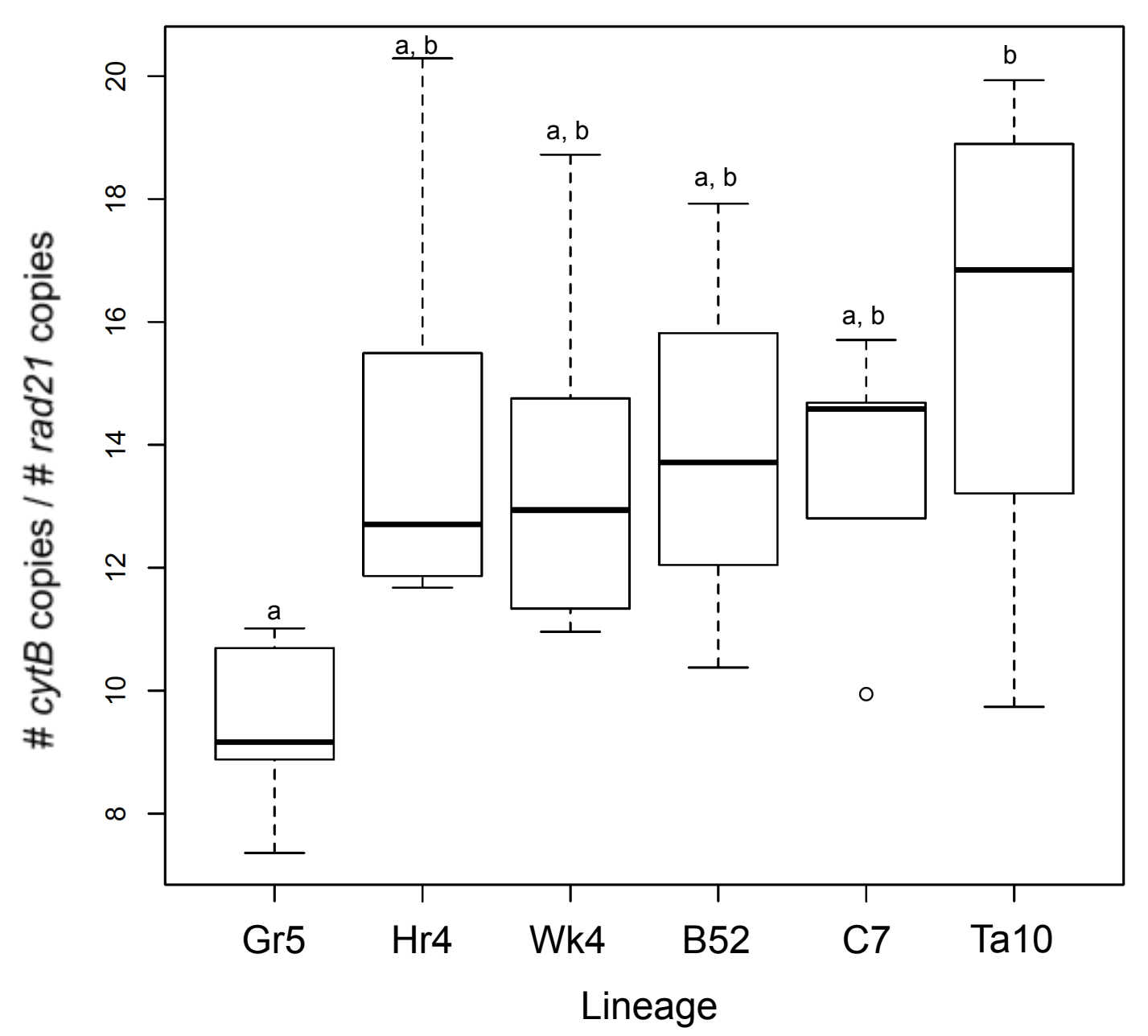
cytB copies / # rad21 copies
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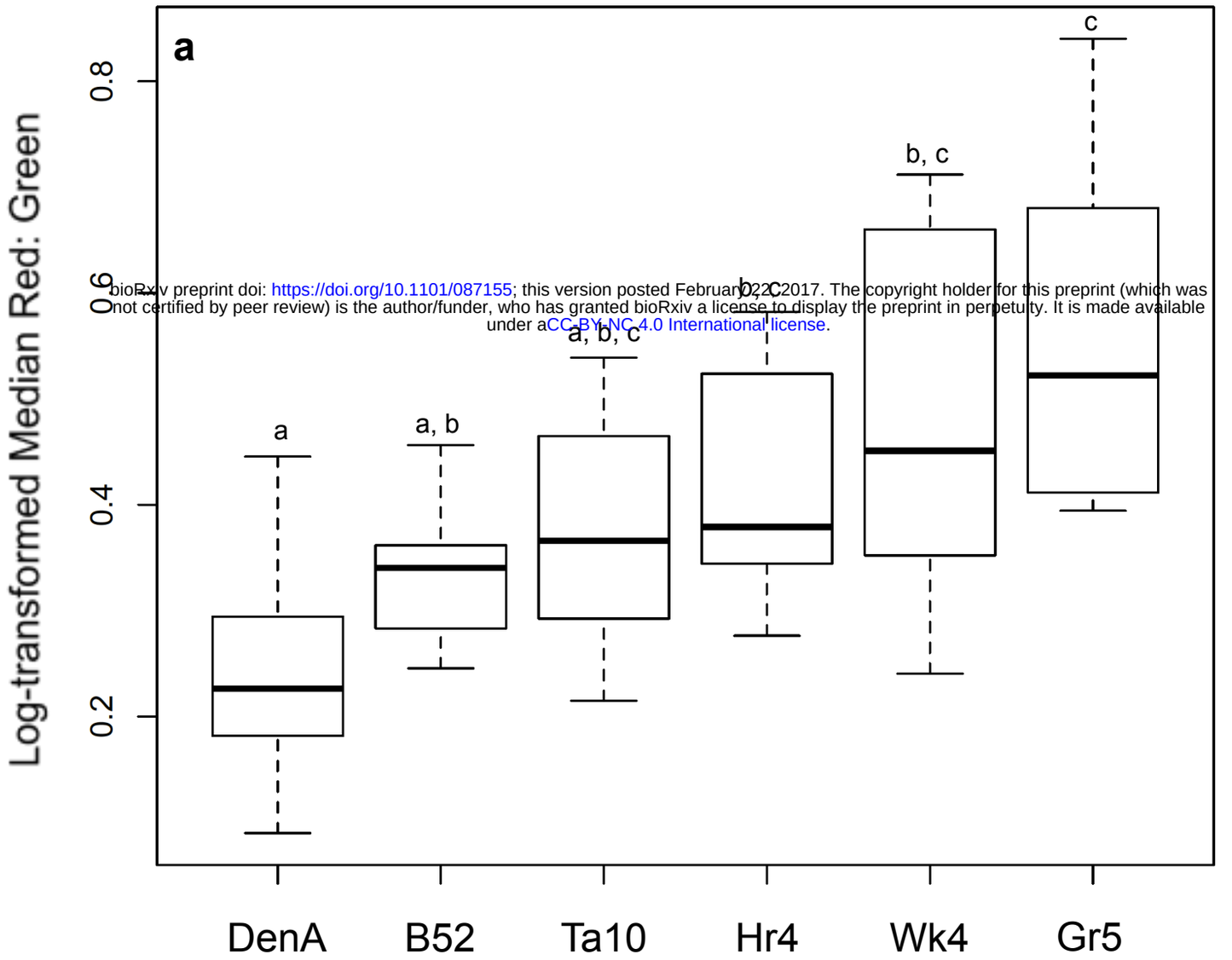

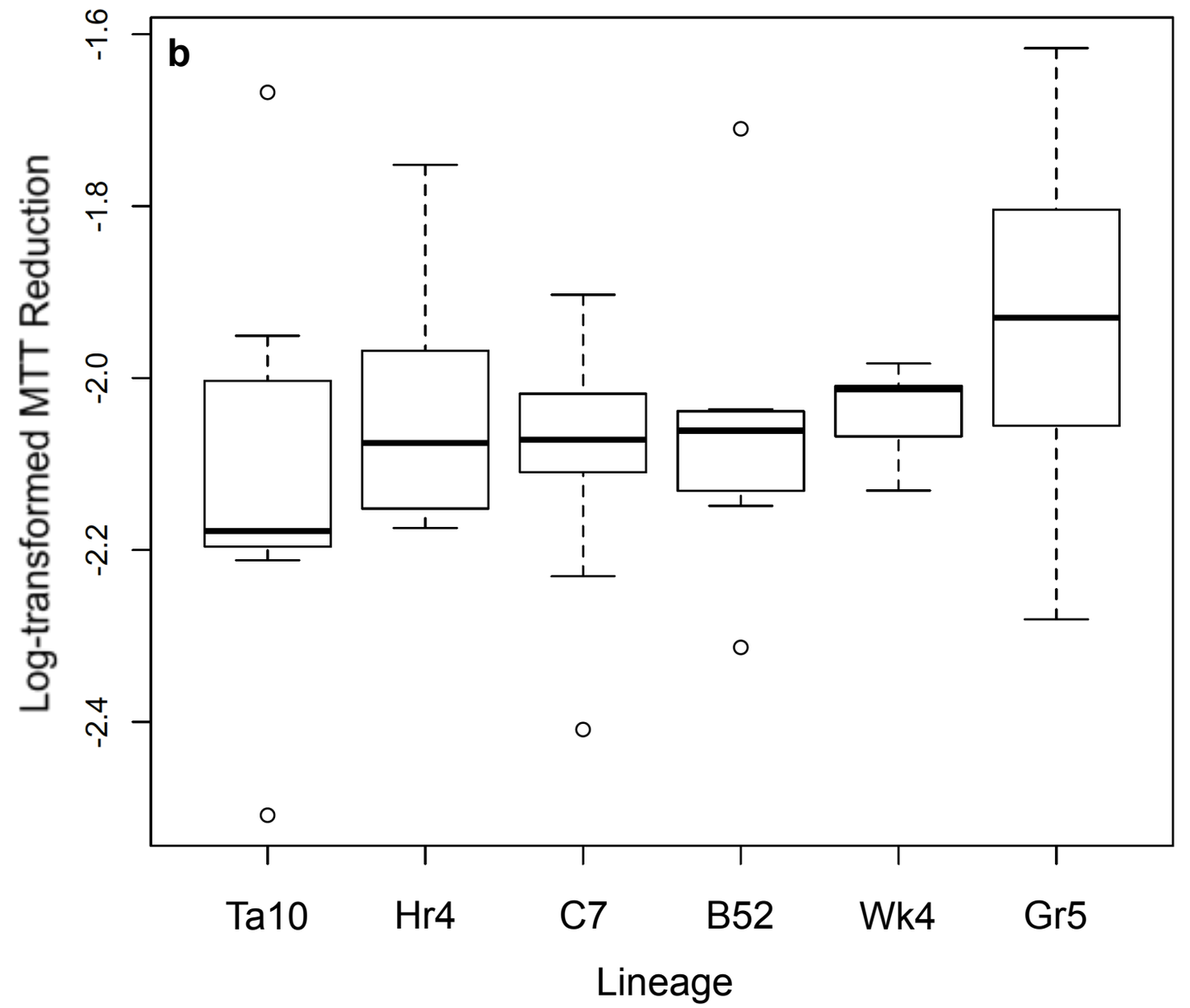

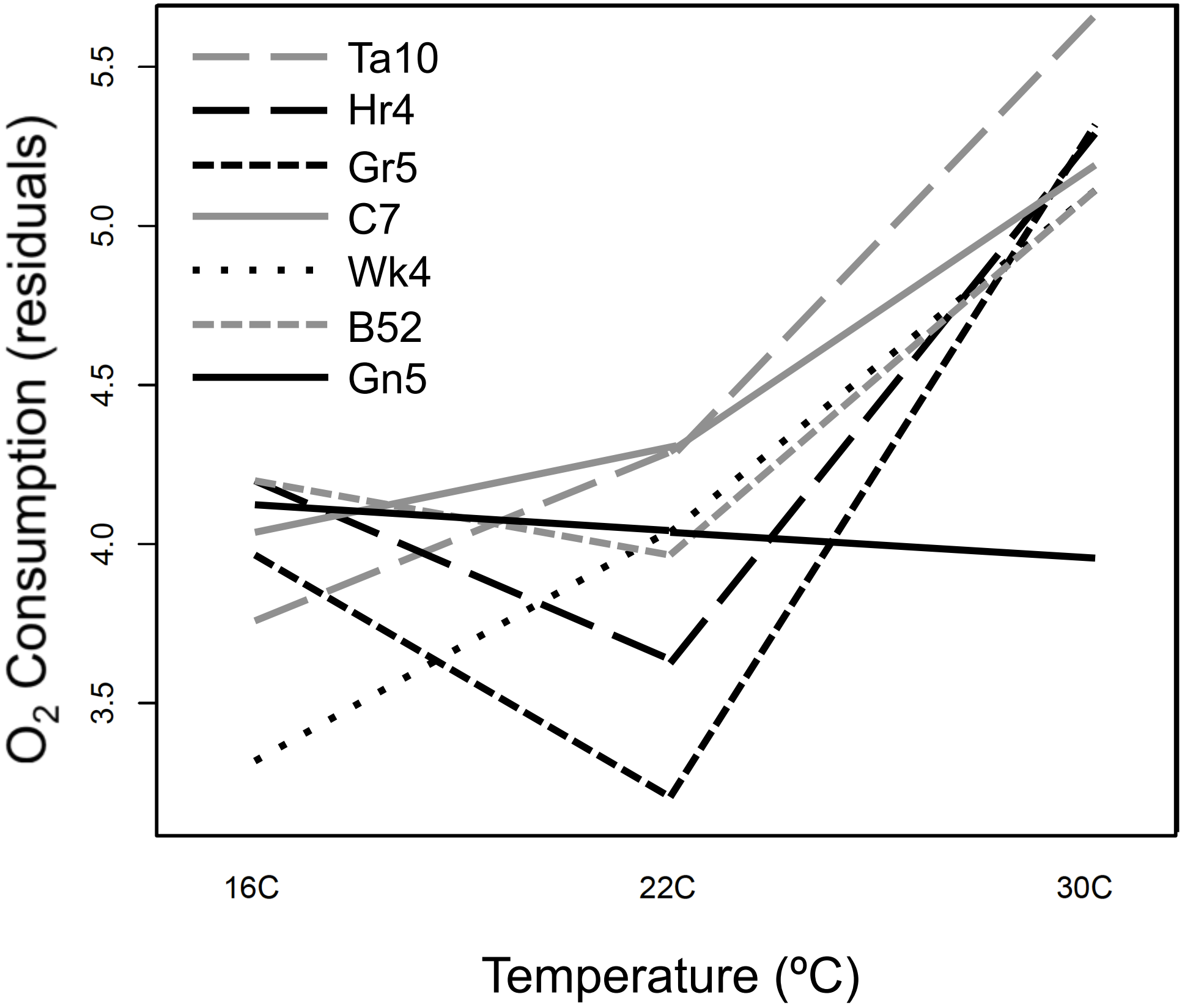
Ta10
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O_2 Consumption (residuals)
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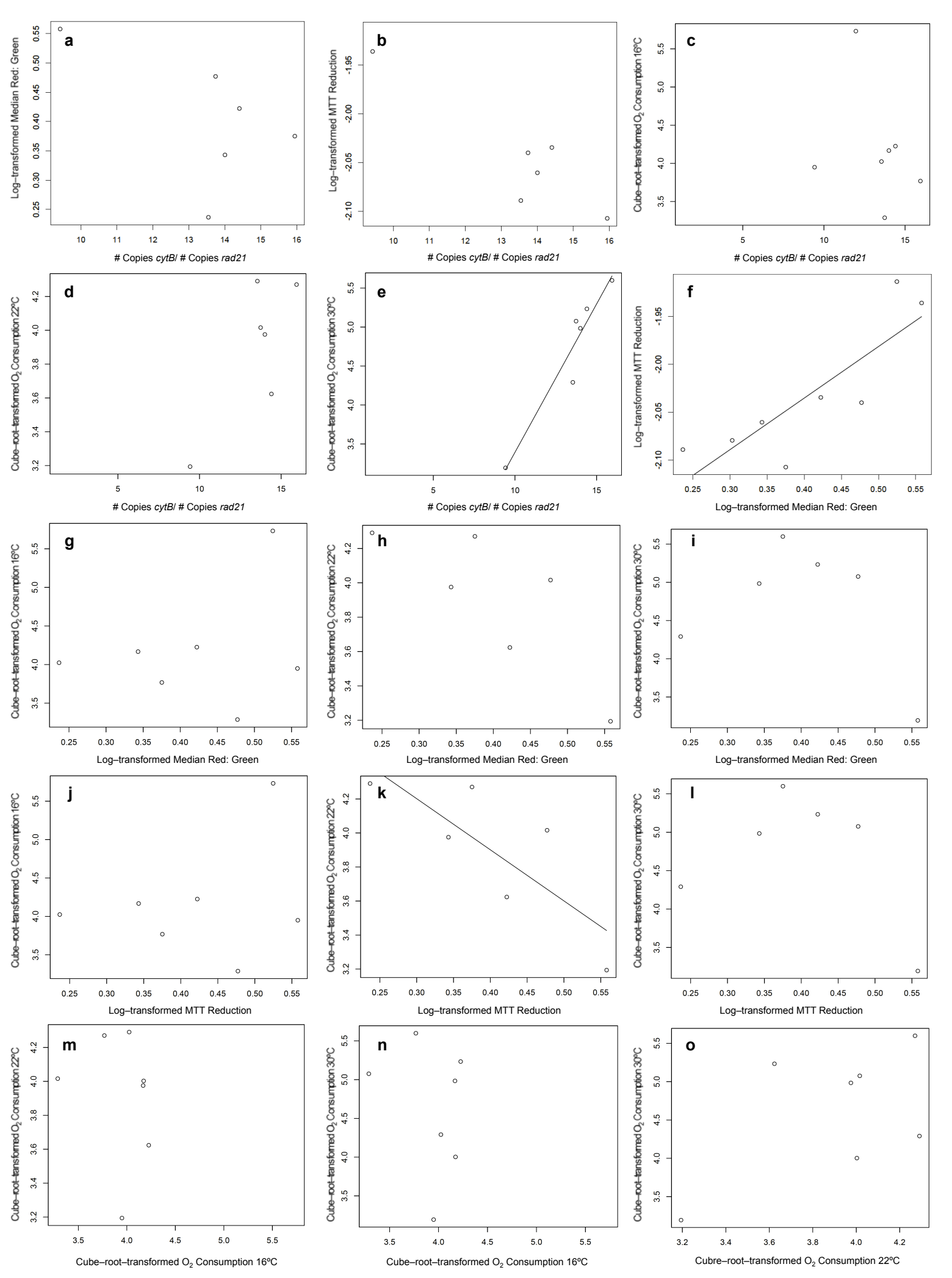
a
Log–transformed Median Red: Green
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Log–transformed MTT Reduction
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Cube–root–transformed O2 Consumption 16ºC
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Log–transformed MTT Reduction
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g
Cube–root–transformed O2 Consumption 16ºC
Log–transformed Median Red: Green
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Cube–root–transformed O2 Consumption 22ºC
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