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Elevated oxidative damage is correlated with reduced fitness in interpopulation hybrids of a marine copepod

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Abstract

Aerobic energy production occurs via the oxidative phosphorylation pathway (OXPHOS), which is critically dependent on interactions between the 13 mitochondrial DNA (mtDNA)-encoded and ~70 nuclear-encoded protein subunits. Disruptive mutations in any component of OXPHOS can result in impaired ATP production and exacerbated oxidative stress; in mammalian systems, such mutations are associated with ageing as well as numerous diseases. Recent studies have suggested that oxidative stress plays a role in fitness trade-offs in life-history evolution and functional ecology. Here, we show that outcrossing between populations with divergent mitochondrial DNA can exacerbate cellular oxidative stress in hybrid offspring. In the copepod *Tigriopus californicus*, we found that hybrids that showed evidence of fitness breakdown (low fecundity) also exhibited elevated levels of oxidative damage to DNA, while those with no clear breakdown did not show significantly elevated damage. The extent of oxidative stress in hybrids appears to be dependent on the degree of genetic divergence between their respective parental populations, but this pattern needs further testing using multiple crosses at different levels of divergence. Given previous evidence in *T. californicus* that hybridization disrupts nuclear/mitochondrial interactions and reduces hybrid fitness, our results suggest that such negative intergenomic epistasis may also increase the production of damaging cellular oxidants; consequently, mtDNA evolution may play a significant role in generating postzygotic isolating barriers among diverging populations.

1. Introduction

Hybrid breakdown is a pattern of postzygotic isolation that occurs during the early stages of allopatric divergence, and it is characterized by markedly reduced fitness in F_2 and later generation hybrids [1]. Hybrid breakdown has been observed in a wide array of phenotypes, including fecundity [2], sperm swimming speed [3], offspring viability [4,5], growth rate [6], and stress response [7]. The genes involved in the early stages of reproductive isolation are likely to be found in the cellular and biochemical pathways underlying these phenotypes.

Hybrid breakdown is often explained by the Dobzhansky–Muller (DM) model; evolution results in coadaptation among interacting sets of alleles within diverging isolated populations, but incompatibilities are revealed in recombinant F_2 genomes of interpopulation hybrids [8,9]. Although most investigations of DM incompatibilities have focused on interactions among nuclear genes [10], epistasis between nuclear and mitochondrial genomes may be particularly relevant in hybrid breakdown because of the organelle's central role in metabolism [11]; small disruptions in mitochondrial function are likely to be reflected across several phenotypes [2,7,12,13]. Traits such as fertility and longevity, for instance, may be affected by certain combinations of mitochondrial haplotype and nuclear background, even at the intraspecific level [14,15]. Furthermore, systems in which mitochondrial DNA (mtDNA) evolves rapidly, such as most animals and yeasts, may be predisposed to intergenomic DM incompatibilities [16,17].

Mitochondrial oxidative phosphorylation (OXPHOS) relies on efficient interactions between nuclear- and mtDNA-encoded proteins, making it a candidate pathway for exhibiting mitonuclear incompatibilities. The intertidal copepod *Tigriopus californicus* has served as an excellent model in which to examine mitonuclear epistasis and hybrid breakdown [2, 18-22]. Geographically isolated populations exhibit very high levels of mtDNA divergence (>18%) [18],

and interpopulation hybrids are viable but frequently exhibit strong fitness breakdown in life-history traits [2,19]. Such low fitness is consistently associated with mitonuclear incompatibilities exposed by hybrid combinations of nuclear and mitochondrial genomes [7,20]. Moreover, recent experiments revealed that *T. californicus* hybrids often suffer from impaired OXPHOS enzyme activities and lowered ATP production capacity [21,22]. These patterns are concordant with results of mitochondrial dysfunction in other hybrid systems (*Nasonia*: [23]; *Saccharomyces*: [24]).

In addition to ATP, the OXPHOS pathway results in the production of reactive oxygen species (ROS). During aerobic respiration, some of the electron-accepting oxygen molecules may remain only partially reduced and form ROS [25]. Controlled ROS leak is an inevitable by-product of OXPHOS activity, but most of the oxidants are quenched by the antioxidant system in healthy individuals [26]. However, a significant imbalance between ROS production and antioxidant capacity may result in cellular oxidative stress [26]. Mutations in proteins of OXPHOS, for instance, have been shown to disturb electron flow and cause oxidative stress [27,28]. Oxidative stress has long been associated with ageing as well as with several mammalian diseases [29], and it has recently received attention from ecologists as a central mechanism driving life-history trade-offs [30-32], providing a link between organismal health and reproductive output [33,34]. Despite its potentially widespread fitness consequences, oxidative stress has been largely ignored as a possible outcome of hybrid incompatibilities.

Here, we present experimental evidence that outcrossing between divergent populations may lead to increased oxidative damage, suggesting this cellular process may be relevant in the establishment of postzygotic isolating barriers. After generating recombinant inbred lines among *T. californicus* populations, we screened lines for evidence of fitness breakdown and quantified

oxidative stress associated with each fitness level, also accounting for genetic divergence among parental populations. Considering the negative epistatic interactions in OXPHOS observed in *T. californicus* [19], we hypothesized that outcrossing between populations with highly divergent mitochondrial genomes may exacerbate ROS leak and oxidative stress in the same way *de novo* deleterious mutations do. Accordingly, we address two questions: 1) Is fitness breakdown across hybrid lines associated with levels of oxidative stress? and 2) Is the level of oxidative stress across hybrid lines related to the degree of divergence between the parental lineages?

2. Materials and Methods

(a) Generation of experimental lines

In order to generate recombinant genomes with different levels of fitness, we crossed populations exhibiting different levels of divergence. *Tigriopus californicus* samples were collected from five sites along the California coast: Ocean Beach, San Diego (henceforth SD: 32°45' N, 117°15' W), Bird Rock, La Jolla (BR: 32°48' N, 117°16' W), Abalone Cove, Los Angeles (AB: 33°44' N, 118°22' W), Santa Cruz (SC: 36°57' N; 122°03' W), and Bodega Marine Laboratory, Bodega Bay (BB: 38° 19' N, 123° 04'W). Animals were kept in large stock cultures in 400-ml beakers (~500-1000 individuals/beaker) containing 300 ml of filtered seawater. All stock and experimental cultures were maintained in 20°C with a 12-hour light:dark cycle, and were fed ground dried *Spirulina* wafers.

Hybrids were generated between two genetically similar populations (SD x BR) and multiple genetically divergent populations (SD or BR x AB, SC, or BB); divergence estimates were based on sequences of a mitochondrial gene (cytochrome *b*) and a nuclear-encoded

mitochondrial-targeting gene (mitochondrial RNA polymerase; electronic supplementary material and Table 1). Experimental crosses were performed in petri dishes and kept in the same conditions as stock cultures. *Tigriopus californicus* females mate only once. Mature males clasp and guard virgin females until they reach their terminal molt (i.e. when they are reproductively mature), at which time the females are inseminated [21]. Mature males and virgin females from each population were obtained by teasing apart clasped pairs with fine needles. Separated individuals were then used to start intrapopulation parental control crosses (e.g. SD x SD) and interpopulation hybrid crosses (e.g. SD♀ x BR♂, and reciprocal). Crosses were initiated with four replicate dishes, each comprising 20-25 pairs. At each generation from F_1 through F_3, clasped pairs were separated and individuals mated with those from replicate dishes at the same stage in order to prevent inbreeding during the first two generations of recombination. As gravid F_3 females appeared, they were isolated in individual dishes and each isofemale line was then propagated through full-sib matings for five generations, with new generations being transferred to fresh culture dishes. A total of 360 hybrid (36 of each cross) and 120 parental (24 of each) isofemale lines were initiated at the F_3 generation, and both hybrid and parental sets of crosses were inbred in the same manner. Once each line reached the F_9 generation, we made no more efforts to maintain discrete generations, allowing the number of individuals to increase. Density of individuals was maintained similar across lines by transferring highly fecund lines to larger containers (e.g. deeper petri dishes or beakers). Water quality, salinity and food were monitored and adjusted as needed.

(b) Assessment of fecundity

The number of hatching larvae (nauplii) has been repeatedly shown to suffer breakdown in F_2 or later generation hybrids of *T. californicus* [2,21]. We used this trait as a measure of fitness in each F_9 line that had sufficient individuals. Clasped pairs from each F_9 inbred line were isolated in fresh culture plates and closely monitored for the hatching of a female's first brood, at which time the number of hatched nauplii was tallied under a dissecting microscope, and the female returned to culture. Fecundity for each inbred line was estimated as the mean number of hatchlings across 6-15 replicate broods. We defined 'parental-level' fitness by the lowest mean fecundity across inbred parental lines. Finally, we screened hybrid lines according to their fitness levels, designating lines with fecundity at or higher than the minimum parental-level as 'high fitness' and lines below that level as 'low fitness' (i.e. exhibiting hybrid breakdown).

(c) Oxidative damage assay

Oxidative stress is the outcome of an imbalance between ROS production and total antioxidant defense capacity, with a net increase in unquenched ROS resulting in damage to proteins, lipids, and DNA [25,26]. The observed level of damage to macromolecules hence provides better quantification of oxidative stress than commonly used assays of antioxidant enzyme activities or gene expression [35]. For each inbred line, we quantified the concentration of 8-hydroxy-2'-deoxyguanosine (8-OH-dG), which is the oxidized derivative of guanine and a highly specific biomarker of ROS-mediated damage to DNA [36]. Tissue samples were obtained by collecting the broods initiated for **Assessment of Fecundity**. After fecundity was measured, broods were allowed to grow to age 21-24 days, when they were transferred to a 1.5-ml centrifuge tube and rinsed twice with fresh filtered seawater. The water was then removed by pipetting, and the

161 copepods were flash-frozen by immersing the tube in a slurry dry-ice:ethanol mixture. Tissues
162 were kept at -80°C until all replicates were obtained for the assay. By maintaining similar culture
163 conditions and by collecting copepods at the same age, we attempted to control for environmental
164 and age effects among lines.

165 We determined through preliminary trials that a minimum of 15 individuals is needed in a
166 sample in order to obtain sufficient genomic DNA (gDNA) for the 8-OH-dG assay. When
167 collecting copepods for tissue, as described above, we aimed to have 15-25 individuals per
168 sample. Since some hybrid lines had consistently low fecundity, we pooled different replicate
169 broods as necessary to obtain the minimum number. Genomic DNA was extracted from frozen
170 tissues using the QIAGEN DNeasy Kit (cat. 69506), extract purity assessed by measuring
171 absorbance ratios A_{260}/A_{280} and A_{260}/A_{230}, and gDNA concentration determined with a sensitive
172 fluorescence method (PicoGreen Assay Kit, Invitrogen cat. P11496). For each line, two to three
173 high-quality gDNA samples were used in an enzyme-linked immuno-sorbant assay (ELISA,
174 Cayman Chemical cat. 589320) to quantify 8-OH-dG, and resulting values were normalized to
175 respective DNA concentrations.

176

177 **(d) Statistical Analyses**

178 All statistical analyses were performed in R 2.6.2 (R Development Core Team). An initial
179 comparison of overall difference in 8-OH-dG levels between parental and hybrid lines was
180 performed with linear mixed-effects models, with cross type (parental/hybrid) included as fixed
181 effect and replicate as a random effect. A model with equal variances not assumed between cross
182 types performed better than a model with equal variances (log-likelihood ratio = 45.2, $P < 0.001$);
183 hence, we report results from the former model. Subsequent comparisons between groups

(parental *vs* hybrids, with hybrid lines categorized by fitness level and degree of interpopulation genetic divergence) for differences in 8-OH-dG and fecundity were performed with *t*-tests, using mean values for each inbred line as individual data points. Finally, Pearson's correlation was employed to examine the association between oxidative damage and fecundity.

3. Results

A total of 12 parental and 28 hybrid lines were still viable at the F_9 generation, after five generations of inbreeding. Hybrid lines exhibited significantly lower fecundity than parental lines ($t_{19.90} = 3.39$, $P < 0.01$; mean ± SEM number of first-brood larvae, parental: 21.3 ± 2.3, hybrid: 13.9 ± 0.8). Variance in fecundity across hybrid lines was high, with 16 lines showing breakdown (i.e. below-parental fecundity) and 12 lines well within the range of parental fitness (figure 1). As expected [2], crosses between genetically close populations yielded proportionately fewer low-fitness lines than those between divergent populations (Fisher's exact test, $P = 0.015$), but this result is tempered by the fact that only a single pair of genetically close populations was used in the study.

ELISA assays revealed that *T. californicus* hybrid lines exhibited, on average, elevated levels of oxidative damage to DNA when compared to parental lines ($F_{1,75} = 7.19$, $P = 0.009$, figure 2; electronic supplementary material, figure S1). Average levels of 8-OH-dG between high and low divergence crosses were very similar, but the variance across low divergence lines was greater than across high divergence lines (figure 2). High-divergence hybrid lines exhibited significantly higher levels of 8-OH-dG than parental lines ($t_{24.6} = 2.30$, $P = 0.015$), while lines in the single low-divergence cross showed no significant difference from parentals ($t_{10.7} = 1.19$, $P =$

0.13). Furthermore, the highest levels of DNA damage were consistently found in lines suffering hybrid breakdown (*t*-test *versus* parental lines: $t_{21.7} = 2.54$, $P = 0.009$), while hybrid lines with parental-level fecundity showed no significant difference in damage when compared to parentals ($t_{14.7} = 0.57$, $P = 0.29$, figure 2). Finally, we detected a significant negative relationship between fecundity and 8-OH-dG levels across all lines ($r = -0.37$, $P = 0.018$, figure 3).

4. Discussion

Experiments with genetic model systems have shown that defects in any of the OXPHOS enzyme complexes exacerbate ROS production, presumably by stalling electron flow and accumulating superoxide anions ($O_2^{-}\cdot$), the precursor of most ROS [37-39]. In many of these systems, the mitochondrial dysfunctions are associated with human diseases and cancers, and have been traced to point mutations in OXPHOS genes [28,40,41]. In *T. californicus*, populations are highly divergent across mtDNA genes [18] and interpopulation hybrids suffer from impaired OXPHOS functions [21,22]. Hence, we hypothesized that mitochondrial dysfunction in *T. californicus* exacerbate levels of cellular oxidative stress and promote increased damage to macromolecules.

(a) Oxidative damage in *Tigriopus californicus*

Overall, our results show that oxidative damage in hybrids was, on average, >30% higher than the background levels in parental populations. Support for our hypothesis, however, does not require this overall difference, since intergenic incompatibilities are expected to occur in only a fraction of recombinant genomes. Indeed, variance in oxidative damage across hybrid lines was large. Although previous studies strongly suggest that hybrid breakdown in *T. californicus* is due

predominantly to mito-nuclear incompatibilities [20-22], the influence of negative epistasis in nuclear-nuclear interactions cannot be ruled out [42]. Nevertheless, our main goal was to test whether recombinant genomes exhibiting these incompatibilities also suffer from elevated oxidative stress. Recombinant lines showing breakdown in fecundity showed the highest levels of DNA damage, even in the cross involving less-divergent populations. With a single cross type within the 'low-divergence' category, our study has limited power to address the influence of divergence. The presence of high oxidative damage in some low-divergence hybrid lines, however, at least suggests that ROS-inducing negative epistasis may appear early in allopatric divergence.

We argue that the observed values of damage in hybrids may be an underestimate. Because of limitations in assay sensitivity, we could not quantify damage in individual copepods, and pooling F_2 hybrids would conceal associations between phenotypes and different recombinant genomes. Our approach was to allow inbreeding of isofemale lines for five generations in order to increase genomic homogeneity within lines, permitting pooling of individuals for quantification of damage as well as fecundity. One consequence of waiting for multiple generations before assaying damage is that recombinant lines with the strongest degree of breakdown were likely lost due to intrinsic selection before reaching the F_9 stage. Therefore, levels of oxidative damage in hybrids are potentially even higher soon after the first generation of recombination.

(b) Oxidative stress and reproduction

Recent ecological studies have suggested that oxidative stress may be a key link in trade-offs between reproduction and other life-history traits, but this relationship is still poorly understood

253 [26,30,31]. For instance, experimental manipulations of reproductive investment in zebra finches
254 (*Taeniopygia guttata*) revealed a decrease in antioxidant capacity with increasing clutch sizes
255 [33], while the reverse was observed in field colonies of a swift species (*Alpus melba*) [43], and
256 no correlations were found between measures of damage and litter size in *Mus musculus* [44].
257 These studies aimed to test the general prediction that elevated oxidative stress is a costly
258 consequence of reproductive efforts [30,31]. Our experiment was not designed to examine
259 increases in oxidative stress due to reproduction, which would require quantifying pre- and post-
260 reproduction oxidative status. Nevertheless, we found a significant negative relationship between
261 fecundity of females and 8-OH-dG (i.e., oxidative damage) levels of their offspring across all
262 lines. While the proximate cause of this correlation cannot be inferred from our data, it is
263 consistent with findings in mice and humans that accumulation of oxidative damage may directly
264 impair reproduction by injuring oocytes, reducing fertilization success, or affecting early
265 embryonic development [45,46].
266
267 **(c) Possible sources and consequences of oxidative stress in *T. californicus***
268 The mitochondrial OXPHOS pathway is the main source of ROS [25,26]. Given previous
269 experimental evidence of mitochondrial dysfunction in *T. californicus* hybrids, at least some
270 genetic incompatibilities responsible for increased oxidative damage in these hybrids are likely
271 components of OXPHOS complexes [20,22]. OXPHOS dysfunction may in turn also be due to
272 disruption of other intergenomic pathways, especially of mtDNA transcription [7] or translation
273 [47], which are required for producing OXPHOS proteins. Elevated oxidative damage in hybrids
274 with mito-nuclear mismatches could result from either a direct increase in basal ROS leakage
275 from a dysfunctional OXPHOS system or from reduced antioxidant capacity. It seems likely that

both mechanisms can contribute to reductions in hybrid fitness. Damage due to increased ROS, in turn, requires the repair or replacement of macromolecules resulting in higher maintenance costs and consequently reduced hybrid fertility and viability. Since previous work has shown that hybrid mitochondria in *T. californicus* have reduced ATP synthetic capacity [21], energy for preventive antioxidant activity and energy to repair damage is possibly reduced in hybrids. In addition to causing damage to cellular components, increased ROS levels activate numerous retrograde signaling pathways from mitochondria to the nucleus, which alter expression of many genes in both genomes and can affect important metabolic processes [48,49]. Moreover, dysfunctional ROS metabolism may undergo a positive feedback loop: increased oxidative damage can increase mitochondrial dysfunction, promoting further ROS accumulation [25,29].

Evolution within populations promotes coadaptation between the nuclear and mitochondrial genomes. We propose that hybridization breaks up this coadaptation, specifically in the OXPHOS pathway, where nuclear- and mtDNA-encoded proteins interact most closely [14,17,50]. Disruption of OXPHOS function results in elevated basal levels of ROS and potentially reduced antioxidant defenses. Besides causing damage to cellular components and increasing maintenance and repair costs, accumulation of ROS has been shown to promote mtDNA rearrangements [38] and to reduce protein translation fidelity [51]. The net impact will be reduced hybrid fecundity, viability, and growth rate. Consequently, our data suggest that elevated oxidative stress resulting from hybridization between genetically differentiated natural populations may be a significant molecular mechanism underlying the establishment of postzygotic isolating barriers.

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Table 1. Amino acid sequence divergence (%) among *Tigriopus californicus* populations. Population abbreviations: SD, San Diego; BR, Bird Rock; AB, Abalone Cove; SC, Santa Cruz; BB, Bodega Bay. Shown are pairwise divergences for cytochrome *b* (above diagonal) and mitochondrial RNA polymerase (below diagonal). Comparisons marked with '-' were not estimated because hybrids between those populations were not included in this study.

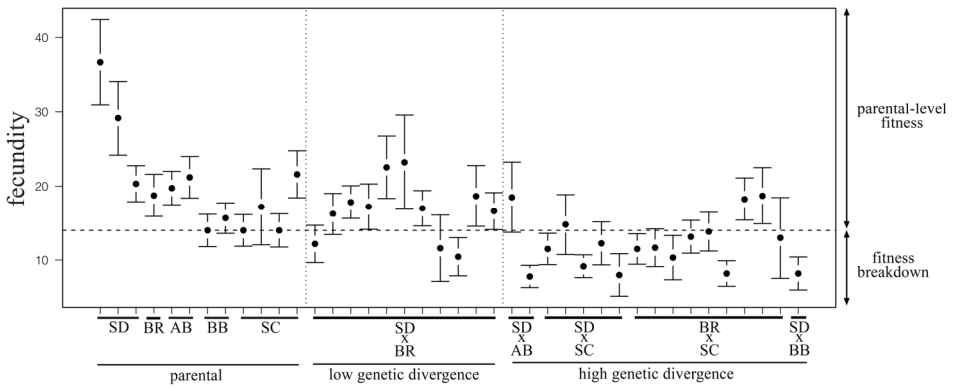
	SD	BR	AB	SC	BB
SD		1.87	7.40	7.16	8.04
BR	0.53		-	7.19	-
AB	1.22	-		-	-
SC	1.39	1.73	-		-
BB	1.56	-	-	-	

Figure legends

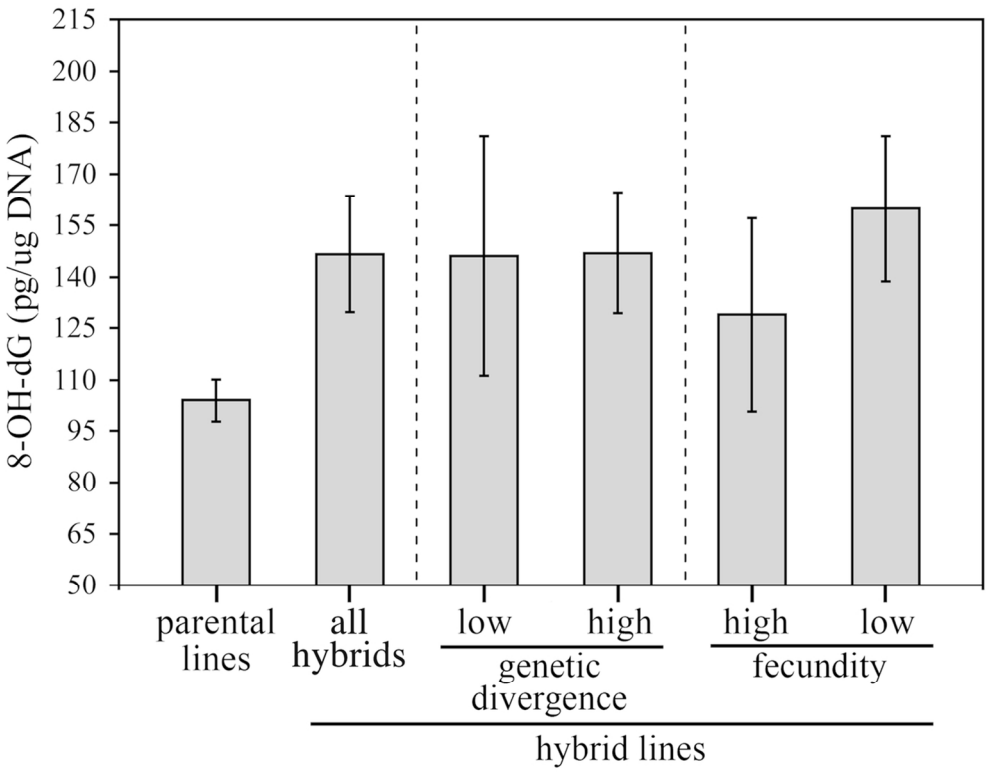
Figure 1. Distribution of fecundity across inbred lines of *Tigriopus californicus*. Fecundity was measured as the number of larvae hatching from a female's first brood. Data points are means ± SEM for each experimental line. Population abbreviations are as listed in Table 1. The horizontal dashed line separates hybrid samples according to their fitness level, using the lowest parental mean (n = 14 hatchlings) as threshold. Reciprocal crosses (e.g. SD♀x SC♂ and SC♀x SD♂) are pooled under the same designation (SD x SC).

Figure 2. Levels of oxidative damage to DNA in *Tigriopus californicus* inbred lines. Shown are means ± SEM picograms of 8-OH-dG, normalized to DNA concentrations, for each category of inbred line. For genetic divergence, reciprocal crosses between SD x BR are considered 'low' while crosses between (SD or BR) x (AB, SC, or BB) are considered 'high'. Fecundity levels were assigned based on the mean fecundity of each hybrid line compared to the parental-level threshold, as described in Figure 1. In other words, hybrid lines showing breakdown were pooled as 'low', and those with parental-level fecundity were pooled as 'high'.

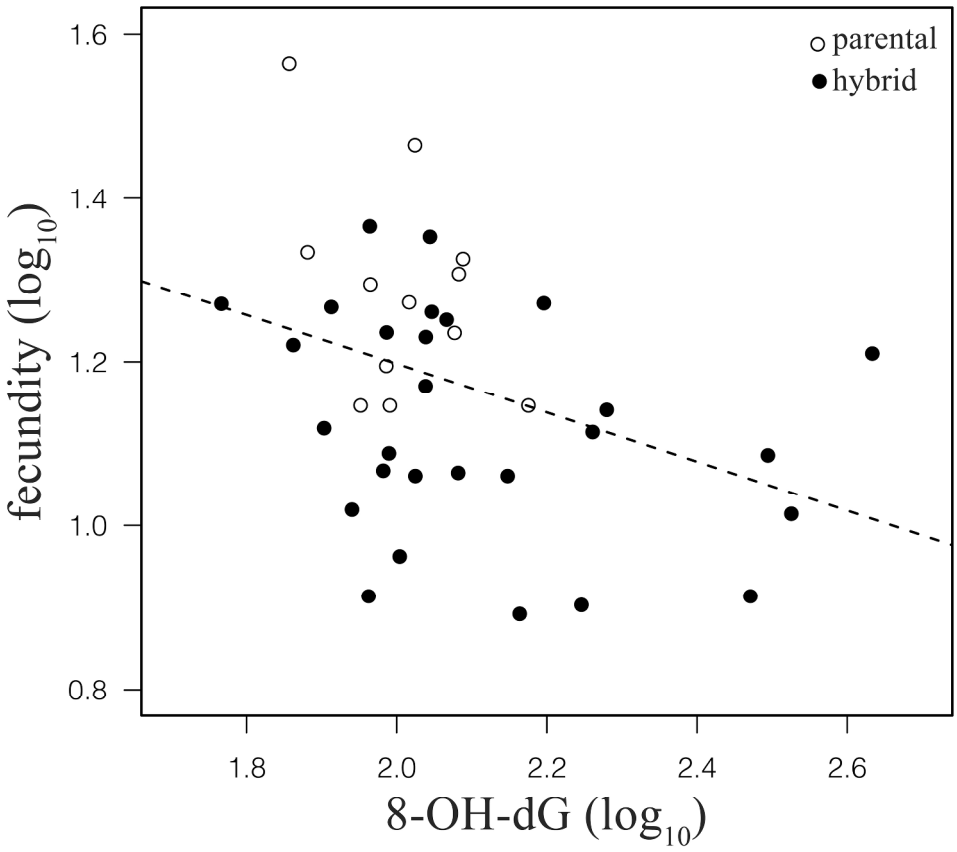
Figure 3. Correlation between fecundity and oxidative damage to DNA across inbred lines of *Tigriopus californicus*. Fecundity was measured in females, while oxidative damage was quantified in their offspring.



Fecundity across inbred lines.
165x67mm (300 x 300 DPI)



Oxidative stress among groups.
117x90mm (300 x 300 DPI)



Correlation between fecundity and oxidative damage.
276x250mm (300 x 300 DPI)