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Title Page

Title:
Developmental rate displays effects of inheritance but not of sex in inter-population hybrids of *Tigriopus californicus*

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Abstract

Coevolved genetic interactions within populations can be disrupted by hybridization resulting in individuals with reduced hybrid fitness. This "hybrid breakdown" may contribute to reproductive isolation as coevolved genes diverge between populations; however, trait variation and breakdown in hybrids may be sex-specific due to differential effects of incompatibilities between females and males. The extent to which this trait variation among hybrids is inherited across generations remains unclear. Here we present two experiments investigating variation in developmental rate among reciprocal inter-population hybrids of the intertidal copepod *Tigriopus californicus*. Developmental rate is a fitness-related trait in this species that is affected by interactions between mitochondrial-encoded and nuclear-encoded genes resulting in variation in mitochondrial ATP synthesis capacities in hybrids. First, we show that F_2 hybrid developmental rate is equivalent in the two reciprocal crosses and is unaffected by sex, suggesting that hybrid breakdown of developmental rate is likely experienced equally by females and males. Second, we demonstrate that variation in developmental rate among F_3 hybrids is heritable; times to copepodid metamorphosis of F_4 offspring of fast-developing F_3 parents (12.25 ± 0.05 d, μ ± SEM) were significantly faster than those of F_4 offspring of slow-developing parents (14.58 ± 0.05 d). Third, we find that ATP synthesis rates in these F_4 hybrids are unaffected by the developmental rates of their parents, but that mitochondria from females synthesize ATP at faster rates than mitochondria from males. Taken together, these results suggest that sex-specific variation of these fitness-related traits varies among traits in these hybrids and that effects likely associated with hybrid breakdown display substantial inheritance across hybrid generations.

Introduction

Compatible interactions between genetic loci are maintained by coevolution within taxa (Rand et al., 2004; Hill, 2015; Burton et al., 2013), but hybridization has the potential to disrupt this coevolution. As a result, hybrids may express genetic incompatibilities that cause loss of fitness (i.e., hybrid breakdown) ~~which is associated with reduced performance in fitness-related traits~~ (Burton & Barreto, 2012; Hill et al., 2019). These reductions in hybrid fitness and performance have the potential to limit gene flow between taxa as selection favours the maintenance of compatible interactions (Burton et al., 2013; Hill et al., 2019; Healy & Burton 2020; Han & Barreto, 2021). At early stages of divergence, incompatibilities may be particularly likely to involve loci in rapidly evolving DNA sequences, such as heterologous sex chromosomes (e.g., Phillips & Edmands, 2012) or the mitochondrial genome (Lynch, 1997; Burton & Barreto, 2012; Wallace, 2010). Given the sex-specific inheritance of these rapidly evolving sequences, there is an increased likelihood of variation in the effects of incompatibilities between hybrid females and males. Therefore, the potential for incompatible interactions to create barriers to gene flow and to contribute to early stages of reproductive isolation depends not only on the heritability of variation in fitness-related traits across hybrid generations, but also on the extent to which this trait variation differs between the sexes.

Tigriopus californicus is a species of intertidal copepod that is ideal for the study of phenotypic variation in inter-population hybrids, and of hybrid breakdown more generally (Burton et al., 2006; Barreto et al., 2018; Burton, 2022). These copepods inhabit supralittoral tidepools found on rocky outcrops along the Pacific coast of North America from Baja California, Mexico to Alaska, USA. There is virtually no migration of *T. californicus* between rocky outcrops (Burton & Feldman, 1981; Burton, 1997), resulting in extremely high genetic

divergence among populations, particularly in the mitochondrial genome (Burton et al., 2007; Barreto et al., 2018). However, laboratory crosses between populations generate offspring that are both viable and fertile (e.g., Burton, 1986), and previously published studies in *T. californicus* have demonstrated hybrid breakdown from the F_2 generation onwards across several fitness-related traits such as fecundity, viability, hatching success, metamorphosis success, developmental rate and mitochondrial performance (Burton, 1986, 1987, 1990; Edmands, 1999; Edmands & Burton, 1999; Willett & Burton, 2001, 2003; Ellison & Burton, 2006, 2008a, 2008b; Healy & Burton, 2020; Han & Barreto, 2021). Signatures of incompatibilities have been detected between nuclear-encoded genes or between mitochondrial-encoded genes and nuclear-encoded, although the latter (mitonuclear) effects tend to dominate overall (Edmands et al., 2009; Pritchard et al., 2011; Foley et al., 2013; Lima et al., 2019; Healy & Burton, 2020; Han & Barreto, 2021; Pereira et al., 2021). This major role of mitonuclear interactions is particularly evident for variation in developmental rate among F_2 hybrids as in crosses between a San Diego, California population and either a Santa Cruz, California population (Healy & Burton, 2020) or a Strawberry Hill Wayside, Oregon (Han & Barreto, 2021), there is strong selection for compatible mitonuclear genotypes in fast-developing hybrids.

Despite the lack of heterologous sex chromosomes in *T. californicus* (Alexander et al., 2015), there may be sex-specific trait variation in hybrids as a result of genetic interactions involving sex-determining loci (e.g., Lopez et al., 2021) or of differential physiological sensitivities to the effects of incompatibilities between females and males (e.g., Foley et al., 2013). Alternatively, for traits affected by mitonuclear incompatibilities, the maternal inheritance of the mitochondrial genome (e.g., Giles et al., 1980) may also lead to sex-specific effects. For example, the ‘mother’s curse’ hypothesis posits that mutations in mitochondrial DNA causing

88 beneficial interactions in females will accumulate even if they cause negative interactions in
89 males, due to the lack of paternal transmission of mitochondrial DNA (Frank & Hurst, 1996;
90 Gemmell et al., 2004). Effects of mitonuclear interactions aligning with the 'mother's curse' in
91 hybrids have been observed for many traits (Rand et al., 2001; Camus et al., 2012; Milot et al.,
92 2017; Carnegie et al., 2021, but see Mossman et al., 2016a, 2016b, 2017; Eyre-Walker, 2017;
93 Rand & Mossman, 2020). Yet, although many fitness-related traits demonstrate sex-specific
94 variation in *T. californicus* hybrids (Willett & Burton, 2001; Foley et al., 2013; Flanagan et al.,
95 2021; Li et al., 2022; Watson et al., 2022), these effects are not always consistent with a
96 "mother's curse" (Watson et al., 2022). For example, previous estimates suggest the times
97 necessary to reach adulthood for juveniles from intra- or inter-population crosses in *T.*
98 *californicus* are equivalent in both sexes (Burton, 1990). However, the potential effects of sex
99 throughout hybrid development are not well characterized, and the extent to which variation in
100 developmental rate is inherited across hybrid generations in unknown.
101 To address these knowledge gaps, we present the results of two experiments that (1)
102 investigate the effects of sex on developmental rate in *T. californicus* inter-population hybrids,
103 (2) examine the inheritance of variation in developmental rate between hybrid generations, and
104 (3) assess mitochondrial ATP synthesis in the offspring of fast or slow developing hybrids. In
105 particular, we focus on these questions: Does developmental rate vary between female and male
106 F_2 hybrids throughout development? Is variation in developmental rate among hybrids dependent
107 on mitochondrial genotype? Is fast or slow development among F_3 hybrids inherited by their F_4
108 offspring? Among F_4 hybrids, are there associations between developmental rate or sex and
109 mitochondrial performance?
110

Materials & methods

Copepod collection and laboratory acclimation

T. californicus were collected from splashpools in the intertidal zone in San Diego, California (SD; 32° 45′ N, 117° 15′ W), and Santa Cruz, California (SC; 36° 56′ N, 122° 02′ W) in the summer of 2018. Copepods were transferred into 1 L plastic bottles containing water from their tidepools using large plastic pipettes and transported to Scripps Institution of Oceanography, University of California San Diego within 24 h. Collected copepods were split among several 250 mL laboratory cultures in 400 mL glass beakers that were held in incubators set at 20 °C and 12h:12h light:dark, and cultures were maintained with filtered (0.44 µm) natural seawater (35 psu). Powdered spirulina (Salt Creek, Inc., South Salt Lake City, UT, USA) and ground TetraMin® Tropical Flakes (Spectrum Brands Pet LLC, Blacksburg, VA, USA) were added as food to the cultures once per week, and copepods also consumed natural algal growth within their cultures. Laboratory cultures were maintained for a minimum of three months (i.e., ~3 generations) prior to the start of experiments.

Developmental rate in female and male F_2 hybrids

Virgin SD and SC females were obtained by splitting mate-guarding pairs with a fine needle (Burton et al., 1981; Burton, 1985), and reciprocal crosses between the populations, SD♀ x SC♂ (SDxSC) or SC♀ x SD♂ (SCxSD), were initiated by placing 40 virgin females from one population in ~200 mL of filtered seawater in a 2.5 x 15 cm petri dish with 40 males from the other population. Females and males paired haphazardly, and when gravid P_0 females (i.e., females carrying an egg sac) were observed they were moved to a new dish. All dishes were maintained under the same conditions as the laboratory cultures. Egg sacs hatched naturally in the new dishes, and when F_1 hybrid offspring were visible to the naked eye, the P_0 females were

134 removed to avoid overlapping generations in the dishes. After maturation, F_1 adults paired and
135 mated haphazardly and F_2 developmental rate trials were initiated one week after gravid F_1
136 females were observed. This avoided using only the fastest-developing F_1 hybrids as parents in
137 the F_2 trials while still preventing any F_2 hybrids from reaching adulthood in the F_1 dishes.
138 Mature (i.e., red) egg sacs were removed from F_1 gravid females using a fine needle and
139 placed individually in wells of Falcon® 6-well plates (Thermo Fisher Scientific, Waltham, MA,
140 USA). This was done for 10 SDxSC and 10 SCxSD egg sacs. Nauplii (i.e., larval copepods)
141 hatched from the egg sacs overnight, and development was tracked daily by observation through
142 a stereo microscope as in Healy & Burton (2020). *Tigriopus sp.* development involves a distinct
143 metamorphosis from the last naupliar stage (N6) to the first copepodid stage (C1; Raisuddin et
144 al., 2007), and developmental rate can be assessed by the time from hatch to metamorphosis. As
145 copepodids appeared, they were transferred individually into wells of Falcon® 24-well plates
146 (Thermo Fisher Scientific) and developmental progress through the copepodid stages (C1, C2,
147 C3, C4, C5 and adult) was monitored as described by Tsuboko-Ishii & Burton (2018). The times
148 between stage transitions were recorded for each individual and developmental rate was scored
149 for a total of 478 F_2 hybrids (273 SDxSC and 205 SCxSD). Throughout these developmental
150 trials the nauplii and copepodids were fed every other day by the addition of powdered spirulina
151 to the 6- or 24-well plates.
152 *Developmental rate in F_3 hybrids and their F_4 offspring*
153 In a separate experiment, three hybrid lines for both reciprocal crosses between the SD
154 and SC populations (i.e., six lines total) were initiated as described above for the F_2 experiment.
155 However, the initial crosses between the SD and SC populations involved 50 virgin females and
156 50 males for each line and rather than dissecting F_2 egg sacs from gravid F_1 females to score

157 developmental rate, 144 mature F_2 egg sacs were deposited into new 2.5 x 15 cm petri dishes
158 (one per line) and allowed to hatch overnight. Within these dishes the F_2 hybrids matured,
159 haphazardly paired and mated. Gravid F_2 females were transferred to a new dish for each line,
160 and F_3 developmental rate trials began approximately two weeks after the transfer of the first
161 gravid F_2 female to the new dish. The increase in interval between the F_2 gravid female transfers
162 and the collection of egg sacs compared to the interval for F_1 females (see above) was to account
163 for generally high levels of variation in developmental rate among F_2 hybrids (e.g., Healy &
164 Burton, 2020).

165 Mature F_3 egg sacs were dissected from 60 gravid F_2 females for each line, and were
166 placed individually into wells of Falcon® 6-well plates to allow the days post hatch (dph) to C1
167 metamorphosis to be scored for each F_3 offspring. In total, developmental rate was determined
168 for 11,263 F_3 copepodids with between 1,360 and 2,537 individuals scored per line
169 (Supplemental Table S1). The F_3 copepodids were divided into two developmental rate groups
170 for each line: fast developers that metamorphosed $\leq$ 10 dph and slow developers that
171 metamorphosed $\geq$ 17 dph. Each group was established in its own 2.5 x 15 cm petri dish, and the
172 number of copepodids establishing each group ranged from 77 to 597 (Supplemental Table S1).
173 As for the F_2 hybrids, the F_3 offspring matured and mated haphazardly and gravid F_3 females
174 were transferred to a new dish when they were observed. Two weeks after the initial transfers of
175 the gravid F_3 females, 30 mature F_4 egg sacs were collected for each group (i.e., 12 groups for
176 each line x F_3 parental developmental rate combination) from the gravid females and time to C1
177 metamorphosis was scored for the individual F_4 offspring. Developmental rate was assessed for
178 672 to 1157 F_4 copepodids per group (Supplemental Table S1) with 10,018 F_4 individuals scored
179 overall.

180 *Mitochondrial ATP synthesis rates in F_4 hybrids*

181 ATP synthesis rates were measured *in vitro* for mitochondria isolated from the F_4 *T.*
182 *californicus* hybrids using an approach similar to those presented in Harada et al. (2019), Healy
183 et al. (2019) and Healy and Burton (2020). In brief, mitochondria were isolated from pools of 15
184 F_4 copepods from each line x F_3 parental developmental rate group once the copepods had
185 reached adulthood. Rates obtained with this number of adults fall within the linear range of the
186 assay (Harada et al. 2019). Copepods were homogenized by hand in 800 µL of ice-cold buffer
187 (400 mmol L^{-1} sucrose, 100 mmol L^{-1} KCl, 70 mmol L^{-1} HEPES, 6 mmol L^{-1} EGTA, 3 mmol L^{-1}
188 EDTA, 1% w/v BSA, pH 7.6) with a Teflon-on-glass homogenizer, and mitochondria were
189 isolated by differential centrifugation, resuspended in 130 µL of an ice-cold assay buffer (560
190 mmol L^{-1} sucrose, 100 mmol L^{-1} KCl, 70 mmol L^{-1} HEPES, 10 mmol L^{-1} KH_2PO_4, pH 7.6),
191 supplied with saturating concentrations of ETS complex I or II substrates and allowed to
192 synthesize ATP under saturating conditions for 10 min at 20 °C. Initial and final concentrations
193 of ATP for each reaction were determined with CellTiter-Glo (Promega, Madison, WI, USA),
194 and reactions were performed with three sets of substrates for each sample. Two of the sets of
195 substrates led to donation of electrons to complex I: (1) 5 mmol L^{-1} pyruvate and 2 mmol L^{-1}
196 malate (PM) and (2) 10 mmol L^{-1} glutamate and 2 mmol L^{-1} malate (GM), and one set led to
197 donation of electrons to complex II: 10 mmol L^{-1} succinate (S). All sets of substrates also
198 included 1 mmol L^{-1} ADP. In general, ATP synthesis reactions were performed for 6 pools of
199 adult females and 6 pools of adult males from each group of F_4 hybrids. However, many F_4
200 individuals from these groups were allocated to other experiments in our laboratory, and some
201 groups demonstrated strongly skewed sex ratios (e.g., Alexander et al., 2015). Consequently, for
202 the line A males from fast-developing F_3 parents and for the line C males from slow-developing

203 F_3 parents only 5 pools were available for ATP synthesis reactions, and for males from line F
204 with slow-developing F_3 parents ATP synthesis reactions were only possible for 36 individuals.
205 In this last case, the males were homogenized in 3 pools of 12, resuspension volumes were
206 adjusted proportionally and GM-substrate reactions were not conducted. Thus, the PM and S
207 ATP synthesis rates for this group (line F males from slow-developing F_3 parents) were
208 stoichiometrically equivalent to the PM and S measurements for the other pools of copepods;
209 however, given the protocol adjustments that were necessary and the relatively small sample size
210 for this group (n = 3), comparisons between this group and others should be interpreted with
211 some caution.

212 ATP synthesis rates were normalized to citrate synthase (CS) reaction rates which were
213 measured *in vitro* for each mitochondrial isolation. CS is a nuclear-encoded enzyme that
214 functions within the tricarboxylic acid cycle and is commonly used as an index of mitochondrial
215 amount to normalize oxidative phosphorylation reaction rates (e.g., Gnaiger, 2020). CS assays
216 were run in duplicate using 6 µL of the mitochondrial isolations in each replicate. Reactions were
217 conducted in flat-bottomed 96-well plates (Corning, Glendale, AZ, USA) at a final volume of
218 120 µL with reactant and buffer component concentrations as follows: 50 mmol L^{-1} Tris pH 8.0,
219 0.1 mmol L^{-1} 5,5′-Dithiobis(2-nitrobenzoic acid), 0.3 mmol L^{-1} Acetyl CoA, 0.5 mmol L^{-1}
220 oxaloacetate and 0.1% vol/vol Triton X-100 (Spinazzi et al., 2012). Prior to the addition of
221 oxaloacetate, background reaction rates were measured by tracking the change in absorbance at
222 412 nm over 5 min using a SpectraMax® iD3 Multi-Mode Microplate Reader (Molecular
223 Devices, LLC., San Jose, CA, USA). After the addition of oxaloacetate to start the reaction, the
224 change in absorbance at 412 nm was again measured for 5 min and reaction rates were
225 determined by regressing the change in absorbance against time (in general $R^2 \geq 0.99$). Final CS

reaction rates were calculated by subtracting the background rate from the reaction rate (i.e., with oxaloacetate) for each replicate, and then taking the average value of the duplicates for each mitochondrial isolation. ATP synthesis rates were normalized to the CS reaction rates by division (see Supplemental Table S2 for CS rates normalized per copepod).

Statistical analyses

All statistical tests were performed in *R* v4.2.0 (R Core Team, 2022) with $\alpha = 0.05$. The majority of analyses in the current study were performed using linear mixed-effects models implemented with the *lmerTest* package v3.1.3 (Kuznetsova et al., 2017). For effects of sex on F_2 development, cross and sex were fixed factors, and egg sac was a random factor. For effects of cross on F_3 developmental rate, cross was a fixed factor, and there were nested random factors with egg sac nested within line. For effects of F_3 parental developmental rate on F_4 developmental rate, an overall model had F_3 developmental rate and cross as fixed factors, and egg sac nested within group (i.e., line x F_3 developmental rate groups) as random factors. Following the overall model, line-specific models were fit with F_3 parental developmental rate as a fixed factor and egg sac as a random factor. For variation in developmental rate between F_3 and F_4 hybrids, cross and line were fixed factors, and egg sac was nested within line as random factors. For F_4 ATP synthesis rates, cross, sex and F_3 parental developmental rate were fixed factors and line was a random factor. In general, final models were simplified by removing non-significant interaction terms hierarchically by the order of interactions, and developmental rate data were log transformed prior to model fitting. Realized heritabilities (h^2) for developmental rate were calculated for each line with the breeder's equation: response to selection = h^2 x selection differential (Lush, 1937). In the current study, the selection differential was equal to the average developmental rate for the fast- or slow- developing F_3 parents minus the average F_3

249 developmental rate, and response to selection was equal to the average F_4 developmental rate
250 minus the average F_3 developmental rate. Relationships between the hatch to copepodid stage 1
251 (C1), and C1 to adult development rates were assessed with a linear model for variation in C1 to
252 adult developmental rate that had hatch to C1 developmental rate as a continuous factor and
253 cross as a fixed factor. Potential effects of density dependence on developmental rate were
254 assessed in F_3 and F_4 hybrids using linear models for variation in the average developmental rate
255 for an egg sac that had the number of copepodids from an egg sac as a continuous factor and
256 either line or line x F_3 parental developmental rate group as a fixed factor (F_3 and F_4 models,
257 respectively). For the F_4 hybrids, additional models were fit within each line x F_3 parental
258 developmental rate group with the number of copepodids from an egg sac as a continuous factor
259 followed by type-III analysis of variance tests utilizing the *R* package *car* v3.0-13 (Fox &
260 Weisberg, 2019).
261

262 **Results**

263 *Developmental rates in female and male F_2 hybrids*

264 There was no variation in developmental rate between female and male F_2 hybrids when
265 assessed by time from hatch to copepodid stage 1 ($p = 0.51$; Figure 1a), copepodid stage 1 to
266 adult ($p = 0.20$; Figure 1b), or hatch to adult ($p = 0.62$; Figure 1c), and variance in developmental
267 rate did not differ between the sexes for any measure of developmental rate in either cross (i.e.,
268 in SDxSC or SCxSD hybrids; Bartlett test $p \geq 0.07$). There were also no effects of cross on any
269 of these metrics ($p \geq 0.44$). Interestingly, there was no significant relationship between the times
270 from hatch to C1 and from C1 to adult across egg sacs ($p = 0.15$; Supplemental Figure S1). In
271 general, approximately 2 to 2.5 d were spent at each copepodid stage with the time at C5 tending

272 to be slightly longer than times spent at other stages (Table 1), and similar to overall
273 developmental rate, there were no effects of sex on rate of development at any specific copepodid
274 stage ($p \geq 0.12$). At most stages there were no differences in developmental rate between
275 SDxSC and SCxSD copepodids ($p \geq 0.66$), but SDxSC copepodids spent less time at stage 1
276 than SCxSD copepodids ($p = 0.009$).

277 *Inheritance of developmental rate between F_3 and F_4 hybrids*

278 Across the F_3 lines in our study, metamorphosis to the C1 stage was observed from 7 to
279 45 dph, and there was no significant difference in developmental rate between the SDxSC and
280 SCxSD lines ($p = 0.09$) with only a small trend for faster development in SDxSC (12.14 ± 0.05
281 d, μ ± SEM) than in SCxSD (13.49 ± 0.04 d; Figure 2). The F_3 copepodids were grouped into
282 fast and slow developers (≤ 10 and ≥ 17 dph, respectively), and among lines 9-33 egg sacs
283 contributed to only the fast-developing groups, 4-25 contributed to only the slow-developing
284 groups and 18-47 contributed to both groups.

285 Developmental rates in the F_4 offspring of parents from the fast- and slow-developing F_3
286 groups were not affected by cross (i.e., SDxSC versus SCxSD; $p = 0.11$), but were affected by
287 the developmental rate group of their F_3 parents ($p = 0.005$; Figure 3). For all lines, the median
288 developmental rates of the F_4 offspring of the fast-developing F_3 hybrids were higher than the
289 median developmental rates of the F_4 offspring of the slow-developing F_3 hybrids, and in all but
290 SCxSD line E, there were significant differences between offspring from the fast- and slow-
291 developing F_3 groups ($p = 0.46$ for SCxSD line E and $p \leq 0.015$ for all other lines).
292 Developmental rates tended to be faster in F_4 offspring from fast-developing F_3 parents than in
293 the F_3 generation on average, but these differences were not significant ($p = 0.12$; Supplemental
294 Figure S2a). In contrast, the developmental rates of F_4 offspring from slow-developing F_3 parents

295 were significantly slower than the F_3 generation on average ($p = 9.4 \times 10^{-14}$; Supplemental Figure
296 S2b). Realized heritabilities for developmental rate between the F_3 and F_4 generations were 0.16
297 ± 0.10 and 0.29 ± 0.06 (μ ± SEM) for the fast and slow developers, respectively (Table 2), or
298 0.24 ± 0.06 and 0.27 ± 0.08 if only the lines displaying significant differences between the
299 offspring of fast or slow developers were considered (lines A, B, C, D and F).

300 In general, egg sacs of females from the slow-developing F_3 groups produced higher
301 numbers of copepodids than did the eggs sacs of females from the fast-developing groups. As a
302 result, we assessed if developmental rates across egg sacs were consistent with effects of density
303 dependence. For the F_3 copepodids (Supplemental Figure S3a-f), average developmental rates
304 across egg sacs were not affected by line ($p = 0.17$) or by the number of copepodids that
305 metamorphosed from an egg sac ($p = 0.28$) and there was no interaction between these effects (p
306 $= 0.58$). For the F_4 copepodids (Supplemental Figure S3g-l), there was significant effect of line x
307 F_3 parental developmental rate group ($p = 3.8 \times 10^{-4}$) and no significant effect of copepodid
308 number per egg sac ($p = 0.18$). However, in this case, there was a significant interaction between
309 these factors ($p = 1.4 \times 10^{-3}$). Thus, effects of the number of copepodids per egg sac were
310 analyzed separately for each line x F_3 parental developmental rate group. For 8 of the 12 groups,
311 there was no significant effect of copepodid number on developmental rate ($p \geq 0.055$), whereas
312 the SDxSC line B:slow-developing parents, SDxSC line C:fast-developing parents, SDxSC line
313 C:slow-developing parents and SCxSD line E:slow-developing parents groups displayed
314 significant effects prior to correction for multiple tests ($p \leq 0.018$). After correction (Bonferroni-
315 corrected $\alpha = 4.17 \times 10^{-3}$), only the line B:slow and line E:slow groups still showed significant
316 effects. To investigate these results further, we repeated these analyses using only egg sacs with
317 ≤ 40 copepodids (308 out of 360 egg sacs; ~86%) to assess if any potential density effects could

318 be traced to large egg sacs specifically. After correction for multiple tests as above, no lines had
319 an effect of copepod number on average egg sac developmental rate ($p = 9.0 \times 10^{-3}$ for SCxSD
320 line E:slow-developing parents, and $p \geq 0.076$ for all other groups). We also repeated our F_4
321 developmental rate analysis using the egg sacs with $\leq$ 40 copepodids and the overall findings
322 were unchanged (cross: $p = 0.098$; F_3 parental developmental rate: $p = 0.035$). Therefore, taken
323 together, our results suggested that any potential density effects on developmental rate in our
324 study were modest, and variation in developmental rate among F_3 *T. californicus* hybrids was
325 inherited by their F_4 offspring.

326 *Variation in ATP synthesis rates among F_4 hybrids*

327 Maximal *in vitro* ATP synthesis rates were measured for two sets of ETS complex I
328 substrates: pyruvate-malate (PM), and glutamate-malate (GM). PM ATP synthesis rates in F_4
329 hybrids were affected by sex ($p < 2 \times 10^{-16}$) and F_3 parental developmental rate ($p = 0.0014$), but
330 not cross (i.e., SDxSC or SCxSD; $p = 0.82$); however, there was also a significant interaction
331 between sex and parental developmental rate ($p = 0.040$; Figure 4a). The general trends in the
332 PM-fueled synthesis rates were similar to those for the GM ATP synthesis rates (Figure 4b), but
333 the statistical results were somewhat different. For the GM-fueled rates, there was a significant
334 effect of sex ($p < 2 \times 10^{-16}$), but not of F_3 parental developmental rate ($p = 0.085$) or cross ($p =$
335 0.40), and the only significant interaction was between parental developmental rate and cross (p
336 $= 0.0015$). Interpretation of these results is complicated by these interactions, but the clearest
337 pattern was that of higher synthesis rates in females than in males for both the PM and GM
338 substrates (PM: 0.0092 ± 0.0003 and 0.0049 ± 0.0001 and GM: 0.0097 ± 0.0003 and 0.0054 ±
339 0.0001, μ ± SEM, ATP rate:CS rate for females and males, respectively; note that data from F_4

340 hybrids from the SCxSD line F:slow-developing parents group were excluded from the GM
341 comparison here, see Methods and materials for details).
342 ATP synthesis rates were also assessed using an ETS complex II substrate: succinate (S).
343 There were significant effects of sex ($p = 5.6 \times 10^{-14}$) and F_3 parental developmental rate ($p = 1.6$
344 $\times 10^{-4}$), but not of cross ($p = 0.14$), on the F_4 S ATP synthesis rates (Figure 4c). Additionally,
345 there was a significant interaction between parental developmental rate and cross ($p = 4.6 \times 10^{-4}$).
346 Again, the clearest pattern was that females tended to have higher S ATP synthesis rates than
347 males (0.0195 ± 0.0003 and 0.0156 ± 0.0004, μ ± SEM, S ATP rate:CS rate for females and
348 males, respectively). However, the difference between females and males was proportionally
349 smaller with the complex II substrate than with the complex I substrates, and the ratio of PM-
350 fueled synthesis rate to S-fueled synthesis rate was higher in females than in males (sex: $p = 5.0$
351 $\times 10^{-14}$, F_3 parental developmental rate: $p = 1.3 \times 10^{-6}$, cross: $p = 0.75$; Table 3). As observed for
352 the ATP synthesis rates independently, there were also Interactive effects on the PM:S rate ratios
353 (sex x F_3 parental developmental rate: $p = 0.026$, sex x cross: $p = 0.028$).
354
355 **Discussion**
356 In the current study, we assess the effects of sex and inheritance on traits known to
357 display hybrid breakdown in inter-population hybrids of *T. californicus*: developmental rate and
358 ATP synthesis rate (e.g., Healy & Burton, 2020). We find that in hybrids between the SD and SC
359 populations of this species, developmental rate does not differ between the reciprocal crosses
360 overall, or between females and males at any stage of development (F_2 generation). We also find
361 that mitochondria isolated from hybrid females synthesize ATP at faster rates than mitochondria
362 isolated from hybrid males (F_4 generation). In comparison to mitochondria from males,

mitochondria from females have higher ~~proportional~~ rates of complex-I fueled ATP synthesis relative to complex-II fueled synthesis. Taken together, these results suggest that the positive relationship between mitochondrial performance and developmental rate previously observed in these hybrids (Healy & Burton, 2020) may also depend on sex-specific balancing of ATP supply to ATP demand. Additionally, we demonstrate that variation in developmental rate displays substantial heritability across hybrid generations (F_3 to F_4), suggesting that genetic incompatibilities are likely maintained across generations consistent with roles in limiting gene flow and contributing to early stages of reproductive isolation between populations.

Phenotypic patterns in female and male inter-population hybrids

Phenotypic differences between females and males have been well characterized for a number of traits in *T. californicus*, including lifespan, viability, fertility, stress tolerances and gene expression patterns (Willett & Burton, 2001; Willett, 2010; Leong et al., 2018; Willett & Son, 2018; Foley et al., 2019; Li et al., 2019, 2020, 2022; Flanagan et al., 2021, 2022; Watson et al., 2022), and at least some of these sex-specific patterns have also been observed in inter-population hybrids. For instance, Li et al. (2022) found that among F_1 hybrids, males had longer lifespans than females under benign conditions, whereas females lived longer than males under food-limited conditions. Furthermore, Foley et al. (2013) showed that differences in nuclear allele frequencies between reciprocal F_2 hybrids (i.e., between alternate mitochondrial genotypes) were sex-dependent, particularly for chromosome 10. However, in the current study there was virtually no variation in developmental rate between the sexes at any stage of development (consistent with the less detailed results of Burton [1990]), and there was also no difference in overall developmental rate between the two reciprocal crosses. The similarities in developmental rate between the SDxSC and SCxSD hybrids are consistent with previous studies

386 in *T. californicus* (Ellison & Burton, 2008b; Healy & Burton, 2020, 2023). Unexpectedly, there
387 was no correlation between the rates of development from hatch to copepodid stage 1 (C1) and
388 from C1 to adult across the F_2 egg sacs in the current study. This suggests that fast developers
389 through one phase of development may not necessarily also be fast developers through the other
390 phase, and that the mechanisms underlying developmental rate, or their effects, likely change
391 throughout development, which has been observed in *Drosophila sp.* (Hoekstra et al., 2018). In
392 any case, as development from hatch to C1 and from hatch to adult have both been used to
393 measure developmental rate in *T. californicus* (e.g., Burton, 1990; Harada et al., 2019), the lack
394 of correlation between the two phases of development is of particular note for considering results
395 across published studies and for designing future experiments with this species.

396 Given the lack of sex-specific hybrid developmental rates observed in the current study, it
397 is somewhat surprising that the ATP synthesis rates for mitochondria isolated from female
398 copepods were higher than those for mitochondria isolated from male copepods in the F_3 to F_4
399 experiment. In contrast, Healy and Burton (2020) demonstrated a positive relationship between
400 developmental rate and ATP synthesis rate in F_2 hybrids between SD and SC *T. californicus*.
401 These potentially contradictory results across studies may simply be a consequence of comparing
402 results from F_2 and F_4 hybrids. Alternatively, it is possible that metabolic demands are higher in
403 female copepods than in male copepods during development, such that elevated ATP synthesis
404 capacities could be a compensatory physiological response in females. However, at least in
405 adults, there is little evidence for higher metabolic rates in females than in males under benign
406 conditions (Powers et al., 2022).

407 Previous studies have detected some evidence for variation in complex IV activities
408 between female and male *T. californicus* (Edmands & Burton, 1998, 1999), and higher ATP

synthesis rates in females than in males in the current study were independent of the use of complex I or II substrates to fuel the ETS. However, the proportional increase in ATP synthesis rates from males to females was greater with complex I than complex II substrates. This is clearly evident through the elevated ratios of PM ATP synthesis rates to S ATP synthesis rates in females compared to males and implies that oxidative phosphorylation may be more reliant on complex I function in female than in male *T. californicus*. Sex-specific differences in the proportional contributions of complex I and II respiration may play a role in variation in viability, lifespan or longevity between females and males in inter-population hybrids of this species reported elsewhere (Willett & Burton, 2001; Li et al., 2022; Watson et al., 2022).

Heritable variation in developmental rate among hybrids

Heritability of developmental rate or growth during development has been observed in several species (e.g., *D. melanogaster* – Chippindale et al., 1997, 2004, and *Crassostrea gigas* – Pace et al., 2006; Meyer & Manahan, 2010), and among the F_3 hybrid individuals in our study there was substantial variation in developmental rate with metamorphosis to stage 1 copepodid occurring between 7 and 45 dph across lines. Note this range of times to metamorphosis is similar to that observed for F_2 hybrids from these crosses by Healy and Burton (2020), whereas in the current study variation in F_2 hybrids was much narrower: 8 to 17 dph. The cause of this difference is unclear as culturing temperatures, salinities and photoperiods were the same across experiments and studies, but it is possible that uncontrolled differences in culture lighting or algal growth contribute to this variation. Regardless, consistent with results from other species, inheritance of developmental rate between F_3 and F_4 hybrids was observed in at least 5 of our 6 lines. This inheritance of developmental rate was asymmetric for fast- and slow-developing hybrids. Across all the hybrid lines, the increase in developmental rate from the average F_3

432 developmental rate was 0.55 ± 0.37 d (μ ± SEM) for F_4 offspring of fast developers compared to
433 a decrease of -1.78 ± 0.55 d for F_4 offspring of slow developers. Moreover, generation only had a
434 significant effect on developmental rate between the F_3 hybrids and the F_4 offspring of slow
435 developers (see Supplemental Figure S2), which is consistent with the trend for higher realized
436 heritability in slow-developing than in fast-developing hybrids. We hypothesize that this
437 asymmetry in response to selection may reflect that fast development requires avoiding multiple
438 mitonuclear incompatibilities in hybrids between the SD and SC populations, whereas a range of
439 possible incompatibilities may result in slow development (Healy & Burton, 2020). This
440 suggests that there are fewer fast-developing genotypes than slow-developing genotypes in
441 hybrids between these populations, which may reduce additive genetic variance in fast
442 developers.

443 Despite the heritability of F_3 parental developmental rates in F_4 offspring, there was no
444 variation in maximal ATP synthesis rates between F_4 offspring of fast- or slow-developing
445 parents. As discussed above, this lack of relationship between developmental rate and
446 mitochondrial performance contrasts with previous studies in *T. californicus* (Healy & Burton,
447 2020; Han & Barreto, 2021). However, there was overlap in the ranges of developmental rates
448 for the F_4 offspring of fast- or slow-developing parents in the current study, and mitochondria
449 were isolated from pools of copepods selected haphazardly from each F_4 group. Therefore, it is
450 possible that we were unable to resolve any potentially subtle differences in mitochondrial
451 performance between the groups after the effects of allelic reassortment between the F_3 and F_4
452 generation.

453 *Potential implications for the effects of mitonuclear incompatibilities in hybrids*

454 Genetic incompatibilities between the mitochondrial and the nuclear genomes are thought
455 to play a role in hybrid breakdown (Hill, 2017; Hill et al., 2019), and these effects are substantial
456 in *T. californicus* (Burton et al., 2006; 2013; Burton, 2022). The negative consequences of these
457 incompatibilities are evident through loss of performance in several fitness-related traits (Ellison
458 & Burton 2006, 2008b; Healy & Burton, 2020; Han & Barreto, 2021). In particular, Healy and
459 Burton (2020) demonstrated that differences in developmental rate and ATP synthesis rate
460 among SDxSC and SCxSD F_2 hybrids are highly dependent on variation in the extent of
461 mitonuclear compatibility. In the current study, we did not re-assess the role of mitonuclear
462 incompatibilities underlying hybrid trait variation specifically. However, building from the
463 strong effects detected for these traits by Healy and Burton (2020) and from other previously
464 published studies in *T. californicus* (Burton et al., 2006, 2013; Ellison & Burton, 2006; 2008b;
465 Han & Barreto, 2021), the data presented here may provide insights into not only the inheritance
466 of mitonuclear incompatibilities across hybrid generations, but also the potential for sex-specific
467 effects of these incompatibilities.
468 The strong influence of mitonuclear incompatibilities on hybrid developmental rate and
469 ATP synthesis rate in SDxSC and SCxSD *T. californicus* (Healy & Burton, 2020) implies there
470 is a genetic basis for this trait variation. Indirect evidence for heritability of fecundity,
471 survivorship, developmental rate and ATP synthesis rate associated with mitonuclear interactions
472 in this species has been shown through studies that have maintained hybrid lines displaying
473 positive relationships between mitonuclear compatibility and variation in these traits (Ellison &
474 Burton, 2006; Edmands et al., 2009; Barreto & Burton, 2013; Pereira et al., 2014, 2021; Powers
475 et al., 2021). Here, we directly quantify this heritability of developmental rate across successive
476 hybrid generations ($h^2 = 0.16 \pm 0.10$ and 0.29 ± 0.06 for fast and slow developers, respectively),

477 which suggests that effects of mitonuclear incompatibilities are likely felt across generations
478 consistent with a potential role in the early stages of reproductive isolation (Hill, 2017; Hill et al.,
479 2019). Additionally, sex-specific mitonuclear effects are often predicted as a result of the
480 "mother's curse" hypothesis (Frank & Hurst, 1996; Rand et al., 2001; Gemmell et al., 2004;
481 Carnegie et al., 2021) or other mechanisms that could produce differential effects between
482 females and males (e.g., interactions with sex-determining loci; Lopez et al., 2021). Despite
483 these possibilities for sex to affect loss of fitness in hybrids, variation in developmental rate
484 between female and male F_2 *T. californicus* hybrids was not observed in the current study,
485 implying that potential negative consequences of incompatible mitonuclear genotypes are likely
486 equivalent between the sexes. In part, this may relate to the lack of heteromorphic sex
487 chromosomes in this species (Alexander et al., 2015); however, sex-specific effects in hybrid *T.*
488 *californicus* are not consistently aligned with the expectations of the 'mother's curse' hypothesis
489 in general (e.g., Watson et al., 2022). Similarly, variation in mitonuclear effects on
490 developmental time between females and males is generally modest or absent in *Drosophila sp.*
491 (Jelić et al., 2015; Mossman et al., 2016a; Erić et al., 2022) despite sex-specific effects of
492 mitochondrial genotype on many other fitness-related traits when evaluated on common nuclear
493 genetic backgrounds (Camus et al., 2012; Kurbalija Novičić et al., 2015; Nagarajan-Radha et al.,
494 2019, 2020; Carnegie et al., 2021).
495
496 **Conclusion**
497 Loss of fitness in hybrids (i.e., hybrid breakdown) is likely a key process contributing to
498 barriers to gene flow between taxa. The effects of breakdown may often be caused by the
499 expression of incompatible genetic interactions following hybridization and have the potential to

vary between the sexes. The results of the current study demonstrate that developmental rate does not show sex-specific variation in inter-population hybrids of *T. californicus*, whereas ATP synthesis rates are higher in females than in males. These patterns suggest that sex-specific effects of hybridization on fitness-related traits varies among traits and levels of biological organization. Despite this, our data suggest there is substantial heritability of variation in developmental rate in hybrids, consistent with effects of hybrid breakdown that span generations, and with a potential role in establishing the early stages of reproductive isolation.

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Data availability

Prior to final publication, all datasets used in the current study will be submitted to the Dryad Digital Respository.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Author contributions

TMH and RSB conceived and designed the experiments; TMH and ACH collected the data; TMH performed the analyses; TMH prepared the tables and figures, and TMH, ACH and RSB wrote the manuscript.

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859 **Tables**

860

861 **Table 1.** Time to metamorphosis at each copepodid stage during *T. californicus* development in
862 female and male F_2 inter-population SDxSC and SCxSD hybrids.

863

Developmental stage transition	Time to metamorphosis (d) (μ ± SEM)			
	SDxSC cross		SCxSD cross	
	Female	Male	Female	Male
C1 to C2†	1.85 ± 0.05	1.80 ± 0.07	2.23 ± 0.07	2.11 ± 0.11
C2 to C3	2.39 ± 0.07	2.26 ± 0.09	2.21 ± 0.06	2.22 ± 0.09
C3 to C4	2.40 ± 0.08	2.41 ± 0.08	2.25 ± 0.06	2.38 ± 0.19
C4 to C5	2.26 ± 0.07	2.28 ± 0.09	2.14 ± 0.08	2.07 ± 0.14
C5 to A	2.69 ± 0.06	2.95 ± 0.11	2.61 ± 0.12	2.65 ± 0.16

864 † significant difference between SDxSC and SCxSD ($p = 0.009$)

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866

867

868 **Table 2.** Realized heritabilities (h^2) for developmental rate between the F_3 and F_4 generations for
869 fast- and slow-developing hybrid *T. californicus*.

870

Cross	Line	Realized heritability (h^2; F_3 to F_4 generation)	
		Fast developers	Slow developers
SDxSC	*A*	0.06	0.39
	B	0.29	0.45
	C	0.28	0.24
SCxSD	*D*	0.18	0.26
	E	-0.26	0.37
	F	0.41	0.01

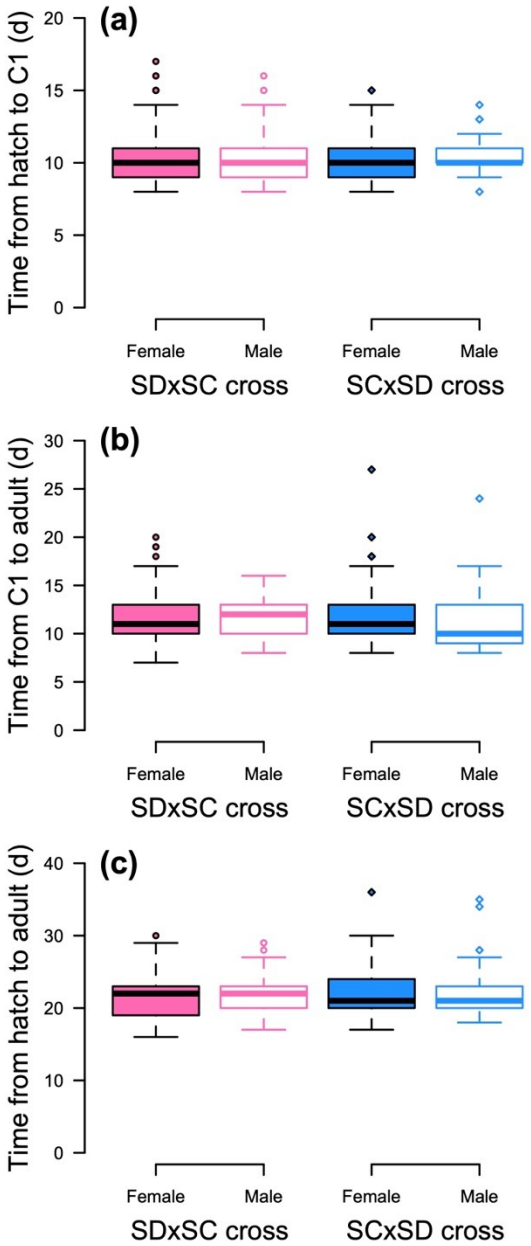
871

872 **Table 3.** Ratios of pyruvate-malate (PM) ATP synthesis rate to succinate (S) ATP synthesis rate
873 for female and male F_4 inter-population SDxSC and SCxSD hybrids that were offspring of either
874 fast- or slow-developing F_3 parents.
875

Cross	Line	F_3 parental developmental rate	PM:S ATP synthesis rate ratio (μ ± SEM)	
			Female	Male
SDxSC	*A*	*Fast*	0.46 ± 0.02	0.39 ± 0.02
		Slow	0.45 ± 0.01	0.32 ± 0.02
	B	*Fast*	0.39 ± 0.02	0.31 ± 0.01
		Slow	0.48 ± 0.03	0.38 ± 0.01
	C	*Fast*	0.45 ± 0.03	0.31 ± 0.02
		Slow	0.60 ± 0.03	0.32 ± 0.02
SCxSD	*D*	*Fast*	0.45 ± 0.01	0.30 ± 0.01
		Slow	0.44 ± 0.02	0.30 ± 0.01
	E	*Fast*	0.43 ± 0.02	0.28 ± 0.01
		Slow	0.48 ± 0.01	0.27 ± 0.01
	F	*Fast*	0.44 ± 0.01	0.26 ± 0.01
		Slow	0.61 ± 0.04	0.45 ± 0.04

876

877 **Figures**
878



879 **Figure 1.** Box plots of developmental rate from (a) hatch to stage 1 copepodid (C1), (b) C1 to
880 adult, and (c) hatch to adult for female (filled boxes) and male (open boxes) F_2 *T. californicus*
881 reciprocal hybrids (SDxSC – pink; SCxSD – blue). No significant effects of sex or cross were
882 detected for any of the measurements of developmental rate.

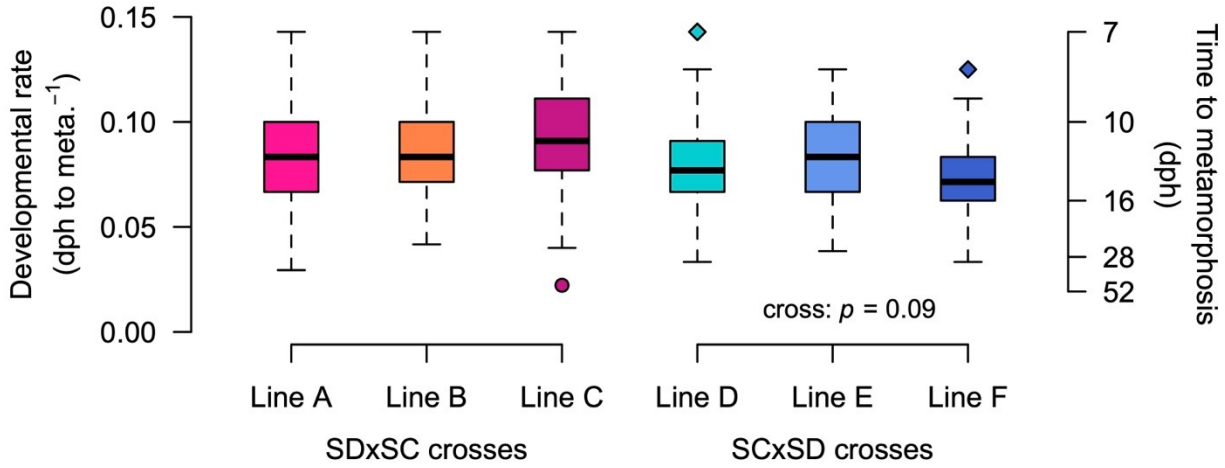


Figure 2. Box plots of developmental rate from hatch to stage 1 copepodid (C1) metamorphosis for F_3 *T. californicus* reciprocal hybrids (SDxSC, lines A-C – warm colors, circles; SCxSD, lines D-F – cool colors, diamonds). Developmental rates are shown as a rate (left axis) and as a time to metamorphosis (right axis). The *p*-value for potential variation between the SDxSC and SCxSD crosses is shown on the graph.

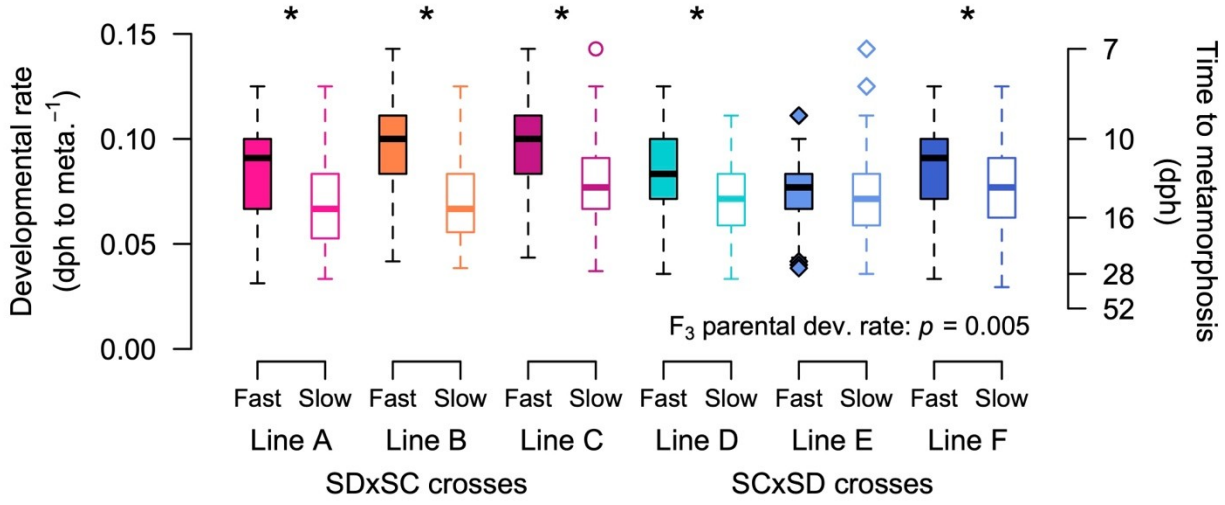


Figure 3. Box plots of developmental rate from hatch to stage 1 copepodid (C1) metamorphosis for F_4 *T. californicus* reciprocal hybrids (SDxSC, lines A-C – warm colors, circles; SCxSD, lines D-F – cool colors, diamonds) that were offspring of 'Fast'- or 'Slow'-developing F_3 parents (filled or open boxes, respectively). Developmental rates are shown as a rate (left axis) and as a time to metamorphosis (right axis). The *p*-value for an effect of F_3 parental developmental rate in

899 general is shown on the graph, and asterisks indicate significant effects of F_3 parental
900 developmental rate that were detected by line-specific tests.

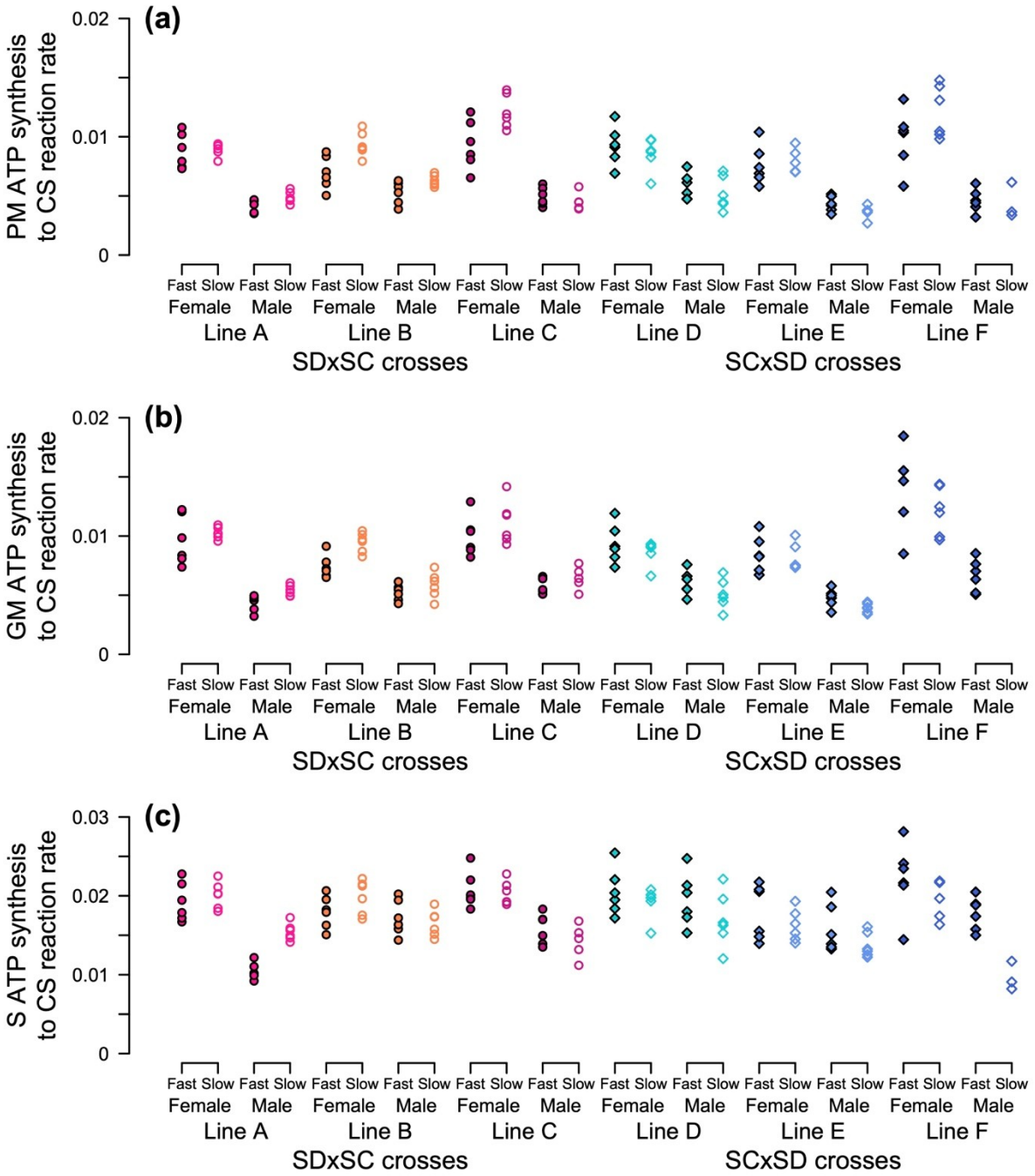


Figure 4. Plots of maximal *in vitro* ATP synthesis rates of mitochondria isolated from F_4 *T. californicus* reciprocal hybrids (SDxSC, lines A-C – warm colors, circles; SCxSD, lines D-F – cool colors, diamonds) that were offspring of 'Fast'- or 'Slow'-developing F_3 parents (filled or open boxes, respectively). Assays were conducted to assess electron transport system complex I-fueled (a: PM – pyruvate-malate; b: GM – glutamate-malate) and complex II-fueled (c: S – succinate) ATP synthesis under saturating conditions. Statistical results are presented in the main text of the Results.