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1 **The contribution of mutation to variation in temperature-dependent sprint speed in**

2 **zebrafish, *Danio rerio***

3

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12 ## Abstract

13 The contribution of new mutations to phenotypic variation, and the consequences of this
14 variation for individual fitness, are fundamental concepts for understanding genetic variation
15 and adaptation. Here, we investigated how mutation influenced variation in a complex trait in
16 zebrafish, *Danio rerio*. Typical of many ecologically relevant traits in ectotherms, swimming
17 speed in fish is temperature-dependent, with evidence of adaptive evolution of thermal
18 performance. We chemically induced novel germline point mutations in males, and measured
19 sprint speed in their sons at six temperatures (between 16°C and 34°C). Mutational effects on
20 speed were strongly positively correlated among temperatures, resulting in statistical support
21 for only a single axis of mutational variation, reflecting temperature-independent variation in
22 speed (faster-slower mode). While these results suggest pleiotropic effects on speed across
23 different temperatures, when mutation have consistent directional effects on each trait,
24 spurious correlations arise via linkage, or heterogeneity in mutation number. However,
25 mutation did not change mean speed, indicating no directional bias in mutational effects. The
26 results contribute to emerging evidence that mutations may predominantly have synergistic
27 cross-environment effects, in contrast to conditionally neutral or antagonistic effects which
28 underpin thermal adaptation. However, aspects of experimental design might limit resolution
29 of mutations with non-synergistic effects.
30

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31 ## Introduction

32 Populations can rapidly evolve in response to environmental changes (Hendry and Kinnison
33 1999; Hairston et al. 2005; Reznick et al. 2019), with strong evidence that local adaptation is
34 common (Leimu and Fischer 2008; Hereford 2009). These observations point to the pervasive
35 presence of heterogeneity among genotypes in their fitness under different environmental
36 conditions, that is, variance due to genotype-by-environment interactions (GxE). In addition
37 to supporting adaptive divergence of trait mean, GxE also represents the evolutionary
38 potential of phenotypic plasticity, the expression of environment-specific phenotypes by the
39 same genotype (Via and Lande 1985; de Jong 1990). GxE has been reported for diverse
40 quantitative traits and environments in populations of a wide range of organisms (Des Marais
41 et al. 2013; Wood and Brodie 2015; Saltz et al. 2018). Furthermore, environment-specific
42 effects of segregating alleles have been characterised for specific loci (e.g., Barrett et al.
43 2009; Li et al. 2014). However, the interaction of evolutionary processes to generate and
44 maintain quantitative genetic variation, including GxE, are not well understood (de Jong and
45 Gavrilets 2000; Josephs 2018; Walsh and Lynch 2018).
46
47 Mutation is the ultimate source of genetic variation. Heterogeneous selection pressures may
48 alter allele frequencies and shape the standing genetic variation available for on-going
49 adaptive evolution, but the distribution of phenotypic effects of mutations that arise could
50 ultimately determine the nature of standing genetic variation (de Jong and Gavrilets 2000;
51 Walsh and Lynch 2018), whether populations persist (Lynch and Gabriel 1990; Gabriel et al.
52 1993), and how they adapt (Lorch et al. 2003; Mee and Yeaman 2019). Broadly, there are
53 two ways that mutation can impact these processes.
54

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55 First, if mutations have a consistent direction of effect on fitness across environments,
56 heterogeneity in the magnitude of effect (strength of selection) may result in the magnitude of
57 standing genetic variation differing among environments. In particular, for deleterious
58 mutations, variation in the magnitude of fitness effect could result in accumulation of greater
59 mutation load under some environments, and rapid purging under environment change
60 (potentially leading to rapid population size declines). Martin and Lenormand (2006) and
61 Agrawal and Whitlock (2010) reviewed studies in which mutational effects under standard
62 rearing conditions were contrasted with effects under stressful levels of chemical toxins,
63 nutritional resources, or temperature. No overall pattern was detected, with mutations having
64 the same effect in both environments, or either stronger or weaker fitness effects. An updated
65 analysis by Berger et al. (2021) arrived at the same conclusion for abiotic stressors generally,
66 but their results suggested elevated temperatures typically increased the strength of selection.
67
68 A second way that mutational effects may impact evolutionary genetic phenomena is through
69 their contribution to GxE associated with local adaptation. In their meta-analysis of nine
70 studies of mutational effects, Martin and Lenormand (2006) identified a consistent pattern of
71 increased variance in mutational effects in stressful relative to benign conditions, concluding
72 that the frequency of beneficial mutations increased as the environmental change shifted the
73 population further from the ancestral adaptive optimum. A shift in the distribution of fitness
74 effects of mutations has long been hypothesised (Fisher 1930; Orr 1998), with accumulating
75 empirical evidence of an increase in the frequency of beneficial mutations as the ancestor
76 becomes less fit (e.g., Silander et al. 2007; Stearns and Fenster 2016). A shift in fitness
77 effects among environments suggests mutations may typically have antagonistic pleiotropic
78 effects (i.e., trade-off) across environments, being beneficial under some conditions, but
79 deleterious under others, consistent with theories of local adaptation (Felsenstein 1976;

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80 Bürger 2000). In contrast, observation of predominantly positive cross-environment
81 mutational correlations (Fry and Heinsohn 2002; Baer et al. 2006; Latimer et al. 2014) as
82 well as direct characterisation of individual mutations under different environmental
83 conditions (Ostrowski et al. 2005; Sane et al. 2018; Stewart et al. 2022), suggest that
84 mutations more frequently have conditionally neutral (i.e., affecting the trait in only one
85 environment) or concordant (synergistic) cross-environment effects.
86
87 Notably, much of the research into environmental dependency of mutational effects has
88 focused on contrasting fitness effects between different types of environments (e.g.,
89 alternative nutrition sources) or between dichotomous, typically extreme, levels of an
90 environmental variable. Levels of many abiotic and biotic factors vary on a continuous scale,
91 with populations inhabiting complex, multidimensional environments. The environmental
92 experience of any given mutation (arising spontaneously in a generation) will therefore be
93 sampled from the distribution of natural conditions a population experiences over both its
94 spatial range and an individual's lifecycle. Mutations may come under fluctuating direction
95 or strength of selection due to environment heterogeneity on these scales, which will
96 influence the nature of standing genetic variation within populations. Studies under natural,
97 field, conditions have revealed heterogeneity in mutational effects between temporally or
98 spatially varying conditions (Roles et al. 2016; Rutter et al. 2018). We suggest that
99 understanding how mutation contributes to standing genetic variation, and evolutionary
100 potential, depends on extending our understanding of the heterogeneity of mutational effects
101 across environmental gradients spanning ecologically relevant values.
102
103 Temperature is a key aspect of the environment, which changes over short (diurnal), medium
104 (seasonal) and long (e.g., climate warming) time scales, as well as over small (e.g., shade vs

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105 sun patches; lake shallows vs depths) and large (latitudinal or elevational) spatial scales.
106 Temperature affects biochemical reactions, which, in organisms with variable body
107 temperatures (ectotherms), will affect physiological rates and all traits dependent on those
108 rates, ultimately encompassing fitness (Huey and Kingsolver 1989; Hochachka and Somero
109 2002; Angilletta 2009). Temperature-dependent traits in ectotherms typically follow a
110 stereotypical pattern of increasing values up to a so-called optimal temperature, followed by a
111 rapid decline in values (Huey and Kingsolver 1989; Izem and Kingsolver 2005). Ecologically
112 relevant modes of variation in this thermal performance curve shape have been identified,
113 particularly variation in optimal temperature (hotter-colder mode) and in the width of the
114 function (i.e., the range of temperatures over which high levels of performance are
115 maintained: the specialist-generalist mode) (Huey and Kingsolver 1989; Izem and Kingsolver
116 2005). There are many examples of divergence among populations and species aligned with
117 these modes of variation, typically reflecting known differences in thermal ecology of the
118 taxa (e.g., Yamahira et al. 2007; Logan et al. 2018). Both hotter-colder and specialist-
119 generalist modes of variation are consistent with antagonistic or conditionally neutral, rather
120 than concordant cross-environment effects of contributing genetic variants (Kingsolver et al.
121 2001; Izem and Kingsolver 2005).
122
123 While several studies have investigated mutational effects on traits that will impact
124 physiological processes (e.g., metabolites and enzymes: Clark et al. 1995; Harada 1995;
125 Davies et al. 2016), few have considered whole-organism performance traits other than
126 fitness itself (Huey et al. 2003; Ajie et al. 2005; Latimer et al. 2014). Locomotor performance
127 is a thermally sensitive trait in ectotherms (e.g., Condon et al. 2010; Latimer et al. 2014;
128 Logan et al. 2018), and contributes to fitness-enhancing functions such as feeding, migration,
129 mating and predator evasion (Jayne and Bennett 1990; Irschick and Garland 2001; Husak and

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130 Fox 2008; Irschick et al. 2008; Careau and Garland 2012). While few studies have supported
131 selection acting directly on performance indices such as maximum speed or endurance
132 (Walker et al. 2005; Irschick et al. 2008; Wilson et al. 2020), repeated (parallel or
133 convergent) evolution of the same performance – environment relationships (e.g., McGuigan
134 et al. 2003; Nelson et al. 2008; Fu et al. 2013; da Silva et al. 2014; Kern and Langerhans
135 2019) is consistent with performance being genetically correlated with fitness. Similarly,
136 thermal performance curves for locomotor phenotypes have diverged among taxa inhabiting
137 different thermal environments (e.g., Logan et al. 2018) consistent with thermal heterogeneity
138 in locomotor performance being an ecologically relevant and heritable phenotype.
139
140 Here, we applied a chemical mutagen to induce mutations in males of a laboratory strain of
141 zebrafish (*Danio rerio*) and investigated the effects of these mutations on swimming
142 performance of their sons. We assayed sprint swimming speed at the constant temperature
143 experienced by these fish throughout their life (28°C; where the zebrafish stock centre system
144 temperature is 28.5°C: Westerfield 2007) and during acute exposure to five other
145 temperatures between 16°C and 34°C. Zebrafish are distributed throughout India and
146 neighbouring countries, from sea level to over 1500m, and are typically found in habitats
147 with temperatures between 16.5°C and 34.0°C, although more extreme temperatures have
148 been reported (12.3°C - 38.4°C) (McClure et al. 2006; Spence et al. 2006; Engeszer et al.
149 2007; Arunachalam et al. 2013). Applying multivariate analyses and contrasting among-
150 family variance estimated from sons of mutated sires to that estimated from sons of control,
151 non-mutated males, we investigated the contribution of new mutation to the variation in this
152 complex, environmentally dependent phenotype. We particularly focus on determining
153 whether mutations have concordant effects on speed across all temperatures, or whether

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154 mutations have conditionally neutral or antagonistic effects, generating heterogeneity in
155 mutational variance among temperatures.
156

157 **Methods**

158 All work was conducted with approval of The University of Queensland's Animal Welfare
159 Unit. Adults of the Wild India Kolkata (WIK: Rauch et al. 1997) strain were imported from
160 the Zebrafish International Resource Centre (Oregon), founding a local population at The
161 University of Queensland, maintained for five generations prior to the current experiment
162 (~30 parents per sex per generation). Following protocols detailed in Rohner et al. (2011) and
163 McGuigan and Aw (2017), males were mutagenized via a 40-minute exposure to 3mM of N-
164 ethyl-N-nitrosourea (ENU). ENU is an alkylating agent that induces point mutations
165 spanning a full spectrum of effects including nonsense, missense, and splice-site mutations
166 (Knapik 2000; Wienholds et al. 2003). The ENU protocols developed for forward genetic
167 screens to determine gene function, 1 hour exposure to 3mM ENU once a week for six
168 weeks, induces ~1400 – 9400 mutations (de Bruijn et al. 2009; Rohner et al. 2011). Repeated
169 exposure to ENU saturates DNA repair mechanisms (Noveroske et al. 2000), resulting in
170 more mutations than a single, higher concentration, dose (Hitotsumachi et al. 1985; Rohner et
171 al. 2011). Our aim, with ~1/9th of the total standard dose, was to induce relatively few
172 mutations, more reflective of typical long-running spontaneous mutation accumulation (MA)
173 studies in invertebrates and plants (Halligan and Keightley 2009; Katju and Bergthorsson
174 2019). We consider the potential consequences of the mutation protocol further in the
175 Discussion.
176

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177 At least two weeks after ENU exposure (ensuring germline mutation transmission: Mullins et
178 al. 1994), each mutagenized male was paired with a non-mutagenized female from the same
179 WIK population to generate full sibling families, referred to as the Mutant treatment. The
180 WIK stock was originally founded by a single pair of fish (Rauch et al. 1997). Reflective of
181 this small founder size, and ongoing maintenance at a small population size (Trevarrow and
182 Robison 2004), WIK has low polymorphism relative to wild populations (Coe et al. 2009;
183 Suurvali et al. 2020), and long runs of homozygosity, consistent with low effective
184 population size (Suurvali et al. 2020). Nonetheless, WIK is far from genetically
185 homogeneous (Coe et al. 2009; Brown et al. 2012; Butler et al. 2015; Suurvali et al. 2020),
186 and thus to infer the effect of new mutations induced by ENU against this background of
187 standing genetic variation, a second set of males from the same WIK population, but not
188 exposed to ENU, were also bred with non-mutagenized females, again generating full-sibling
189 families. These Control treatment families, reared and assayed under identical conditions to
190 the Mutant families, allowed us to estimate both the increase in heritable phenotypic variance
191 due to new mutations, and changes in trait mean, reflecting bias in the average direction of
192 effect of mutations.
193
194 Due to logistical constraints on rearing larvae, breeding was conducted in two blocks (3.5
195 weeks apart), with 27 and 23 families per treatment bred in the first and second block,
196 respectively. Rates of natural spawning declined over time; to obtain the desired number of
197 families (50 per treatment) and minimise age differences among families, we utilised in-vitro
198 fertilisation (IVF) protocols on freshly collected gametes (Ransom and Zon 1999) to generate
199 10 mutant and four control full-sib families.
200

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201 Offspring from each of the 100 families (50 per Mutant and Control treatments) were reared
202 in two replicate 3.5L tanks, with ~30 fish per tank at 28°C, following husbandry protocols
203 detailed in (Conradsen et al. 2016). The 200 tanks were connected via a recirculating water
204 system, with tank position randomised among families and mutagenesis treatment. At ~70-
205 100 days post fertilisation (dpf), three males from each tank (six from each family; 600 in
206 total) were injected with a coloured elastomer tag (Northwest Marine Technology, Inc, Shaw
207 Island, WA, USA) on either their left or right dorsal side to allow for individual identification
208 (protocol detailed in Conradsen and McGuigan 2015). Tagged males were then transferred to
209 new 3.5L tanks, each of which contained six Mutant and six Control males, randomly
210 sampled from the 50 families per treatment.

211 *Swimming performance*

212 Swimming speed was assayed for each of the 600 tagged males at each of six temperatures:
213 16°C, 20°C, 24°C, 28°C, 31°C, and 34°C. All males were first assayed at temperatures 16°C
214 and 28°C in a semi-random order that ensured no bias in the order in which sons of the 100
215 families encountered the test temperatures, the time of day (morning or afternoon) they
216 swam, or the test apparatus (two swimming flumes were used). Fish were assayed at the
217 remaining four temperatures following the same semi-randomised design. Offspring from the
218 first block of breeding (27 families per treatment) completed all swim trials prior to the first
219 trails for fish from the second breeding block. In Block 1 (2), fish were 90 - 119 (114 – 149)
220 dpf at their first trial, and 152 – 212 (183 – 219) dpf at their last trial.
221

222 Swimming speed was assayed in either a 10L (swim chamber 38cm x 10cm x 9cm length x
223 width x height) or 30L (46cm x 14cm x 13cm) flume (Loligo Systems, Tjele, Denmark).
224 Water in the flumes was maintained at temperatures 28°C and above using heaters (200 W or

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225 1500 W, Quian Hu, Singapore) and at temperatures 24°C and below using chillers (440 W
226 TECO, Ravena Italy). Fish were fasted for 12 – 24h prior to swimming. Prior to trials at non-
227 ambient temperatures (above or below 28°C), all 12 fish from a single holding tank were
228 placed individually in small tanks submersed in a larger tank at 28°C, which was then heated
229 or cooled at a rate of 0.2°C per minute to bring the fish to their test temperature.
230
231 Speed was measured using a short-duration (<500 second) stepped velocity test (Brett 1964),
232 a metric referred to as U_{sprint} (Starrs et al. 2011; Widrick et al. 2018). U_{sprint} has been shown to
233 be strongly positively correlated with speed measured in more commonly used prolonged
234 (>30 minute) stepped velocity tests (Reidy et al. 2000; Starrs et al. 2011), suggesting that
235 these metrics capture similar information about individual swimming capability. The shorter-
236 duration U_{sprint} was preferred in the current study due to the large number (3,600) of planned
237 assays. To measure U_{sprint}, fish were placed into the swimming chamber at a flow rate of
238 16cms^{-1} for two minutes. A pilot study confirmed this time was adequate for fish to exhibit
239 routine behaviours. Flow was then increased by 4cm^{-1} at intervals of 15 seconds. The trial
240 was complete (and water flow stopped) when the fish was unable to hold station and was
241 swept to the grid at back of the chamber. U_{sprint} was calculated following Brett (1964) as:
242 $U_{sprint} = U_i + (U \times (T_{ii} / T))$, where U_i was the maximum velocity (cm^{-1} second) maintained for
243 the full 15 second interval, U was the water speed increment (here, 4cm^{-1} second), T_{ii} was the
244 time (seconds) the fish swam in their final velocity before tiring, and T was the time interval
245 at each step (here, 15 seconds).

246 *Data Analyses*

247 Two approaches have been widely used to study variation in traits that change value as a
248 function of the environment: functional or multivariate (character-state) (Kirkpatrick and

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249 Heckman 1989; Griswold et al. 2008; Gomulkiewicz et al. 2018). Data from the current
250 experiment, where all individuals were assayed at the same set of temperatures, is well suited
251 to a multivariate approach (Gomulkiewicz et al. 2018). Implementing a multivariate
252 analytical approach also directly supported comparison to results from the only other study,
253 that we are aware of, investigating how mutational effects on locomotion vary with
254 temperature (Latimer et al. 2014), and allowed us to explicitly address questions about
255 heterogeneity in the magnitude of genetic (mutational) variance among temperatures, as well
256 as the correlation in mutational effects across temperatures.
257
258 Swimming speed (U_{sprint}) was assayed for a total of 594 males, with data at all six
259 temperatures available for 576 of these. Estimates of quantitative genetic parameters are
260 sensitive to extreme (outlier) values. Here, 27 observations of U_{sprint} were greater than 3.0
261 standard deviations (SD) from the mean and excluded from all analyses. If these observations
262 reflected large effect mutation, their exclusion would under-estimate mutational effects.
263 However, outlier individuals were widely distributed across 22 families, 12 Control and 10
264 Mutant, suggesting that they do not reflect mutational effects.
265
266 We first investigated whether induced mutations affected either the mean speed, or the
267 average relationship between temperature and speed. We fit the following model using
268 maximum likelihood within PROC Mixed in SAS (SAS Institute Inc. 2012):
269
270 $$y = \mu + \mathbf{XB} + \mathbf{Z}_F\boldsymbol{\delta}_F + \mathbf{Z}_R\boldsymbol{\delta}_R + \mathbf{Z}_I\boldsymbol{\delta}_I \qquad \text{Model (1)}$$
271
272 where y was the vector of U_{sprint} observations, μ was the global mean U_{sprint} and $\mathbf{X}$ was the
273 design matrix relating observations to their level of the categorical fixed effects, $\mathbf{B}$. The fixed

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274 effects of interest were mutation treatment (Mutant or Control), temperature, and the
275 interaction between mutation treatment and temperature. Several other fixed effects were fit
276 to account for additional potential sources of variation in speed. Trial, the six repeated
277 measures of speed per individual, captured variation due to age, experience, and any changes
278 in general laboratory conditions over the duration of the experiment. Time, categorised as
279 AM or PM, was fit to account for potential diurnal effects on speed. Flume and block were fit
280 to, respectively, account for differences in swimming performance between the two different
281 flumes (10L versus 30L), or breeding blocks (containing fish from 27 and 23 families per
282 treatment, respectively). $\mathbf{Z}_F$, $\mathbf{Z}_R$ and $\mathbf{Z}_I$ were the design matrices for the variance in speed
283 attributable to the random effects of Family, replicate Rearing tank (nested within Family),
284 and Individual (nested within tank), respectively. Reflecting the repeated measures nature of
285 the experimental design, these random effects were each modelled as unconstrained
286 (co)variances in speed around temperature-specific intercepts ($\boldsymbol{\delta}_F$, $\boldsymbol{\delta}_R$ and $\boldsymbol{\delta}_I$). To assess the
287 null hypotheses of no effect of mutation treatment on mean speed or temperature-specific
288 speed, the Satterthwaite degrees of freedom correction was applied. Among-family variance
289 was heterogenous between mutation treatments (see Results), and we investigated other
290 models to ensure results were robust. We modified model (1) to fit treatment-specific random
291 effects; to accommodate zero estimates of the hypothesis *F*-ratio denominator (due to no
292 among-family variance in Control treatment), we applied a log-likelihood ratio test to the
293 nested models in which the fixed effect of interest was fit versus not fit. The interaction
294 between mutation treatment and temperature was tested first, and this no-interaction model
295 was the reference model against which the main effects of mutation treatment and
296 temperature were assessed. Both approaches supported the same conclusion and we therefore,
297 report only the results from model (1).

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298 Second, we investigated how mutagenesis affected the among-family (co)variance in
299 swimming speed across the six temperatures. Mutagenesis of sires can contribute to
300 differences between the Mutant and Control populations in both the among-family (where
301 brothers inherit the same mutation from their father) and within-family (where brothers
302 inherit different mutations) variance. However, variation between the two replicate rearing
303 tanks per family, and among the three brothers sampled from each tank will reflect not only
304 these genetic differences, but also the micro-environmental variation between and within
305 tanks, respectively, which cannot be further partitioned out given our design. Therefore, we
306 focus our investigation on among-family variation, which can be unambiguously assigned to
307 genetic causes.

308 To estimate among-family variance, data was first centred (mean = 0) on the respective level
309 of each of the fixed effects included in model (1). This approach is equivalent to fitting fixed
310 effects in the analysis but improves model efficiency. Using REML in PROC MIXED to fit a
311 modified version of model (1) (no fixed effects) to the data for each mutation treatment
312 separately, we applied a factor analytic modelling approach (Hine and Blows 2006) to
313 determine the statistically supported axes of the among-family covariance matrix. The
314 among-family variation in swimming speed was constrained to zero (no among-family
315 variance, implemented by not fitting the among-family effect) through to six dimensions
316 (implemented using TYPE=FA0(n), where n was the number of dimensions, ranging from 1
317 to 6). A log-likelihood ratio test (LRT) was applied to test whether adding a dimension
318 improved model fit; the difference in log-likelihood between nested models follows a chi-
319 square distribution with the degrees of freedom equal to the difference in the number of
320 estimated parameters.

321

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322 We then analysed data from both treatments within the same model, estimating treatment-
323 specific random effects (implemented using the GROUP statement), to test the null
324 hypothesis that mutagenesis affected genetic variation. We used a LRT to compare a model
325 in which treatment-specific among-family variance was estimated to a model in which a
326 common, pooled, among-family variance was estimated; given evidence of low-
327 dimensionality (see Results), this model was fit with one dimension of among-family
328 variance, but results were consistent for higher-dimension models.
329
330 To further investigate the nature of the among-family variance in speed, we estimated the
331 unconstrained (TYPE=UN) treatment-specific among-family covariances. We placed robust
332 confidence intervals on model estimates using the REML-MVN sampling approach (Meyer
333 and Houle 2013; Houle and Meyer 2015). We used the MASS package (Venables and Ripley
334 2002) in R (R Core Team 2020) to draw 10,000 random samples from the distribution $N \sim (\hat{\theta}$,
335 **V**) where $\hat{\theta}$ was the vector of covariance parameter estimates, and **V** was the asymptotic
336 variance-covariance matrix from the REML model. While the REML variance estimates
337 were constrained to be positive, the REML-MVN samples were not (i.e., were on the G-
338 scale: Houle and Meyer 2015). We therefore interpreted the statistical significance of
339 individual parameter estimates based on whether the confidence intervals (CI) included zero,
340 which is equivalent to applying a LRT (Dugand et al. 2021). For variances, this is a one-
341 tailed test (as variances cannot be negative; 90% CI), while for covariances it is a two-tailed
342 test (95% CI). We used the 'eigen' function in base R (R Core Team 2020) to decompose the
343 unconstrained REML estimates of among-family covariance to their major axes, and
344 projected these axes ($V_{F_T} = e^t V_{F_T} e$, where V_{F_T} is the among-family variance in treatment T, e
345 is an eigenvector of the REML among-family matrix, and ^{t} indicates the transpose)

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346 (McGuigan and Aguirre 2016) through the 10,000 REML-MVN samples to place CI on the
347 eigenvalues.

348 **Results**

349 Mutation did not change mean sprint speed (main effect of mutation treatment: $F_{1, 99.8} = 0.31$,
350 $p = 0.5767$), or the response of sprint speed to temperature (mutation treatment x temperature
351 interaction: $F_{5, 99.3} = 0.48$, $p = 0.7909$) (Figure 1A). U_{sprint} depended on temperature (main
352 effect of temperature: $F_{5, 115} = 1861.76$, $p < 0.0001$), exhibiting the classical pattern of
353 thermal performance curves, with a rapid increase in speed until ~28°C (maintenance
354 temperature), although there was little decline in speed by the maximum assayed temperature
355 (34°C) (Figure 1).
356
357 In the Control treatment, the estimated among-family (genetic) variation in swimming speed
358 was zero at four of the six assayed temperatures, ranging up to a maximum of 3.08 (Table
359 1A). Consistent with expectations, among-family variance was greater in the Mutant
360 treatment at every temperature, ranging from 2.55 up to 11.70, although only at 24°C was the
361 among-family variance statistically distinguishable from zero (Table 1B). We rejected the
362 null hypothesis that Mutant and Control treatments had the same among-family variance
363 (Log-likelihood ratio test of fit of models estimating one-dimension of treatment-specific
364 versus pooled among-family variance: $\chi^2 = 13.63$, df = 6, $P = 0.0341$). Thus, the data
365 supported the hypothesis that the ENU mutagenesis introduced new genetic variation in
366 swimming speed.
367
368 We compared the magnitude of among-family variance following mutagenesis (Mutant
369 minus Control) to published estimates from spontaneous mutation accumulation studies on

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370 traits classified by Conradsen et al. (2022) as physiological; these traits have an average
371 magnitude of variance intermediate between fitness (life-history) and morphological traits
372 (Figure 4b of Conradsen et al. 2022). We expanded the dataset to include estimates of
373 *Drosophila serrata* locomotor activity at all temperatures assessed by Latimer et al. (2011).
374 Mutational variance in U_{sprint} in the current study was within the range observed for these
375 published estimates but was biased toward higher values (Figure 2).
376
377 The factor-analytic test of dimensionality best supported zero dimensions of among-family
378 variance in the Control treatment (Table 2A). However, the REML-MVN CI of the
379 eigenvalues supported two non-zero eigenvalues (Table 2A). Sztepanacz and Blows (2017)
380 demonstrated REML-MVN CI are less robust to sampling error than factor analytic
381 modelling and can lend statistical support to spurious covariance. While non-zero among-
382 family variance in speed was estimated at two temperatures (24°C and 31°C: Table 1A), the
383 model was unable to estimate (positive) variance at the other four temperatures and, further,
384 the 90% CI of the among-family variance at both 24°C and 31°C spanned a wide range of
385 negative through positive values (Table 1A) suggesting no statistical support for among-
386 family variance in speed at any temperature. Reflecting this, the among-Control-family
387 matrix was very ill-conditioned: the first eigenvalue (9.9; Table 2A) was substantially larger
388 than the trace of the matrix (sum of the diagonal in Table 1A = 4.3), and there was a negative
389 eigenvalue of nearly the same magnitude as the first (positive) eigenvalue (-9.3; Table 2).
390 Therefore, we conclude that the weight of evidence is consistent with no statistical support
391 for genetic variation in swimming speed (U_{sprint}) in this WIK population.
392
393 In the Mutant treatment, there was statistical support for one-dimension of among-family
394 variance (Table 2B), suggesting either that mutation had introduced variance in U_{sprint} at only

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395 one assayed temperature (consistent with GxE), or that the effects of mutations were
396 concordant across all assayed temperatures (consistent with no GxE). Although among-
397 family variance was only statistically distinct from zero at 24°C, 90% CI estimates at three
398 other temperatures were strongly skewed toward positive values, while the 90% CI of
399 variance estimates substantially overlapped across the six temperatures (Table 1B). All
400 pairwise covariances were positive (although only 26% were statistically distinct from zero:
401 Table 1B). The first eigenvalue (35.2; Table 2B) was three times larger than the largest
402 variance at any individual temperature and accounted for 95% of the total among-family
403 variance. The contribution of speed at each temperature to this axis of variation (i.e., the
404 eigenvector loadings: Table 3) were all in the same direction and of similar magnitude.
405 Therefore, we suggest the data provide evidence that mutations have consistent effects on
406 speed irrespective of the assay temperature, with no evidence of environment-specific effects.
407

408 **Discussion**

409 We detected phenotypic effects of mutation on a complex trait, swimming speed, but did not
410 detect any mutational variance for the plasticity of speed in response to heterogeneity in
411 water temperature. Mutations had concordant effects across the 18°C thermal gradient over
412 which speed itself varied by more than 35cm^{-1} second. Our results are consistent with
413 mutation introducing variation along a vertical shift, or faster-slower mode of thermal
414 performance variation (Huey and Kingsolver 1989; Izem and Kingsolver 2005). In the only
415 other study that we are aware of investigating mutational variance in locomotor thermal
416 performance, Latimer et al. (2014) characterised the contribution of spontaneous mutation to
417 variance in locomotor activity of *Drosophila serrata*, and similarly reported that most (76%
418 or 70% in males or females, respectively) mutational variance was associated with a faster-

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419 slower mode. This mode of variation is interpreted as reflecting pleiotropic mutations with
420 consistent direction of effects across all temperatures (Kingsolver et al. 2001), where
421 selection for increased (or decreased) performance at one temperature would lead to
422 correlated evolution at all temperatures, and under thermally heterogeneous conditions,
423 consistent selection would effectively fix (eliminate) variants.
424
425 In contrast to mutation predominantly influencing average performance (Latimer et al. 2014),
426 <1% of standing genetic variation in *D. serrata* was associated with the faster-slower mode;
427 rather, most variation was aligned with a specialist-generalist mode (where the width of the
428 performance curve varies) (Latimer et al. 2011). Other studies of standing genetic variation of
429 thermally dependence in non-locomotor traits have also suggested little genetic variation
430 associated with the faster-slower mode (e.g., Izem and Kingsolver 2005). Notably, mutational
431 correlations among life-history traits are typically more strongly positive than the
432 corresponding standing genetic correlation, reflecting selective elimination of concordantly
433 deleterious mutations (Houle et al. 1994; Estes and Phillips 2006; McGuigan et al. 2011).
434 Latimer et al. (2014) similarly suggested that the mismatch of mutational and standing
435 genetic variation might indicate that most mutations affecting performance are deleterious,
436 and do not persist in standing genetic variation. However, other studies of standing genetic
437 variation in thermal performance curves for individual or population-level growth have
438 provided contradictory evidence, suggesting that most (rather than least) variation is
439 associated with a faster-slower mode (Yamahira et al. 2007; Moghadam et al. 2020), and
440 several recent studies of locomotor performance found statistical support for heritability only
441 of curve height (i.e., faster-slower variation) and not optimal temperature or curve width
442 (Logan et al. 2018; Martins et al. 2019). To resolve the contrary predictions that standing
443 genetic variation reflects selective process (Latimer et al. 2014) versus mutational limits

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444 (Yamahira et al. 2007) further data on the phenotypic variation introduced by mutation to
445 thermal performance traits will be required. The distribution of mutations may influence
446 molecular (Cano et al. 2022) and phenotypic (Houle et al. 2017) adaptation, and
447 understanding whether standing genetic variation reflects limited input of the type of
448 variation that would support local versus global adaptation is necessary for predicting
449 adaptive responses to changing thermal conditions.
450
451 We observed no evidence that mutations had a biased direction of effect on swimming speed,
452 consistent with a previous investigation of the effects of ENU-induced mutation on WIK
453 zebrafish prolonged swimming speed at 28°C (McGuigan and Aw 2017). For fitness,
454 mutation is predicted to be, and empirically supported as, typically biased toward lower
455 values (Keightley and Lynch 2003; Keightley and Eyre-Walker 2007; Halligan and Keightley
456 2009). However, theoretical models of the maintenance of genetic variance in non-fitness
457 traits typically assume no overall directional trend in the effects of mutations (Barton 1990;
458 Kondrashov and Turelli 1992; Johnson and Barton 2005; Martin and Lenormand 2006);
459 deviation from this expectation could result in substantial directional selection on traits
460 (Zhang and Hill 2008). ENU-induced mutations in guppies decreased the rate of courtship
461 displays (Herdegen and Radwan 2015), while spontaneous mutations decreased *D.*
462 *melanogaster* larval crawling speed, adult heat tolerance, overall coordination (Huey et al.
463 2003) and male escape speed (Shabalina et al. 1997), and decreased velocity of
464 *Caenorhabditis elegans* (Ajie et al. 2005). In contrast, spontaneous mutations decreased *D.*
465 *serrata* locomotor activity only at the hottest assayed temperatures, increasing activity across
466 a wide range of cooler temperatures (Latimer et al. 2014). Other performance traits (e.g.,
467 feeding rate and adult walking speed: Huey et al. 2003) showed no shift in trait mean under
468 mutation accumulation. Given that these studies of whole-organism performance traits have

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469 reported mutational effects that span the full spectrum, further empirical and theoretical
470 consideration of the evolutionary genetic consequences of non-symmetrical mutations for
471 non-fitness traits is required. Differences among traits and studies may simply reflect
472 unpredictable stochastic effects of sampling from the same, complex, distribution of effects.
473 However, several factors, including study design, the traits themselves, genetic background,
474 distribution of dominance, and the environmental range considered, may influence the
475 distribution of phenotypes generated by mutation.
476
477 A challenge faced by classical multi-generational mutation accumulation experiments is
478 evolution of the ancestor (discussed in Lynch et al. 1999). Some heterogeneity in direction of
479 effect may reflect estimation errors arising from changes in the ancestor. This problem is
480 avoided using mutagenesis, where the unmutated comparison population has not had time to
481 evolve. Notably, while classical mutation accumulation experiments are typically initiated
482 from a single ancestral genotype (inbred line), mutagenesis experiments introduce mutation
483 to multiple (here, 50) genetic backgrounds simultaneously. Spontaneous mutation rate (Sharp
484 and Agrawal 2012; Schrider et al. 2013; Huang et al. 2016) and distribution of mutational
485 fitness effects (Fisher 1930; Orr 1998; Silander et al. 2007; Stearns and Fenster 2016) are
486 influenced by ancestral genotype. Whether bias in the direction of mutational effects on non-
487 fitness traits can be (partially) explained by genetic background, or the contribution of the
488 trait to fitness, remains to be determined.
489
490 Observation of both thermally dependent and directionally biased effects of mutations may
491 also be influenced by dominance. We focused here on heterozygous effects: new mutations
492 will be expressed only in heterozygotes due to their rarity, and it is therefore their
493 heterozygous effect that will determine the selection they experience. Our observation that

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494 mutation significantly increased genetic (among-family) variance in speed indicates that at
495 least some induced mutations were not fully recessive in their effects on the focal trait.
496 Mutations with larger homozygous deleterious effects on fitness tend to be more recessive
497 (Agrawal and Whitlock 2011), but the joint distribution of dominance coefficients of
498 pleiotropic mutations affecting fitness and other traits is not well characterised. Mutations
499 causing notable defects in larval swimming performance have been reported to be typically
500 homozygous lethal (Granato et al. 1996), suggesting directional dominance on fitness is
501 correlated with directionality of effects on speed for these large-effect mutations. Similarly, a
502 study of a large cohort of racehorses found evidence of a negative correlation between an
503 individual's inbreeding coefficient and race performance, consistent with correlation of
504 directional dominance of fitness with effects on performance (Todd et al. 2018). Correlated
505 environment-dependent reversals in dominance (recessive to dominant) and fitness
506 (deleterious to beneficial) effects of mutations are predicted to accelerate adaptation
507 (Muralidhar and Veller 2022). Here, the observed concordance of mutational effects on speed
508 are not consistent with environment-dependent changes in the dominance coefficient.
509 Nonetheless, our data cannot exclude the possibility of an unobserved class of undetected
510 mutations with recessive effects that had directionally biased, or non-concordant pleiotropic
511 effects (antagonistic or conditionally neutral), shifting mean speed and generating genotype-
512 by-environment interaction variation.
513
514 We assayed a set range of temperatures (16°C – 34°C), and while speed varied markedly with
515 temperature, it was nonetheless notable that average speed changed very little between 28°C
516 and 34°C (Figure 1). We cannot exclude the possibility that the effects of the sampled
517 mutations on swimming speed were different outside the considered thermal range. The
518 frequency distribution of fitness effects may be shifted under hotter temperatures (Xu 2004;

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519 Chu et al. 2020). However, direct effect of temperature on population growth rate in
520 microbes, where population size limits (bottlenecks) are not imposed per generation, may
521 allow greater opportunity for selection to shift the observed frequency of mutations at hot
522 relative to cool temperatures (Wahl and Agashe 2022), with stronger selection on deleterious
523 mutations under hotter conditions (Berger et al. 2021).
524
525 Consistent with Latimer et al. (2014), we assessed adult phenotypes under acute exposure to
526 different temperatures. The timing and duration of exposure to heterogeneous environments
527 can affect thermal performances (Rezende et al. 2014; Kellermann et al. 2019; Pottier et al.
528 2022), and the observed distribution of mutational effects may likewise change. Fully
529 replicating multiple rearing temperatures to impose long-term exposure, (e.g., through larval
530 development) is logistically limiting. Further, temperature-dependent effects on larval
531 viability may result in the phenotypic effects of different mutations being sampled at different
532 temperatures. Selection acting among siblings within a family of an ENU mutated sire can
533 affect the frequency of mutations within families and observation of their phenotypic effects
534 (Walsh and McGuigan 2018). Temperature also affects development rate in ectotherms,
535 potentially result in confounding of the effects of mutation, development and temperature.
536 We previously showed swimming speed in adult zebrafish changes with age, independent of
537 size or known maturation and senescence boundaries (McGuigan and Aw 2017). While
538 natural populations encounter varying temperatures throughout life, disentangling how
539 genetic effects cause plastic responses to that variation is challenging.
540
541 Our results by no means suggest the absence of any class of mutations with non-concordant
542 pleiotropic effects across temperatures. Quantitative genetic parameters are notoriously
543 difficult to accurately estimate, given their relatively large sampling error (Klein et al. 1973;

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544 Klein 1974). Notably, standing genetic variance for thermal performance traits was below
545 statistical detection limits in several recent studies (Driessen et al. 2007; Logan et al. 2018;
546 Martins et al. 2019; Logan et al. 2020; Bodensteiner et al. 2021). Our results (limited
547 statistical support for heritable variation per temperature, but strong support for heritable
548 variation in speed when considering all data) suggest that, given the pervasive presence of
549 temperature-independent mutational effects, we benefited from repeated measures of the
550 same phenotype to improve estimation. A recent investigation suggested that sampling error
551 is likely to make a non-negligible contribution to heterogeneity among estimates of
552 mutational variance (Conradsen et al. 2022). Similarly, a recent meta-analysis of population
553 means of thermal physiological limit traits suggested that < 8% of 428 estimates were
554 supported by sufficient sample sizes for the mean to be estimated with a high-level of
555 accuracy (Duffy et al. 2021). Thermally dependent traits are likely to remain the focus of
556 understanding how populations can persist under and adapt to changing conditions, but we
557 suggest that the experimental effort involved in obtaining useful and robust parameter
558 estimates is far from trivial, while the risk of inaccurate estimation must also be carefully
559 considered. In the current study, we collected a total of 3,497 measures of speed, which took
560 677 person hours to record (without accounting for time taken for acclimating fish prior to
561 trials, or any aspect of breeding and husbandry). Without additional information on the
562 relative frequency and effect size of mutations with thermally dependent effects it is not
563 simple to predict how much larger an experiment will be required to detect, above
564 measurement error, and characterise other, non-concordant patterns of variation across
565 temperatures. The apparent benefit of repeated measures suggests that improved precision of
566 measurement of performance (via within individual and within family replication) will be
567 important, along with increasing the number of genotypes (families), which would support
568 sampling of more mutations.

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569

570 Mutagenesis could greatly expand the range of taxa in which the distribution of mutational
571 effects on phenotype (and fitness) can be explored. The mutational variance we observed for
572 sprint speed was greater than the per-generation rate of mutational input reported from
573 classical mutation accumulation (MA) studies of related traits (Figure 2), suggesting that we
574 had induced more mutations than typically sampled in long-running MA experiments
575 (although differences in effect size or the greater mutational target size of speed than an
576 individual enzyme or metabolite may also have contributed). Ideally, as is possible in
577 microbial systems, phenotypic effects of individual mutations would be considered.
578 However, isolating individual mutations to characterise their effect remains challenging in
579 higher eukaryotes. Genome sequencing of multicellular eukaryote MA lines, maintained over
580 tens to hundreds of generations, typically reveal multiple (~20 – 90) mutations per line
581 accumulating over these time frames (Schrider et al. 2013; Huang et al. 2016; Assaf et al.
582 2017; Flynn et al. 2017). When MA lines (or here, families) diverge from one another at
583 multiple loci, strong correlations among traits (here, speed at the different temperatures) can
584 arise through both heterogeneity of mutation number among lines or via linkage, but such
585 spurious correlations depend on mutations having biased, unidirectional, effects on the traits
586 (Keightley et al. 2000). However, co-segregation of mutations having opposing effects on the
587 same traits may have obscured patterns arising from more complex (antagonistic or
588 conditionally independent) pleiotropic effects. More generally, epistatic interactions among
589 mutations may influence the observed patterns of phenotypic variation, as apparent in the
590 effect of genetic background on the frequency of beneficial mutations (Silander et al. 2007;
591 Perfeito et al. 2014; Stearns and Fenster 2016). In the current experiment, there was potential
592 for epistatic interactions to influence observed patterns of phenotypic variation both due to

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593 among-sire differences in their genotype (i.e., genetic background), and due to the specific set
594 of mutations induced in each sire.
595
596 Further information is also required on how well the induced mutations reflect the spectrum
597 of naturally arising mutations (Katju and Bergthorsson 2019). The zebrafish genome is large
598 (1400 Mb) relative to other multicellular eukaryotic taxa for which extensive mutational data
599 exist (100 – 200 Mb for *D. melanogaster*, *Daphia pulex*, *Caenorhabditis* species and
600 *Arabidopsis thaliana*), and, in contrast to these other models in which a genetically
601 homogeneous ancestor can be rapidly established, the longer generation time of zebrafish
602 (and other vertebrates) makes this more challenging. Novel mutations must therefore be
603 accurately detected against a background segregating genetic variation (Coe et al. 2009;
604 Brown et al. 2012; Butler et al. 2015; Suurvali et al. 2020). Thus, while studies of zebrafish
605 (and other vertebrate models) can exploit considerable genomic tools, dissecting
606 contributions from overall mutation number versus effect sizes will be challenging.
607
608 Heterogeneity in allelic effects among environmental contexts contribute to phenotypic
609 diversity and adaptive potential of natural populations. The role of mutation in shaping the
610 distribution of this diversity has the potential to influence direction and rate of evolution of
611 populations. The limited amount of evidence to date suggests that mutations with concordant
612 effects across thermal gradients are more frequent or of larger effect than mutations with
613 antagonistic or conditionally neutral effects. However, the patterns of intra and inter-specific
614 genetic diversity of thermally dependent traits suggest major contributions from genetic
615 variants with non-concordant (antagonistic or conditionally neutral) effects. Further
616 investigation of mutational effects is required to reconcile these observations. Studies
617 addressing this knowledge gap will need to be large, ensuring that effects can be accurately

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618 determined. Mutagenesis may facilitate studies across more taxa, and reduce the logistical
619 burden of long-term maintenance.

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992 **Table 1 The among-family variance-covariance matrix for Control (A) and Mutant (B)**
993 **treatments.** The REML-MVN confidence intervals are reported below their respective
994 estimates (90% CI for the variances on the diagonal, a one-tailed test against zero; 95% for
995 covariances on the lower off-diagonal, a two-tailed test against zero). Parameters whose CI
996 do not include zero are shown in bold. Estimates are from a model fitting an unconstrained
997 among-family variance-covariance matrix. No CI could be estimated where the REML
998 estimate was zero.

999

A. Control						
	16°C	20°C	24°C	28°C	31°C	34°C
16°C	0					
20°C	1.382 (-3.72, 6.45)	0				
24°C	1.506 (-3.46, 6.64)	4.611 (-2.06, 11.48)	3.077 (-5.82, 11.8)			
28°C	0.293 (-3.25, 3.74)	-2.645 (-7.70, 2.38)	-4.649 (-9.93, 0.62)	0		
31°C	0.492 (-4.19, 5.03)	-2.639 (-8.35, 3.03)	-0.537 (-7.32, 6.14)	-1.597 (-6.23, 3.03)	1.183 (-5.64, 7.76)	
34°C	-1.168 (-5.26, 2.88)	0.230 (-4.98, 5.44)	-4.109 (-10.09, 1.77)	-3.832 (-7.99, 0.39)	-4.964 (-9.99, 0.03)	0
B. Mutant						
	16°C	20°C	24°C	28°C	31°C	34°C
16°C	2.551 (-0.76, 5.83)					
20°C	3.102 (-1.65, 7.70)	6.867 (-0.52, 14.47)				
24°C	**5.966** (0.14, 11.86)	8.204 (-0.57, 16.96)	**11.697** (0.55, 22.90)			
28°C	**6.255** (1.33, 11.23)	6.452 (-0.61, 13.78)	**10.175** (1.53, 18.74)	5.519 (-2.39, 13.29)		
31°C	4.421 (-0.33, 9.23)	4.532 (-2.75, 11.88)	6.860 (-2.19, 15.92)	2.076 (-5.23, 9.34)	4.973 (-2.82, 12.64)	
34°C	**4.577** (0.12, 8.88)	3.371 (-2.80, 9.59)	6.710 (-1.07, 14.20)	5.084 (-1.56, 11.52)	3.275 (-3.08, 9.66)	5.616 (-0.23, 11.27)

1000

Table 2. Dimensionality of among-family covariance matrices for A) Control and B) Mutant treatments. The results from fitting a nested series of reduced rank co-variance matrices at the among-family level are reported, where the Factor number corresponds to the number of dimensions modelled. Differences in the log-likelihood ratio between sequential models follows a chi-square distribution, with the degrees of freedom (df) corresponding to the difference in the number of parameters between the models. The Akaike Information Criterion (AIC) is also reported (with the smallest AIC, indicating the best fit model, shown in bold). The eigenvalues of the unconstrained among-family matrix estimated in each treatment (Table 1) are also reported, along with the REML-MVN 90% CI. In the Control treatment, the model constrained to six dimensions did not properly converge.

Factor	# Parameters	−2log-likelihood	AIC	χ^2	df	*P*-value	Eigenvector	Eigenvalue	90% CI
				A. Control					
0	42	12043.4	**12121.4**						
1	48	12031.9	12121.9	11.5	6	0.0740	1	9.884	(3.54, 16.33)
2	53	12021.5	12121.5	10.4	5	0.0647	2	6.996	(2.81, 11.16)
3	57	12021.3	12127.3	0.1	4	0.9979	3	1.655	(-2.07, 5.37)
4	60	12021.3	12133.3	<0.0	3	0.9995	4	-0.770	(-3.72, 2.09)

5	62	12021.3	12135.3	<0.0	2	>0.9999	5	-4.175	(-8.78, 0.54)
6	63	*NA*	*NA*	*NA*	*NA*	*NA*	6	-9.331	(-18.85, -0.18)
				B. Mutant					
0	42	11913.6	11997.6						
1	48	11900.1	**11992.1**	13.5	6	0.0364	1	35.201	(11.57, 58.6)
2	53	11898.2	12000.2	2.0	5	0.8550	2	3.462	(-1.20, 8.14)
3	57	11896.8	12004.8	1.4	4	0.8475	3	3.173	(-0.69, 7.03)
4	60	11896.8	12012.8	<0.0	3	0.9993	4	0.361	(-3.29, 4.06)
5	62	11896.8	12016.8	<0.0	2	>0.9999	5	-0.502	(-4.94, 4.00)
6	63	11896.8	12014.8	<0.0	1	0.9261	6	-4.474	(-9.52, 0.46)

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Table 3. Major axes of among-family variance in swimming speed. The eigenvector loadings for the first and second axes of the unconstrained estimate of among-family variance in the Control (i.e., g_{max} and g_2) and Mutant (i.e., m_{max} and m_2) treatments are shown. The corresponding eigenvalues are reported in Table 2.

	Control		Mutant	
	g_{max}	g_2	m_{max}	m_2
16°C	0.184	-0.149	0.317	0.252
20°C	0.482	0.232	0.394	-0.537
24°C	0.737	-0.130	0.591	-0.064
28°C	-0.410	-0.239	0.434	0.274
31°C	-0.022	-0.622	0.306	-0.478
34°C	-0.147	0.681	0.336	0.584

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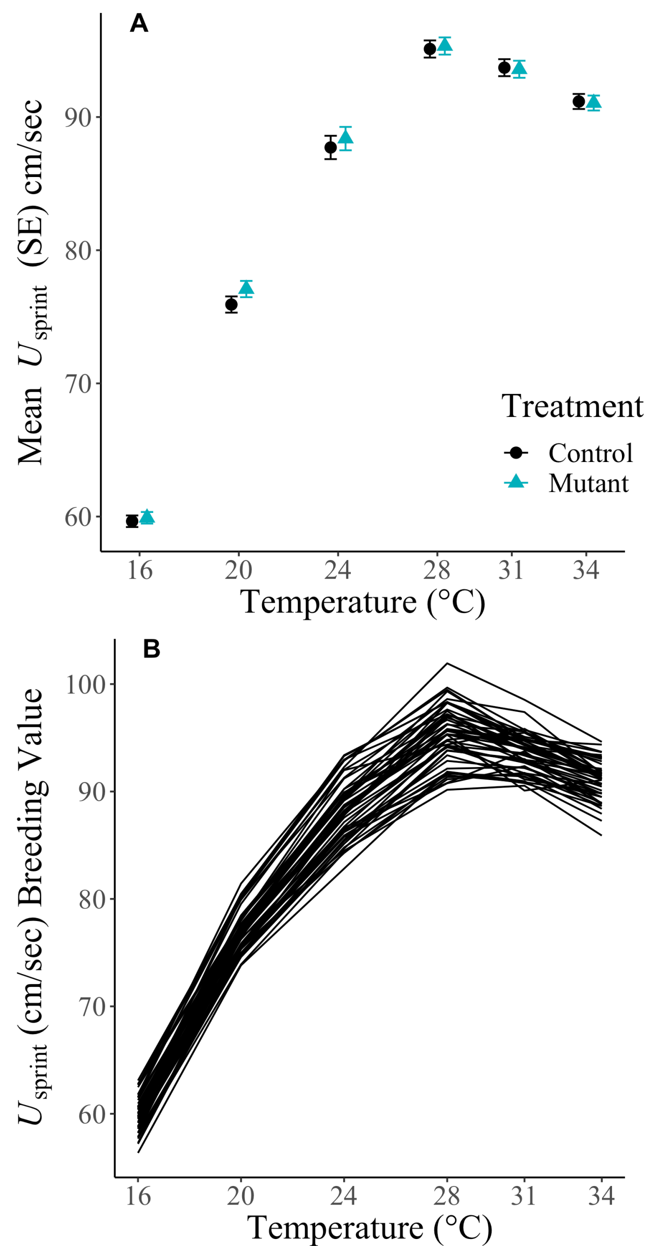

Figure 1. **Variation in swimming speed with temperature.** A) Treatment mean swimming speeds. Plotted are the least squares means (± SE) from model (1) at each of the six temperatures for the Control (black circles) and Mutant (blue triangles) treatments. B) Among Mutant family variation in swimming speed. Plotted are the Best Linear Unbiased Predictors (BLUPs) at each of the six temperatures from model (1) (fit to centred data) for the

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Mutant treatment, with lines connecting the point estimates for each family. The Control treatment BLUPs are not presented as they were invariant at most temperatures (Table 1).

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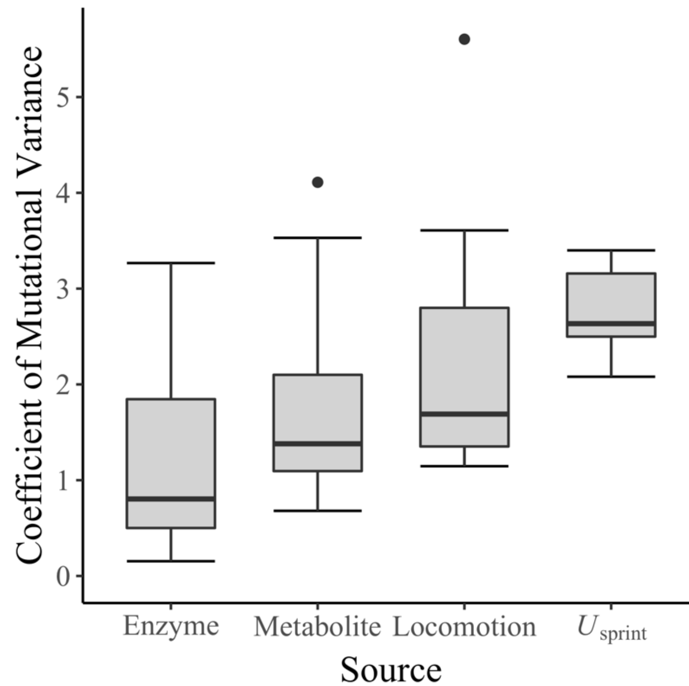

Figure 2. Comparison of the magnitude of mutational variance. Estimates of mutational variance were placed on a coefficient of variance scale: CV = 100 x $\sqrt{V_M}/\bar{X}$ where V_M was the mutational variance and $\bar{X}$ was the trait mean. Plotted are the median (black band), interquartile range (IQR; box) and three times the IQR (whiskers) plus any more extreme observations (circles). The 27 estimates of enzyme activity come from two studies in *Drosophila melanogaster* (Clark et al. 1995; Harada 1995), while the 27 estimates of metabolite pool size come from one study in *Caenorhabditis elegans* (Davies et al. 2016), derived as detailed Table S1 of Conradsen et al. (2022). The 16 locomotion estimates include male and female *D. serrata* activity measured at each of six temperatures (Table 1 of Latimer et al. 2014) as well as turn rate and velocity in *C. elegans* (Ajie et al. 2005; Estes et al. 2005) (derived as described in Table S1 of Conradsen et al. 2022). The six U_{sprint} estimates from the current study are the difference in among-family variance between Mutant and Control treatments, scaled by the Mutant treatment mean speed at that temperature.