1 Article Type: Letter

2 Article Title: **Thermal tolerance and preference are both consistent with the clinal**

3 **distribution of house fly proto-Y chromosomes**

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13

Abstract

Selection pressures can vary within localized areas and across massive geographical scales. Temperature is one of the best studied ecologically variable abiotic factors that can affect selection pressures across multiple spatial scales. Organisms rely on physiological (thermal tolerance) and behavioral (thermal preference) mechanisms to thermoregulate in response to environmental temperature. In addition, spatial heterogeneity in temperatures can select for local adaptation in thermal tolerance, thermal preference, or both. However, the concordance between thermal tolerance and preference across genotypes and sexes within species and across populations is greatly understudied. The house fly, *Musca domestica*, is a well-suited system to examine how genotype and environment interact to affect thermal tolerance and preference. Across multiple continents, house fly males from higher latitudes tend to carry the male-determining gene on the Y chromosome, whereas those from lower latitudes usually have the male-determiner on the third chromosome. We tested whether these two male-determining chromosomes differentially affect thermal tolerance and preference as predicted by their geographical distributions. We identify effects of genotype and developmental temperature on male thermal tolerance and preference that are concordant with the natural distributions of the chromosomes, suggesting that temperature variation across the species range contributes to the maintenance of the polymorphism. In contrast, female thermal preference is bimodal and largely independent of congener male genotypes. These sexually dimorphic thermal preferences suggest that temperature-dependent mating dynamics within populations could further affect the distribution of the two chromosomes. Together, the differences in thermal tolerance and preference across sexes and male genotypes suggest that different selection pressures may affect the frequencies of the male-determining chromosomes across different spatial scales.

37 **Impact Statement**

38 Genetic variation within species can be maintained by environmental factors that vary
39 across the species' range, creating clinal distributions of alleles responsible for ecologically
40 important traits. Some of the best examples of clinal distributions come from temperature-
41 dependent phenotypes, such as thermal tolerance and preference. Although genotype and
42 developmental temperature strongly affect physiological and behavioral traits in ectotherms, the
43 correlation between these traits across genotypes and sexes within species is greatly understudied.
44 We show that two different male-determining chromosomes found in natural populations of
45 house flies affect both thermal tolerance and preference in a way that is concordant with their
46 clinal distributions across latitudes. This provides strong evidence that temperature variation
47 across the species range contributes to the maintenance of the polymorphism. Furthermore, we
48 find evidence that thermal preference is sexually dimorphic, suggesting that temperature-
49 dependent mating dynamics could further affect the distribution of genetic variation in this
50 system. Therefore, at a macro-geographical scale, the differences in thermal tolerance and
51 preference across male genotypes likely contributes to the maintenance of the cline. Within
52 populations, differences in thermal preference likely affect sexual selection dynamics, which may
53 further affect the frequencies of the chromosomes.

54 **Introduction**

55 Ecological variation across a species' range can select for local adaptation within
56 populations, which can contribute to the maintenance of genetic variation by favoring different
57 alleles across the range (Levene, 1953; Felsenstein, 1976; Hedrick *et al.*, 1976; Kawecki & Ebert,
58 2004). In addition, heterogeneous selection pressures that are distributed as a gradual continuum
59 from one end of the species' range to another can create a cline of genetic variation responsible
60 for phenotypes under selection (Slatkin, 1973; Endler, 1977). Some of the best examples of
61 latitudinal clines come from temperature-dependent phenotypes (e.g., body size, developmental
62 rate, and thermal tolerance) that have been well-documented in flies (Partridge *et al.*, 1994;
63 Eanes, 1999; Robinson & Partridge, 2001; Hoffmann *et al.*, 2002). Moreover, heterogeneous
64 selection pressures across a cline may affect males and females differently (Connallon, 2015;
65 Connallon *et al.*, 2019), although the empirical evidence for such variation in sex-specific
66 selection across geographic ranges is mixed (Delcourt *et al.*, 2009; Delph *et al.*, 2011; Allen *et*
67 *al.*, 2017; Lasne *et al.*, 2018).

68 Thermal adaptation within populations and across a species range can occur via selection
69 on physiological, anatomical, or behavioral traits. For example, north-south gradients in heat and
70 cold tolerance have been observed in *Drosophila* (Hoffmann *et al.*, 2002), suggesting
71 physiological adaptation to thermal environments. In addition, ectotherms, such as flies, rely on
72 behavioral mechanisms of thermoregulation by avoiding suboptimal temperatures in search of
73 more optimal ones (Dillon *et al.*, 2009; Kearney *et al.*, 2009), and thermal preference may be
74 correlated with optimal thermal performance (Dawson, 1975; Angilletta *et al.*, 2002).

75 Concordance across genotypes between different thermal traits could reinforce the
76 response to selection, whereas negative correlations could constrain adaptation (Etterson & Shaw,

77 2001). However, it is not clear if physiological and behavioral thermal traits are genetically
78 correlated within a species, between sexes, or across populations (Dawson, 1975; Angilletta *et*
79 *al.*, 2002; Gilbert & Miles, 2017). For example, experiments in *Drosophila subobscura* identified
80 individual chromosomes that affected thermal tolerance or temperature preference, but no single
81 chromosome affected both physiological and behavioral phenotypes (Dolgova *et al.*, 2010; Rego
82 *et al.*, 2010; Castañeda *et al.*, 2019). Furthermore, temperature-dependent traits can affect
83 assortative mating and male reproductive success (Dolgin *et al.*, 2006; Keller & Seehausen,
84 2012), suggesting inter-sexual differences in thermoregulation could affect genetic variation
85 within populations via sexual selection. These sex-specific selection pressures could also
86 contribute to the maintenance of genetic variation via inter-sexual conflict or context-dependent
87 selection (Kotiaho *et al.*, 2001; Rostant *et al.*, 2015; Meisel, 2018). Despite the importance of
88 inter-sexual differences, previous work did not test for differences in the genetic correlation of
89 thermal traits between males and females.

90 We used a sex chromosome polymorphism in the house fly, *Musca domestica*, to
91 investigate the concordance of thermal tolerance and preference across clinally distributed male
92 genotypes. House fly has a polygenic sex determination system, in which a male-determining
93 gene has been mapped to all six chromosomes, some males can carry multiple male-determining
94 chromosomes, and a female-determining allele segregates on one chromosome (McDonald *et al.*,
95 1978; Inoue & Hiroyoshi, 1986; Dübendorfer *et al.*, 2002; Hediger *et al.*, 2010). The *M.*
96 *domestica male determiner* (*Mdmd*) gene is most commonly found on either the third
97 chromosome (III^M) or what was historically referred to as the Y chromosome (Y^M) (Hamm *et al.*,
98 2014; Sharma *et al.*, 2017). Both III^M and Y^M are very young proto-Y chromosomes that are
99 minimally differentiated from their homologous proto-X chromosomes (Meisel *et al.*, 2017; Son

100 *et al.*, 2019; Son & Meisel, 2020). Y^M and III^M are distributed along latitudinal clines on multiple
101 continents in the Northern Hemisphere (Tomita & Wada, 1989; Hamm *et al.*, 2005; Kozielska *et*
102 *al.*, 2008). Y^M is most frequently found at northern latitudes, and III^M is more common at
103 southern latitudes (Figure 1A). This distribution suggests that the Y^M chromosome confers higher
104 fitness in colder climates, and, conversely, III^M confers higher fitness in hotter climates.
105 Therefore, variation in temperature across the species range may create heterogeneous selection
106 pressures that maintain the proto-Y chromosome cline in house fly. Consistent with this
107 hypothesis, seasonality in temperature is the best predictor of the frequencies of the proto-Y
108 chromosomes across natural populations (Feldmeyer *et al.*, 2008).
109 We tested the hypothesis that the Y^M chromosome confers cold-adaptive phenotypes and
110 III^M confers heat-adaptive phenotypes in house fly males, which would be consistent with their
111 latitudinal distributions (Tomita & Wada, 1989; Hamm *et al.*, 2005; Feldmeyer *et al.*, 2008;
112 Kozielska *et al.*, 2008). To those ends, we first evaluated if males carrying the III^M chromosome
113 (hereafter III^M males) have greater tolerance to extreme heat and if males carrying the Y^M
114 chromosome (Y^M males) have greater cold tolerance. Second, we tested if III^M males prefer
115 warmer temperatures than Y^M males, and if males and females differ in their thermal preference.
116 We performed all experiments using flies raised at multiple developmental temperatures because
117 thermal acclimation strongly affects temperature-dependent phenotypes in flies and other
118 ectotherms (Krstevska & Hoffmann, 1994; Dillon *et al.*, 2009). Together, we evaluated if thermal
119 preference and tolerance are aligned for sex-linked genetic variants, tested if this alignment is
120 consistent with the geographic distribution of the proto-Y chromosomes, and then discuss how
121 these temperature-dependent phenotypes could affect the access of males to female mates.

Materials and methods

Fly strains and rearing

We performed our experiments using five nearly isogenic house fly strains, three with III^M males and two with Y^M males (Supplementary Methods). All five strains have a common genetic background from an inbred III^M strain that was produced from a mixture of flies collected across the United States (Scott *et al.*, 1996; Hamm *et al.*, 2005). Each of the three III^M strains carries a different III^M chromosome from a separate wild-derived line, and, likewise, the two Y^M strains carry different Y^M chromosomes. Each strain is fixed for its proto-Y chromosome (either III^M or Y^M), and no other sex determiners, such as the female-determining *Md-tra*^{D} allele (Hediger *et al.*, 2010), segregate within these strains.

We reared each strain at 18°C, 22°C, and 29°C for two generations in order to evaluate how thermal acclimation affects thermal tolerance (Chown & Terblanche, 2006) and thermal preference (Krstevska & Hoffmann, 1994; Dillon *et al.*, 2009). Flies from each developmental temperature were assayed at equivalent physiological ages estimated by accumulated degree days (Barnard & Geden, 1993; Wang *et al.*, 2018). For our heat and cold tolerance assays, we used flies 22–50 total degree days after eclosion. For thermal preference assays, we used flies 96–115 total degree days after eclosion. Additional details and calculations are provided in the Supplementary Methods.

Thermal tolerance

We measured heat and cold tolerance in individual male and female house flies. To measure heat tolerance, lightly anaesthetized individual flies were transferred to a 1.5 ml centrifuge tube that was sealed with fabric. We placed the 1.5 ml tube in a heat block set to 53°C.

144 This temperature was selected because it is the lowest at which heat tolerance could be measured
145 in a reasonable period of time. The time at which a fly fell to the bottom of the tube and could not
146 make its way back to the top was considered the knockdown time. To measure cold tolerance,
147 lightly anaesthetized flies were transferred to a fabric-sealed 20 ml glass vial individually, and the
148 vials were placed in a 4°C refrigerator with a transparent door. Knockdown occured when a fly
149 fell on its back to the bottom of the vial. We gently tapped the assay vial every 2–3 minutes to
150 ensure flies were active.

151 For both heat and cold tolerance assays, we performed an analysis of variance (ANOVA)
152 using the lmer() function in the lme4 (v1.1) R package (Bates *et al.*, 2015) to model the effect of
153 genotype (G: Y^M vs III^M), developmental temperature (T: 18°C or 29°C), and their interaction on
154 knockdown time (K):

155 $$K \sim G + T + G \times T + B + S,$$

156 with experimental batch (B) and strain (S) treated as random effects. We also constructed another
157 model excluding the interaction term:

158 $$K \sim G + T + B + S.$$

159 We then used a drop in deviance test to compare the fit of the models with and without the
160 interaction term using the anova() function in R. We also compared heat and cold tolerance
161 between males raised at 22°C and 29°C, using the same approaches as described above. As the
162 thermal tolerance comparisons between flies raised at 18°C and 29°C and between flies raised at
163 22°C and 29°C were conducted in separate experimental batches, we analyzed each comparison
164 separately. Additional details are provided in the Supplementary Methods.

165 ***Thermal preference***

166 We measured thermal preference as the position of individual flies along a 17–37°C
167 thermal gradient (Figure S1), following a slightly modified version of previous protocols
168 (Anderson *et al.*, 2013; Lynch *et al.*, 2018). For each individual fly, we report mean thermal
169 preference (T_{pref}) as the average position during a 10 minute assay window (measured once per
170 minute). We also report thermal breadth, $T_{breadth}$ (Carrascal *et al.*, 2016), as the coefficient of
171 variation of individual-level T_{pref} during the assay window. $T_{breadth}$ provides an estimate of how
172 individuals utilize thermal space within their environment (Slatyer *et al.*, 2013). Choosier
173 individuals show a lower $T_{breadth}$ value and, thus, would be expected to occupy a narrower range
174 of temperatures within a given thermal habitat.

175 To determine the effects of developmental temperature (18°C, 22°C, and 29°C), genotype
176 (Y^M and III^M), and their interaction on mean T_{pref} across sexes, we created a mixed-effects model
177 using the lme4 package (v1.1) in R (Bates *et al.*, 2015). Developmental temperature, genotype,
178 and their interaction were included as fixed effects, and strain, batch, and lane in the thermal
179 gradient (L) were included as random effects:

180 $$T_{pref} \sim G + T + G{\times}T + B + S + L.$$

181 We did the same for $T_{breadth}$. We then determined whether groups significantly differed in T_{pref} or
182 $T_{breadth}$ using Tukey contrasts with the multcomp package (v1.4) in R (Hothorn *et al.*, 2008).
183 Within developmental temperature treatments, we used Bayesian information criterion (BIC)
184 scores from the mclust (v5.4.5) package in R (Scrucca *et al.*, 2016) to determine whether the
185 distribution of individual measures of T_{pref} within a group are best explained by one or multiple
186 normal distributions.

187 **Results**

188 ***Thermal tolerance depends on developmental temperature and male genotype***

189 We measured extreme heat (53°C) and cold (4°C) tolerance as a readout of differences in
190 physiological thermal adaptation between Y^M and III^M house fly males. We observed the
191 expected effect of acclimation on both heat and cold tolerance (Chown & Terblanche, 2006): flies
192 raised at 18°C tolerate cold longer than the flies raised at 29°C, and flies raised at 29°C tolerate
193 heat longer than flies raised at 18°C (Figure 1). We also find that Y^M males are more cold
194 tolerant, and III^M males are more heat tolerant, consistent with the latitudinal distributions of Y^M
195 and III^M males in nature (Tomita & Wada, 1989; Hamm *et al.*, 2005; Feldmeyer *et al.*, 2008;
196 Kozielska *et al.*, 2008). However, the effect of genotype on thermal tolerance depends on
197 acclimation temperature. Specifically, a linear model with an interaction between genotype (Y^M
198 or III^M) and developmental temperature fits the cold tolerance data significantly better than a
199 model without the interaction term ($\chi^2_1 = 19.3$, $p = 1.1 \times 10^{-5}$). This provides evidence for a G×T
200 effect on cold tolerance—Y^M males are more cold tolerant than III^M males, but only if they are
201 raised at 18°C (Figure 1B). There is also a significant G×T interaction affecting heat tolerance
202 ($\chi^2_1 = 4.71$, $p = 0.030$ comparing models with and without the interaction term): III^M males are
203 more heat tolerant than Y^M males, but only if raised at 29°C (Figure 1D).

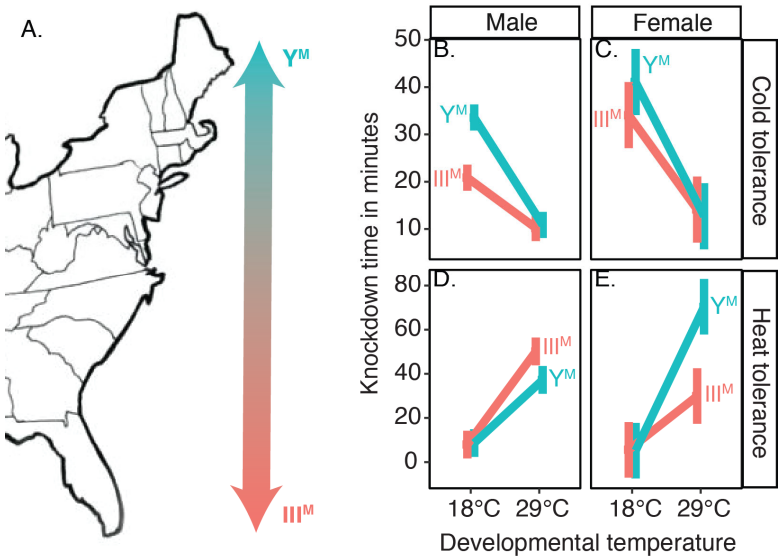
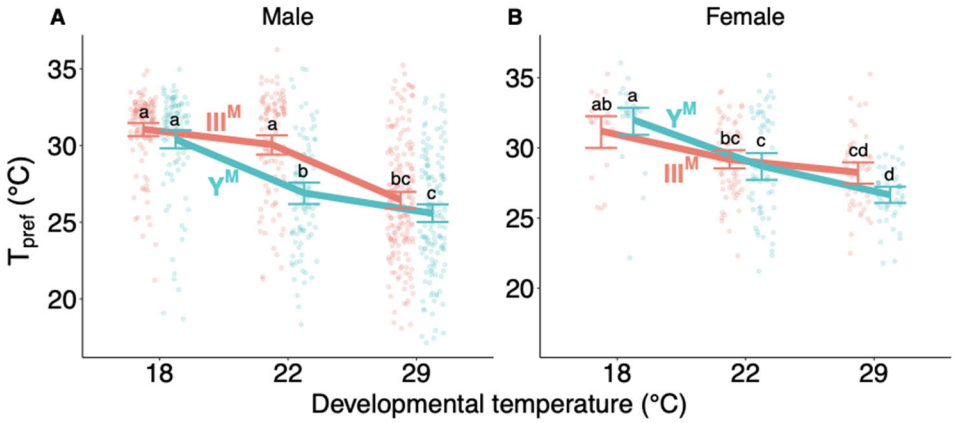


Figure 1 - Thermal tolerance in males and females. (**A**) Map of the eastern United States, showing the cline of Y^M (more common in the north) and III^M (more common in the south). (**B–E**) Graphs show the effect of developmental temperature on knockdown time at either 4°C (cold tolerance) or 53°C (heat tolerance) for Y^M (turquoise) and III^M (salmon) male flies. Proto-Y chromosome labels for females reflect whether males from the strain carry the Y^M or III^M chromosome. Mean knockdown time is plotted for each combination of genotype and temperature. Error bars represent standard error.

We next attempted to identify a threshold temperature for the genotype-specific benefits of acclimation by comparing heat and cold tolerance of flies raised at 22°C and 29°C (instead of 18°C and 29°C). We did not observe a significant effect of the interaction between developmental temperature and male genotype on extreme cold tolerance ($\chi^2_1 = 0.947$, $p = 0.331$ comparing models with and without an interaction term) (Figure S2). We therefore hypothesize that there is a threshold temperature between 18°C and 22°C, below which Y^M males experience a greater

219 benefit of cold acclimation than III^M males. In contrast, there is a significant interaction between
220 genotype and developmental temperature on heat tolerance when comparing males raised at 22°C
221 and 29°C ($\chi^2_1 = 11.02$, $p = 9.0 \times 10^{-4}$ comparing models with and without the interaction term)
222 (Figure S2). Therefore, the threshold for a genotype-specific benefit from heat acclimation lies
223 between 22°C and 29°C.

224 We do not expect any difference in heat or cold tolerance across females from our
225 different strains because all females have the same genotype, regardless of the male genotype in
226 the strain. Indeed, a model with an interaction between developmental temperature and male
227 genotype does not fit the female cold tolerance data better than a model without the interaction
228 term ($\chi^2_1 = 1.46$, $p = 0.23$) (Figure 1C). There is a significant effect of developmental temperature
229 on cold tolerance in females ($\chi^2_1 = 43.5$, $p = 4.3 \times 10^{-11}$ comparing a model with and without
230 developmental temperature), demonstrating that females benefit from cold acclimation regardless
231 of male genotype (Figure 1C). Surprisingly, there is a significant interaction between male
232 genotype and developmental temperature on heat tolerance in females ($\chi^2_1 = 10.4$, $p = 0.0013$
233 comparing a model with and without the interaction term). In general, females raised at warmer
234 temperatures are more heat tolerant (Figure 1E). However, the interaction of male genotype and
235 developmental temperature is in the opposite direction from what would be expected based on the
236 latitudinal distribution of Y^M and III^M: females from strains with Y^M males that are raised at 29°C
237 are more heat tolerant than females from III^M strains raised at 29°C (Figure 1E). We thus
238 conclude that the heat and cold tolerance differences between Y^M and III^M males are specific to
239 males and/or the proto-Y chromosomes (i.e., not genetic background) because we do not observe
240 the same heat or cold tolerance differences in females from those strains (who do not carry the
241 proto-Y chromosomes).



242

243 **Figure 2** - Thermal preference (T_{pref}) of (**A**) male and (**B**) female house flies according to male
244 genotype (III^M = salmon points and line, Y^M = turquoise points and line) and developmental
245 temperature. Each point depicts the mean thermal preference for an individual fly, with lines and
246 error bars denoting means within groups and standard errors of the mean, respectively.
247 Significant differences between groups are denoted by letters, with differing letters highlighting
248 significantly different mean thermal preferences within each graph (Tukey's *post hoc* test, $p <$
249 0.05).

250 ***Thermal preference depends on developmental temperature and male genotype***

251 We next tested if genotype and developmental temperature affect thermal preference
252 (T_{pref}). First, we find that T_{pref} is inversely proportional to developmental temperature (Figure 2),
253 with house flies that develop at a warmer temperature preferring cooler temperatures (and *vice*
254 *versa*), regardless of sex (male: $F_{2, 742.7} = 138.4$, $p < 1.0 \times 10^{-5}$; female: $F_{2, 245.3} = 37.1$, $p = 1.19 \times$
255 10^{-4}; Figure 2). This is consistent with how developmental acclimation affects T_{pref} in *Drosophila*
256 (Dillon *et al.*, 2009).

257 We also find that male proto-Y chromosome genotype (Y^M vs III^M) affects T_{pref} ($F_{1, 756.2}=$
258 44.5, $p < 1.0 \times 10^{-5}$). There is also a significant interaction effect between developmental

259 temperature and genotype on T_{pref} in males ($F_{2, 756.3} = 8.47$, $p = 2.31 \times 10^{-4}$, Figure 2A). Male T_{pref}
260 is similar across genotypes when they develop at either 18°C or 29°C. However, when reared at
261 22°C, III^M males prefer warmer temperatures than Y^M males (Tukey's *post hoc* test, $p < 0.001$).
262 This is consistent with III^M males being more common at lower latitudes (where average
263 temperatures are warmer), and Y^M males more common at higher latitudes (Tomita & Wada,
264 1989; Hamm *et al.*, 2005; Feldmeyer *et al.*, 2008; Kozielska *et al.*, 2008). We do not expect
265 differences in T_{pref} in females across strains because all females have the same genotype. Indeed,
266 the genotype of males in a strain (Y^M vs III^M) and the interaction between male genotype and
267 female developmental temperature showed no significant effect on T_{pref} in females (ANOVA, all
268 $p > 0.1$ in Figure 2B). We assayed more males than females in our thermal preference
269 experiments, and so we repeated our analysis by down-sampling the data to have equal numbers
270 of individuals across treatments. The down-sampled data give equivalent results to the full data
271 set (Supplementary Results).

272 ***Thermal breadth depends on sex and thermal preference***

273 We used thermal breadth ($T_{breadth}$) as a measure of the specificity of T_{pref}. Male $T_{breadth}$ was
274 not significantly affected by either developmental temperature, genotype, or the interaction
275 between genotype and developmental temperature (ANOVA, all $p > 0.1$, Figure S3A). In
276 contrast, developmental temperature ($F_{2, 236.9} = 16.5$, $p < 1.0 \times 10^{-5}$), as well as the interaction
277 between developmental temperature and male genotype ($F_{2, 243.6} = 5.35$, $p = 0.005$), had
278 significant effects on $T_{breadth}$ in females. However, the significant interaction is of small effect, as
279 females from strains with differing male genotypes do not significantly differ in $T_{breadth}$ within
280 any developmental temperature treatment (Figure S3B).

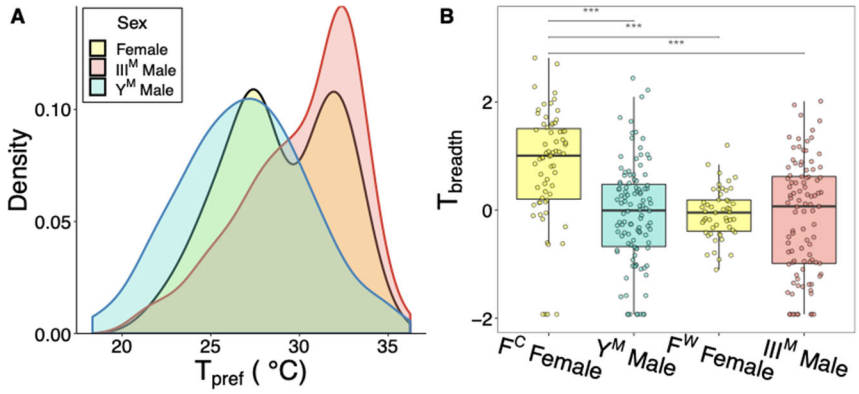


Figure 3 - Thermal breadth ($T_{breadth}$) depends on male genotype and sex. (**A**) Distribution of individual-level mean thermal preferences (T_{pref}) of III^M males, Y^M males, and pooled females that developed at 22°C. Y-axis represents relative density of data points and is analogous to frequency of data points for a given T_{pref} value. (**B**) $T_{breadth}$ of individuals raised at 22°C according to group (F^C = cold-preferring females, F^W = warm-preferring females). Boxplots denote median values and lower- and upper- quartiles. Asterisks denote significant differences in $T_{breadth}$ between groups (***: Tukey's *post hoc* test, $p < 0.01$).

The effect of developmental temperature on $T_{breadth}$ in females is driven by increased variance in T_{pref} when females develop at 22°C. The increased variance in female T_{pref} can be explained by a mixture of two normal distributions (Figure 3A, see Table S1 for statistics). This bimodal distribution is not a result of differences across strains because the same pattern was observed among females separately analyzed based on male genotype (Figure S4). In comparison, a single normal distribution best fit Y^M male T_{pref} when developed at 22°C, and two normal distributions best explained the III^M male T_{pref} when developed at 22°C. Upon inspection, however, the two distributions representing III^M male T_{pref} likely correspond to the tail (mean of

299 28.7°C and large variance of 10.4°C) and peak (mean of 32.6°C and small variance of 0.4°C) of a
300 single skewed distribution, which we are unable to detect using the mclust package we used to fit
301 distributions to our data.

302 We used our model-based clustering analysis of T_{pref} to classify individuals that developed
303 at 22°C into one of four groups: Y^M males (lower T_{pref}), III^M males (higher T_{pref}), females with
304 cooler T_{pref} (F^C females, 59.3% of females tested), and females with warmer T_{pref} (F^W females,
305 40.7% of females tested). The mean T_{pref} of F^C females (26.90°C) is nearly equal to the mean T_{pref}
306 of Y^M males (26.87°C; Figure 3A). Similarly, the mean T_{pref} of F^W females (32.2°C) is near the
307 mode of the T_{pref} of III^M males (32.0–32.5°C; Figure 3A).

308 We further find that T_{pref} is predictive of $T_{breadth}$ for flies that develop at 22°C. We
309 considered flies from our four T_{pref} groups (Y^M males, III^M males, F^C females, and F^W females),
310 and we found a significant effect of group on $T_{breadth}$ ($F_{3, 32.9} = 9.40$, $p = 1.24 \times 10^{-4}$). Specifically,
311 F^C females have significantly greater $T_{breadth}$ than all other groups (Tukey's *post hoc* test, all $p <$
312 1.0×10^{-5}, Figure 3B). Therefore, if we consider $T_{breadth}$ as a measure of the strength of T_{pref}, adult
313 house flies can be summarized by one of three phenotypes related to thermal behavior when
314 developed at 22°C: a relatively strong preference for warm temperatures (III^M males and F^W
315 females, which have high T_{pref} and low $T_{breadth}$), a strong preference for cooler temperatures (Y^M
316 males, with low T_{pref} and low $T_{breadth}$), and a relatively weak preference for cooler temperatures
317 (F^C females, with low T_{pref} and high $T_{breadth}$). Down-sampling the data gives similar results as the
318 full data set (Supplementary Material).

319 **Discussion**

320 We tested if thermal tolerance and preference depend on sex and male genotype in house
321 flies. We find that males carrying the Y^M chromosome (which is common in the northern end of
322 the species' range) are more cold tolerant and prefer colder temperatures. Conversely, males
323 carrying the III^M chromosome (which is common in the southern end of the species' range) are
324 more heat tolerant and prefer warmer temperatures. Our results are therefore consistent with the
325 general trend that temperate populations are typically more cold-tolerant than (sub-) tropical ones
326 (Gibert & Huey, 2001; Hoffmann *et al.*, 2002). The differences in thermal preference are
327 consistent with the idea that behavioral thermoregulation can weaken selection for thermal
328 tolerance, as predicted by the "Bogert Effect" (Huey *et al.*, 2003; Huey & Pascual, 2009;
329 Castañeda *et al.*, 2013). However, the fact that thermal preference and tolerance are both
330 predicted by male genotype provides evidence that these traits are responsive to selection,
331 suggesting any Bogert effects are not sufficient to overwhelm thermal adaptation. These
332 differences in thermal tolerance and preference in males depend on developmental temperature,
333 and they are not observed in congener females from the same strains (who do not carry the Y^M or
334 III^M chromosome). However, females exhibit a bimodal T_{pref}, with females from each of the two
335 subgroups overlapping with one of the male genotypes.

336 ***Thermal tolerance and preference depend on developmental temperature, genotype, and sex***

337 Our results demonstrate, to the best of our knowledge, the first documented example of
338 concordant temperature preference, cold tolerance, and heat tolerance across genotypes within a
339 species. We find that Y^M males both have greater cold tolerance and prefer colder temperatures,
340 whereas III^M males have greater heat tolerance and prefer warmer temperatures (Figures 1 and 2),

341 consistent with their latitudinal distributions (Tomita & Wada, 1989; Hamm *et al.*, 2005;
342 Feldmeyer *et al.*, 2008; Kozielska *et al.*, 2008). Previous work has identified concordant T_{pref} and
343 heat tolerance differences across species (Qu *et al.*, 2011), or found no clear relationship between
344 thermal tolerance and preference across genotypes within species (Yang *et al.*, 2008; Rego *et al.*,
345 2010; Castañeda *et al.*, 2019). Body size is also predicted to vary with thermal traits (Leiva *et al.*,
346 2019). In our study, we did not measure insect body size. While we did not observe any obvious
347 differences between strains, it is possible that some of the genotypic effect on thermal tolerance
348 or preference we observed is due to (temperature-dependent) morphological differences between
349 Y^M and III^M males. Future studies should directly test this hypothesis.

350 We observed strong effects of developmental temperature on both thermal tolerance and
351 preference that depend on both genotype and sex. Acclimation effects on heat and cold tolerance
352 (Figure 1) are well-documented for ectotherms, including flies and other insects (Bowler &
353 Terblanche, 2008). An inverse relationship between developmental temperature and thermal
354 preference has also been observed in other flies (Dillon *et al.*, 2009; Castañeda *et al.*, 2013).
355 Behaviorally navigating towards compensatory temperatures could serve as a means of mitigating
356 the costs of thermally suboptimal development (i.e., too hot or too cold). The observed
357 relationships between thermal tolerance and developmental temperatures are likely to be caused
358 by acclimation and unlikely to be the result of natural selection within our experiment for two
359 reasons. First, there is unlikely to be sufficient genetic variation in these inbred strains for
360 selection to generate these results within 2 generations. Second, prior attempts at selecting for
361 thermal tolerance in house flies resulted in negligible differences in tolerance across
362 developmental temperatures (Geden *et al.*, 2019) However, it is worth noting that the males used
363 by Geden et al. (2019) were likely all III^M based on their geographic origin. Had the experimental

364 population consisted of both III^M and Y^M males, a response to tolerance may have been detected.
365 We conclude that the differences in thermal tolerance (and preference) between Y^M and III^M
366 males have evolved across the natural populations from which we sampled the Y^M and III^M
367 chromosomes.

368 There are important methodological implications for our observation that variation in
369 thermal preference across genotypes depends on developmental temperature. We only observe
370 warmer (colder) thermal preferences in III^M (Y^M) males when developed at 22°C; thermal
371 preference did not differ between male genotypes when raised at more extreme (18°C, 29°C)
372 temperatures (Figure 2A). Previous studies attempting to estimate genetic variance in thermal
373 preference within or among populations of *Drosophila* have had mixed results. While some
374 studies identified genetic variance among populations within species (Good, 1993; Castañeda *et*
375 *al.*, 2013), others did not detect substantial variance within (Krstevska & Hoffmann, 1994) or
376 among species (MacLean *et al.*, 2019). Our results show that the phenotypic presentation of
377 genetic variation for thermal preference can depend on the environmental conditions experienced,
378 which could explain why this variance was not detected in other experiments. In addition, while
379 the genetic mechanisms that regulate thermal tolerance in other systems have been extensively
380 studied (Svetec *et al.*, 2011; Königer & Grath, 2018; Königer *et al.*, 2019), it is possible that
381 some of the molecular pathways involved will only be revealed through experiments conducted
382 across developmental temperatures.

383 We identify multiple differences between males and females in their thermal tolerance
384 and preferences. The strain differences we observed are primarily limited to males, which is
385 expected because the males differ in genotypes (Y^M and III^M) but females are isogenic (Meisel *et*
386 *al.*, 2015). However, there is a difference in heat tolerance between females from strains with Y^M

387 males and females from strains with III^M males (Figure 1). While we can rule out certain
388 genotypic explanations for this difference (i.e., all females are isogenic and do not carry *Md-*
389 *tra*^{D}), we do not yet have a mechanistic explanation on why females show the opposite
390 developmental heat tolerance from males. Nevertheless, the difference in heat tolerance observed
391 between females from different strains is in the opposite direction as between Y^M and III^M males
392 from those strains. This helps us to conclude that differences between Y^M and III^M males are
393 indeed a result of different proto-Y chromosomes rather than their genetic backgrounds. In other
394 words, the difference in heat tolerance between females is an exception that proves the rule with
395 respect to the effects of proto-Y chromosomes on male thermal tolerance and preference.
396 We identified a female-specific plasticity for thermal preference that does not map to male
397 genotype. In females, we found that neither thermal tolerance nor thermal preference differ
398 predictably between strains where males carry different proto-Y chromosomes (Figures 1C, E and
399 2B). However, there is a bimodal thermal preference for females that develop at 22°C (Figure
400 3A), regardless of congener male genotype. In addition, females that had colder T_{pref} when
401 developed at 22°C also had a larger $T_{breadth}$ (Figure 3B). In small ectotherms with little thermal
402 inertia, measures of movement along a thermal gradient (such as $T_{breadth}$) are predicted to be
403 positively correlated with environmental temperature (Anderson *et al.*, 2007). However, we
404 observe the opposite relationship between mean environmental temperature (T_{pref}) and $T_{breadth}$ in
405 females (Figure 3), suggesting that the difference in $T_{breadth}$ cannot be explained by thermal
406 inertia. Our results suggest that, in nature, females with colder temperature preferences may
407 occupy a wider range of temperatures than females with warmer temperature preferences.
408 Because all females in our experiment are expected to have the same genotype, we hypothesize
409 that these differences in T_{pref} and $T_{breadth}$ are conferred by a plastic response to some yet to be

410 characterized factor (e.g., microclimates within larval rearing containers). Alternatively, this
411 plasticity could have a stochastic origin that is intrinsic to the development of thermal preference
412 (Honegger & de Bivort, 2018; Jensen, 2018).

413 The correlation between thermal preference and thermal breadth at 22°C is female-
414 specific: Y^M and III^M males have similar $T_{breadth}$ values when raised at 22°C despite their
415 differences in T_{pref}. Although general sex differences in thermal tolerance (Hoffmann et al. 2005)
416 and thermal preference (Krstevska and Hoffmann 1994) have been documented, this is the first
417 study, to our knowledge, to identify sex differences in the relationship between thermal
418 preference and thermal breadth. Our results suggest that male and female house flies exhibit
419 different thermoregulatory behavioral patterns which may further be influenced by genotype.
420 Directly identifying a sex-by-genotype-by-environment interaction is beyond the scope of this
421 study because sex and genotype are confounded in our experimental design (the females in our
422 experiment have a different genotype from either male, characterized by a lack of either the III^M
423 or Y^M chromosome). Nonetheless, the house fly is a tractable system for directly testing for sex-
424 specific genotype-by-environment interactions on thermoregulation. For example, future work
425 could test for sex-specific effects of Y^M and III^M by measuring phenotypes in females carrying a
426 proto-Y chromosome along with the epistatic female-determining *Md-tra*^{D} allele (Hediger *et al.*,
427 2010; Hamm *et al.*, 2014).

428 ***Environmental heterogeneity and the maintenance of polygenic sex determination***

429 Sex determination pathways rapidly diverge across species, driving evolutionary turnover
430 of sex chromosomes (Bull, 1983; Beukeboom & Perrin, 2014). Polygenic sex determination
431 systems, in which more than one master sex determining locus segregate independently on

432 different chromosomes, have been observed in multiple animal species (Moore & Roberts, 2013).
433 Most population genetic models that attempt to explain the stable maintenance of polygenic sex
434 determination focus on sexually antagonstic effects of sex determining loci or linked alleles on
435 sex chromosomes (Rice, 1986; van Doorn & Kirkpatrick, 2007; Kozielska *et al.*, 2010; Meisel *et*
436 *al.*, 2016). Less attention has been given to ecological factors that can maintain polygenic sex
437 determination (Pen *et al.*, 2010; cf. Bateman & Anholt, 2017).

438 Our results demonstrate how spatially variable ecological factors can maintain polygenic
439 sex determination. Specifically, thermal tolerance and preference phenotypes conferred by the Y^M
440 and III^M chromosomes (Figures 1 and 2) are consistent with the clinal and temperature-dependent
441 distributions of the Y^M and III^M chromosomes (Tomita & Wada, 1989; Hamm *et al.*, 2005;
442 Feldmeyer *et al.*, 2008; Kozielska *et al.*, 2008). Previous experiments identified multiple fitness
443 advantages conferred by the III^M chromosome over Y^M at warmer temperatures, including an
444 increase in frequency of III^M over generations in a laboratory population (Hamm *et al.*, 2009).
445 However, these fitness differences can only explain the invasion or fixation of the III^M
446 chromosome, not the maintenance of the polymorphism. In contrast, differences in thermal
447 tolerance and preference could maintain proto-Y chromosome polymorphism across the species'
448 range, similar to how selection maintains other clinal variation (Slatkin, 1973; Endler, 1977).

449 The house fly system reveals how temperature variation can contribute to the maintenance
450 of polygenic sex determination independently of selection on the sex-determination pathway
451 itself. Temperature is an important contributor to the evolution of sex determination pathways in
452 vertebrates (Bull & Vogt, 1979; Holleley *et al.*, 2015). However, the effects of the house fly
453 proto-Y chromosomes on thermal tolerance and preference likely act independently of the sex
454 determination pathway because there are not differences in the expression of sex determination

455 genes across house fly male genotypes raised at different temperatures in a way that is consistent
456 with their clinal distribution (Adhikari *et al.*, n.d.). This suggests that the effects of the Y^M and
457 III^M chromosomes on thermal phenotypes is a result of alleles on proto-Y chromosomes that are
458 genetically linked to the male-determining locus, as opposed to the male-determiner itself.
459 Therefore, our results highlight how temperature can be important for the evolution of sex
460 determination independently of temperature-dependent activity of the sex determination pathway.
461 Future theoretical work should consider the effect of spatially heterogeneous selection pressures
462 on the maintenance of polygenic sex determination, similar to how temporal heterogeneity can
463 create fluctuating selection pressures that maintain polygenic sex determination (Bateman &
464 Anholt, 2017).

465 ***Selection on thermal phenotypes may depend on geographical scale***

466 Our results suggest that selection on thermal traits differs between macro-geographic
467 species ranges and at a micro-geographical scale within populations. Similar differences in
468 selection pressures according to geographic scale have been documented before in other species
469 (Richter-Boix *et al.*, 2010; De Block *et al.*, 2013; Tüzün *et al.*, 2017). Thermal tolerance and
470 preference in male house flies depend on proto-Y chromosomes genotype in a way that is
471 consistent with the latitudinal distribution of the Y^M and III^M chromosomes (Figures 1 and 2).
472 This suggests that, at the macro-geographic scale, selection is operating on male physiology and
473 behavior to create or maintain the clinal distribution of Y^M and III^M (Figure 4A). It is also worth
474 noting that our study focuses on only two male genotypes (III^M and Y^M). While these are the most
475 prevalent genotypes in the eastern United States, other genotypes exist (including males with
476 multiple proto-Y chromosomes, and females with proto-Y and proto-W chromosomes) and are

477 common in other populations (Franco *et al.*, 1982; Feldmeyer *et al.*, 2008; Hamm & Scott, 2009;
478 Hamm *et al.*, 2014). Future studies should characterize thermal tolerance and preference of these
479 other genotypes in order to determine whether their geographical distribution is similarly
480 explained by thermal biology.

481 At an intermediate developmental temperature (22°C), female thermal preference is
482 bimodal for a reason that we have yet to determine (Figure 3A). This raises the possibility that
483 within populations near the center of the cline (i.e., at a micro-geographic scale), where Y^M and
484 III^M both segregate (e.g., Hamm & Scott, 2008; Meisel *et al.*, 2016), sexual selection may favor
485 males that can preferentially obtain access to the two different female phenotypes. While
486 differences in thermal preference probably did not evolve in response to sexual selection, these
487 differences do likely have important consequences on the reproductive success of III^M and Y^M
488 males where they co-occur. III^M males may disproportionately benefit from differences in T_{pref}
489 and $T_{breadth}$ between males and females. F^C females that prefer colder temperatures have greater
490 $T_{breadth}$ than warm preferring F^W females and both male genotypes (Figure 3B), suggesting that F^C
491 females occupy a wider range of thermal habitats. Thus, III^M males may gain an advantage by
492 having greater access to F^W females, as well as occasional access to F^C females, in contrast to Y^M
493 males who would only be likely to encounter F^C females (Figure 4B). This raises the possibility
494 that differences in thermal preference across genotypes and sexes could affect the dynamics of
495 sexual selection.
496

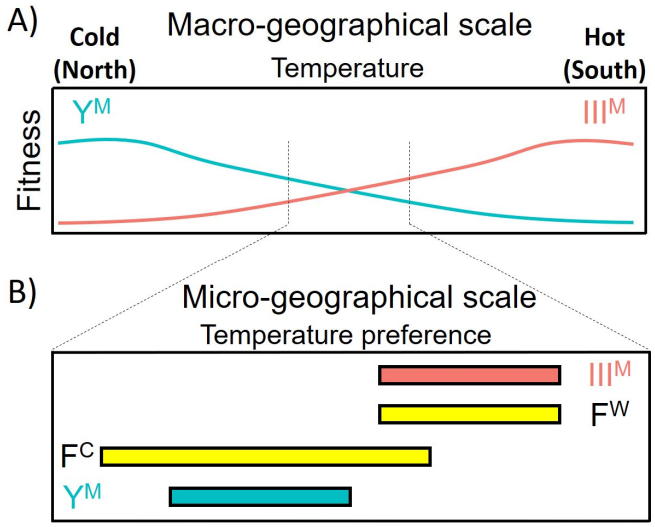


Figure 4 - Selection on the III^M and Y^M chromosomes likely differs across geographic scales. (**A**) At the macro-geographical scale, selection for thermal tolerance and/or thermal preference results in the clinal distribution of the Y^M (turquoise) and III^M (salmon) chromosomes. (**B**) At intermediate developmental temperatures, male genotypes (Y^M vs III^M) differ in thermal preference, which may create asymmetrical mating opportunities because of variation in female thermal preference and breadth (F^C vs F^W). The asymmetry of the overlap of males and females at the intermediate developmental temperature could affect sexual selection in populations where Y^M and III^M both segregate.

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Author Contributions

PD, KA, and RM conceived and designed the study. KA, OH, VS, and JC collected, and KA analyzed, all thermal tolerance data. PD, RP, JT, and AM collected, and PD analyzed, all thermal preference data. PD, KA, and RM wrote the manuscript, and all authors reviewed the manuscript prior to submission.

Data Accessibility

All data files used for analyses described in this manuscript have been deposited in Dryad (doi:10.5061/dryad.n2z34tmvs). Raw video and image files from thermal preference assays are available from the authors upon request.

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