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1 Title: **Female fruit flies cannot protect stored sperm from high temperature damage**

2

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10 **Abstract**

11 Recently, it has been demonstrated that heat-induced male sterility is likely to shape
12 population persistence as climate change progresses. However, an under-explored
13 possibility is that females may be able to successfully store and preserve sperm at
14 temperatures that sterilise males, which could ameliorate the impact of male infertility on
15 populations. Here, we test whether females from two fruit fly species can protect stored
16 sperm from a high temperature stress. We find that sperm carried by female *Drosophila*
17 *virilis* are almost completely sterilised by high temperatures, whereas sperm carried by
18 female *Zaprionus indianus* show only slightly reduced fertility. Heat-shocked *D. virilis*
19 females can recover fertility when allowed to remate, suggesting that the delivered heat-
20 shock is damaging stored sperm and not directly damaging females in this species. The
21 temperatures required to reduce fertility of mated females are substantially lower than the
22 temperatures required to damage mature sperm in males, suggesting that females are
23 worse than males at protecting mature sperm. This suggests that female sperm storage is
24 unlikely to ameliorate the impacts of high temperature fertility losses in males, and instead
25 exacerbates fertility costs of high temperatures, representing an important determinant of
26 population persistence during climate change.

27 **Keywords:** fertility, female sperm storage, heat stress, climate change

28 **1. Background**

29 Anthropogenic climate change poses a significant challenge to global biodiversity. We
30 urgently need to understand how rising average temperatures, and an increasing number of
31 short-term extreme temperature events (Perkins-Kirkpatrick and Lewis, 2020), will affect
32 natural populations. Understanding how high temperatures affect organisms can allow
33 researchers to predict the vulnerability of species and inform conservation efforts, revealing
34 which temperature-sensitive traits are particularly important for determining species
35 persistence. Initial research focused on temperatures required to kill or incapacitate
36 individuals, and it has been shown that species' physiological temperature limits correlate
37 with the maximum temperatures species experience in the wild (Kellermann et al., 2012). It
38 has been known for around a century that high temperatures can sterilise individuals
39 (Cowles, 1945; David et al., 2005; Young and Plough, 1926). Recent work has found that the
40 temperature that sterilises over 80% of males in a species, named a species' upper thermal
41 fertility limit (TFL), correlate more strongly with maximum temperatures that species
42 experience in the wild than lethal limits (Parratt et al., 2021; van Heerwaarden and Sgrò,
43 2021). This indicates that upper TFLs are significant determinants of current species
44 distributions, and are therefore likely to shape population persistence as climate change
45 progresses.

46 Temperature-induced sterility occurs across a wide-variety of taxonomic groups (David et
47 al., 2005; Hurley et al., 2018; Karaca et al., 2002; Sage et al., 2015; Walsh et al., 2019b).
48 Sterility is used here to describe an individual that is unable to produce viable offspring,
49 which could be driven by one or more of the many different components of reproduction
50 (Walsh et al., 2019b). A study of 43 *Drosophila* fruit fly species found that males from nearly

51 half the species (19/43) are sterilised at temperatures significantly lower than temperatures
52 required to kill them (Parratt et al., 2021). Male fertility generally seems more sensitive to
53 high temperatures when directly compared with female fertility (Iossa, 2019; Sales et al.,
54 2018; Walsh et al., 2020), although the converse is possible (Janowitz and Fischer, 2011).
55 The relative sensitivity of male fertility in animals has been attributed to disruption of
56 spermatogenesis or death of mature sperm as a result of thermal stress (Rohmer et al.,
57 2004; Sales et al., 2018). Typically, the effect of temperature on fertility is measured by
58 directly heating males, and subsequently measuring the reproductive capacity of focal males
59 when paired with females following heat-stress (Jørgensen et al., 2006; Karaca et al., 2002;
60 Parratt et al., 2021; Sales et al., 2018; Walsh et al., 2020; Zwoinska et al., 2020) or by
61 measuring other traits linked to fertility (Hurley et al., 2018; Paxton et al., 2016). Likewise,
62 studies measuring female fertility generally stress females prior to mating (Walsh et al.,
63 2020; Walsh et al., 2019a), in order to isolate the effect of temperature on female
64 reproductive physiology, such as oocytes. However, while it is clearly important to measure
65 the effect of thermal stress prior to mating, the effect of high temperatures on females
66 post-mating has been largely ignored (but see McAfee et al., 2020; Sales et al., 2018). This is
67 important because sperm can spend a significant proportion of time within the female
68 reproductive tract prior to fertilisation.

69 Sperm storage is characterised by temporal delays between insemination and fertilisation,
70 during which sperm is maintained within a female's reproductive tract. Female sperm
71 storage is common across taxa, including mammals, birds, reptiles, fish and insects (Holt,
72 2011; Sever and Hamlett, 2002). The time that sperm can be kept viable inside a female
73 varies substantially. In birds and reptiles, sperm storage durations range from seven days up
74 to seven years, in mammals for less than a day up to six months in some bat species,

75 amphibians from four to thirty months, in fish from only days to around two years, and over
76 a decade in some eusocial hymenoptera (Birkhead and Møller, 1993; Holt and Lloyd, 2010;
77 Holt, 2011; Keller, 1998; Levine et al., 2021; Pamilo, 1991). The method of sperm storage
78 can also vary substantially, and phylogenetic evidence suggests long-term storage of sperm
79 has arisen independently across taxa (Holt and Lloyd, 2010). For example in birds and some
80 reptiles, inseminated spermatozoa are stored in microscopic sperm storage tubules (SSTs)
81 embedded in the infundibulum, which allow sperm to survive for extended periods of time
82 (Holt, 2011; Sasanami et al., 2013). Females from the majority of insects and some other
83 arthropods store sperm in a highly chitinised specialised organ called the spermatheca.
84 Most insects have one spermatheca, but some insects have two or three (Pascini and
85 Martins, 2017). However, while female sperm storage for extended durations is
86 taxonomically widespread (Birkhead and Møller, 1993), the impact of high temperatures on
87 sperm stored within mated females is currently understudied. The few efforts to examine
88 the impact of high temperatures on sperm stored within females include mated females of
89 the red flour beetle (*Tribolium castaneum*), which show a 33% reduction in offspring
90 production when exposed to a heatwave treatment (Sales et al., 2018). Also, a four hour
91 heat-stress at 42°C significantly reduces the viability of sperm stored by honey bee queens
92 (McAfee et al., 2020), although in this study the authors do not directly test whether this
93 reduces female offspring production. Given the urgency of understanding the consequences
94 of rising temperatures, we need a better understanding of the thermal robustness of female
95 sperm storage.

96 Fruit flies from the family Drosophilidae provide a useful model group to explore this
97 question. Female *Drosophila* typically possess a pair of spermathecae and a seminal
98 receptacle, the latter of which is a thin extended tubule arising from the uterus (Pitnick et

99 al., 1999). *Drosophila* have been proposed as a model system for studying sperm-female
100 interactions, in order to better understand fertilisation across taxa (Heifetz and Rivlin, 2010).
101 *Drosophila* are also a model taxon for studying thermal reproductive physiology, including
102 examining how high temperatures affect fertility of both males and females prior to mating
103 (David et al., 2005; Parratt et al., 2021; Sgrò et al., 2016; Walsh et al., 2020). However, to
104 our knowledge there has been no substantial effort to examine how high temperatures
105 affect the capacity of mated females to produce offspring in *Drosophila*.

106 Here, we explore the impact of heat stress on sperm storage in females from two
107 Drosophilidae species. We test the tropical pest species *Zaprionus indianus*, and a more
108 temperate species *Drosophila virilis*. Parratt et al. (2021) showed that males of both species
109 die when exposed to ~38°C for 4 hours, and that immediate male fertility is compromised
110 when male *D. virilis* are exposed to ~37°C for 4 hours, but male *Z. indianus* remain fertile.
111 This indicates that mature sperm stored by *D. virilis* males are damaged by thermal stress,
112 but *Z. indianus* maintain fertility. In contrast, the same study found that developing sperm
113 appear to be damaged by high temperatures in both species. Males of both species are
114 sterile 7 days after being heated at ~35°C for 4 hours, indicating that developing sperm are
115 damaged by increased temperatures. However, we do not know the effect of high
116 temperatures on sperm stored within mated females.

117 We test three components of female fertility across time. Firstly, we test the expectation
118 that female fertility will be more robust to high temperatures than male fertility. Secondly,
119 we test whether sperm stored in mated females are more or less sensitive to high
120 temperatures than sperm stored in a males seminal vesicles and developing sperm within
121 the testes, investigated previously. Finally, we explore whether mated females that are

122 heated to a point that sterilises them can recover fertility, after being presented with new
123 male partners. If sterilised mated females can recover by remating, this would suggest that
124 heat induced sterility of mated females is caused by damage to sperm and not direct
125 damage to females.

126 **2. Material and Methods**

127 **2.1 Animal stock maintenance**

128 Stocks of *Drosophila virilis* (Cambridge Fly Facility StrainvS-4, isolated in 1991) and *Zaprionus*
129 *indianus* (DSSC Stock #: 50001-0001.05 ISOFEMALE, isolated in 2004), were kept in a
130 temperature-controlled incubator (LMS 600NP Series 4) at 23°C, 12:12 L:D and ambient
131 humidity. Stocks were maintained at moderate density (50 – 100 flies per 300ml bottle
132 culture). *D. virilis* were kept on standard cornmeal-molasses-agar media, and *Z. indianus*
133 were kept on banana medium. Ovipositing adults for both species were tipped to new food
134 every week to prevent overlapping generations, and were replaced with fresh sexually
135 mature adult flies every 4-6 weeks.

136 **2.2 Experimental treatments**

137 Experimental treatments are summarised in Figure 1. We assessed whether heat stress
138 influences fertility of females when delivered before mating (Experiment 1). We then
139 completed an experiment with two more treatment combinations (Experiment 2a & 2b) to
140 address the outstanding question of whether mated females can protect stored sperm from
141 temperature damage experienced post-mating, and isolate effects on stored sperm from
142 changes to female egg-laying behaviour.

143 We chose to mate females at 7 days old when fully sexually mature, and kept this consistent
144 between experiments. Therefore, females from Experiment 1 are 7 days old at heat-stress,
145 whereas females from Experiment 2 are 14 days old at heat-stress. Prior to heat stress,
146 females from Experiment 1 were separated at emergence and kept as virgins in groups of 10
147 per vial for 7 days, to standardise density prior to the experiment. Females from Experiment

148 2 were separated as virgins and kept in groups of 10 for 7 days, then provided with sexually
149 mature males (7 days old) at a 1:1 sex ratio for a further 7 days prior to heat-stress. This
150 produced an 'assumed' mated treatment, where females would have many opportunities to
151 mate with a variety of males.

152 Immediately following heat stress, females were transferred to individual fresh food vials. In
153 Experiment 1, virgin females were immediately placed with 4 7 day old virgin males. This
154 mating group was moved to fresh vials twice, creating 3 'time-points' where fertility was
155 recorded. Females in Experiment 2 were isolated and transferred to fresh vials giving 3 time-
156 points over 7 days. Experiment 2 was then split into two treatments. Females from
157 Experiment 2a were kept in isolation for an additional 7 days, producing 3 more time-points
158 where females were isolated. Females from Experiment 2b were placed with 4 males
159 following the first 7 days of isolation. This mating group was transferred onto new vials
160 twice more, giving 3 time-points where the females were isolated, followed by 3 recorded
161 time-points where females were paired with males. Females were deemed as qualitatively
162 fertile at a given time-point if there was evidence of larvae present in their vial (1/0),
163 measured by directly observing larvae or their distinctive tracks in the food. We use a binary
164 fertile/infertile measure rather than counting pupae or adults because our methods were
165 likely to result in many sterile vials, producing a dataset of offspring counts with many zeros.
166 Quantitative models typically have difficulty with such data.

167 **2.3 Heat-stress**

168 Groups of 10 females were transferred to fresh 25 x 95mm plastic vials, containing 25ml of
169 'ASG' medium (10g agar, 85g sucrose, 20g yeast extract, 60g maize, 1000ml H₂O, 25ml, 10%
170 Nipagin) to prevent desiccation and keep humidity consistent. These vials were randomly

171 assigned to pre-heated water-baths (Grant TXF200) for four hours at either control: 23°C, or
 172 two stress temperatures: 35°C & 36°C. The chosen temperatures do not affect survival or
 173 immediately sterilise mature adult males of either species, but result in substantial delayed
 174 sterility of males, likely due to the destruction of developing sperm (Parratt et al., 2021).
 175 Immediately following temperature-treatment, flies were returned to benign temperatures
 176 for the remainder of the experiment (23°C). Sample sizes are given in table 1.

177 Table 1: Sample sizes of experimental treatments as summarised in Figure 1.

	<i>Drosophila virilis</i>			<i>Zaprionus indianus</i>		
	23°C	35°C	36°C	23°C	35°C	36°C
Experiment 1	29	29	30	25	25	25
Experiment 2	27	26	18	23	23	24
Experiment 2b	27	26	18	22	23	23

178

179 **2.4 Statistical analyses**

180 Species and experiments were analysed separately due to inherent differences in
 181 methodological design as summarised in Figure 1. Treatment of females in Experiment 2 are
 182 identical from the start of the experiment until the experiment is split after 7 days into the
 183 post stress treatment. Therefore, data from Experiment 2 over the first 3 time-points were
 184 analysed together. The final 3 time-points of Experiment 2a after splitting were not
 185 statistically analysed, as all flies of both species in these final 3 time-points were completely
 186 sterile with only one exception, making these data uninformative. Experiment 2b was

187 analysed after the treatments were split and females were presented with new males, in
188 order to assess differences in fertility recovery across temperature treatments.

189 To assess the effect of temperature on fertility we used generalised linear mixed models
190 with Bernoulli error distributions. We fitted fertility as a binary response variable,
191 temperature and time-point and their interaction as fixed effects, and focal fly ID as a
192 random effect to account for repeated measures. We did model selection using Wald Chi-
193 squared likelihood ratio-tests, removing non-significant interactions. We retained all main
194 effects and reported statistics of these from type II likelihood ratio tests using the 'Anova'
195 function from the 'car' package, in the statistical software 'R' (version 3.5.0). We then
196 reported any pairwise comparisons in which $p < 0.05$ by using the Wald statistic and p-value
197 from the model summary(). To do this we ran the model multiple times, setting each level in
198 turn as the baseline compared with the other levels.

199 3. Results

200 3.1 Experiment 1: Virgin/Heat/Mated

201 Experiment 1 exposed virgin females to benign or stressful temperatures and subsequently
202 mated them. There was no significant interaction between temperature and time on fertility
203 of *D. virilis* from Experiment 1 ($\chi^2_{(2)} = 3.977$, $p=0.137$; Figure 2). There was no main effect of
204 temperature ($\chi^2_{(2)} = 0.093$, $p=0.954$; Figure 2a), or time ($\chi^2_{(1)} = 0.301$, $p=0.583$; Figure 2) on
205 fertility of *D. virilis*. Fertility was initially high, and remained so for the three time-points
206 measured.

207 There was also no significant interaction between temperature and time on fertility of *Z.*
208 *indianus* from Experiment 1 ($\chi^2_{(2)} = 3.946$, $p=0.139$; Figure 2). While the absolute proportion
209 of fertile females heated at 36°C was consistently lower than controls, there was no overall
210 main effect of temperature on fertility of *Z. indianus* ($\chi^2_{(2)} = 4.469$, $p=0.107$; Figure 2).
211 However, there was a significant effect of time on fertility ($\chi^2_{(1)} = 10.911$, $p<0.001$; Figure 2),
212 where flies from all temperatures show increased fertility rates over time.

213 3.2 Experiment 2: Mated/Heat/Isolated

214 Experiment 2 exposed mated females to benign or stressful temperatures, then isolated
215 individuals immediately following heat-stress. Not all females from the pre-stress 'mating'
216 treatment produced offspring, with controls producing a baseline fertility of around 70% in
217 *D. virilis* and around 80% in *Z. indianus* (Figure 3).

218 There was a significant interaction between temperature and time on fertility of *D. virilis* in
219 Experiment 2 prior to treatment splitting ($\chi^2_{(2)} = 9.943$, $p<0.007$; Figure 3). Fertility of
220 controls started high immediately following heat treatment and fell over time, whereas

221 fertility at stress temperatures started low and remained low for the duration. There was a
222 main effect of temperature ($\chi^2_{(2)} = 21.146$, $p < 0.001$; Figure 3) and time ($\chi^2_{(1)} = 17.352$,
223 $p < 0.001$; Figure 3) on fertility of *D. virilis* in Experiment 2. Both stress temperatures showed
224 lower fertility than controls, and all treatments showed a decline in fertility over time.
225 There was no significant interaction between temperature and time on fertility of *Z.*
226 *indianus* from Experiment 2 ($\chi^2_{(2)} = 1.777$, $p = 0.411$; Figure 3). However, there was a
227 significant overall effect of temperature ($\chi^2_{(2)} = 80.161$, $p < 0.001$; Figure 3) and time ($\chi^2_{(1)}$
228 $= 99.756$, $p < 0.001$; Figure 3) on fertility of *Z. indianus*. In this species the highest temperature
229 of 36°C results in significantly lower fertility than both controls ($p < 0.001$) and the stress
230 temperature of 35°C ($p < 0.001$). All temperatures result in a loss of fertility over time.

231 **3.3 Experiment 2b: Mated/Heat/Isolated/Remated**

232 A subsection of females from experiment 2 were given the chance to remate 1 week
233 following heat-stress. There was no significant interaction between temperature and time
234 on fertility of *D. virilis* after females were given the chance to remate in Experiment 2b ($\chi^2_{(2)}$
235 $= 3.549$, $p = 0.170$; Figure 3). However, we found a significant effect of temperature on
236 fertility in *D. virilis* ($\chi^2_{(2)} = 9.520$, $p = 0.009$; Figure 3). Specifically, fertility of females exposed
237 to the stress temperature of 36°C was significantly lower than fertility from the control 23°C
238 ($p = 0.002$) and stress temperature of 35°C ($p = 0.046$). There was no significant effect of time
239 on fertility ($\chi^2_{(1)} = 0.515$, $p = 0.473$; Figure 3).

240 There was no significant interaction between temperature and time on fertility of *Z.*
241 *indianus* when females were given the opportunity to remate in Experiment 2b ($\chi^2_{(2)} =$
242 1.049 , $p = 0.592$; Figure 3). There was also no main effect of temperature on fertility ($\chi^2_{(2)}$

243 =4.250, p=0.119; Figure 3). However, there was a significant effect of time on fertility ($\chi^2_{(1)}$

244 =4.775, p=0.029; Figure 3), where fertility slightly increases over time.

245 **4. Discussion**

246 We found little evidence that virgin females are susceptible to fertility loss at high
247 temperatures. Heat-stress did not influence fertility of virgin *D. virilis* or *Z. indianus* females
248 that were then mated after heat-stress, although it should be noted that females were not
249 heated up to their lethal limit. Fertility of *Z. indianus* females was initially lower at the first
250 time-point measured post heat-stress, and increased over the duration of the experiment.
251 Conversely, fertility of *D. virilis* females was consistently high over the duration, suggesting
252 that *Z. indianus* females were slower to mate and produce offspring with their paired males
253 than *D. virilis*.

254 Mated females given no opportunity to remate used up their viable sperm reserves within
255 the first week of laying. However, we found that heat-stress reduced the number of fertile
256 females of both species, likely through destruction of stored mature sperm. This is curious
257 because a previous study found that mature sperm in males of both species from the same
258 experimental lines appear to be largely unaffected by the same temperature treatments
259 (Parratt et al., 2021). We find that mated females are sterilised at temperatures around 2°C
260 lower than those required to completely sterilise 80% of males from our study species
261 (Parratt et al., 2021). Hence our results suggest that females of both species are worse at
262 protecting mature sperm from high temperatures than males. However, as we did not
263 directly observe sperm death within females, it is also possible that there is an alternative
264 explanation for female sterility, such as embryonic death.

265 We found that the temperatures required to sterilise mated females differ between the two
266 species. Four hours at either 35°C or 36°C almost completely sterilise *D. virilis* females (~90%
267 of females produce no offspring), whereas mated *Z. indianus* females are mostly fertile

268 when stressed at 35°C and only a small majority are sterilised when exposed to 36°C for four
269 hours (~60% of females produce no offspring). The finding that mature sperm from *Z.*
270 *indianus* is likely more resilient than sperm from *D. virilis* is consistent with our previous
271 study that heated adult males of each species, although it should be noted that these
272 experiments were not conducted together. Males of *D. virilis* require temperatures of no
273 less than 37°C for 4h to immediately sterilise the majority of males, whereas males of *Z.*
274 *indianus* are fertile up to their lethal temperature of ~38°C (Parratt et al., 2021). While the
275 absolute temperatures required to sterilise males and mated females appear to be
276 different, these results combine to suggest that mature sperm from *Z. indianus* are generally
277 more thermally robust than those from *D. virilis*. It is unclear exactly why this may be the
278 case, however *Z. indianus* tend to live in slightly warmer areas than *D. virilis*. The
279 temperature experienced by individuals at the upper edge of their thermal range in the
280 hottest month of the year (Tmax+1sd: WorldClim.org BIO05) is 36.1°C for *Z. indianus*,
281 whereas it is 32.6°C in *D. virilis* (Parratt et al., 2021). Therefore, *Z. indianus* sperm may
282 better adapted to high temperatures than *D. virilis*, although this is beyond the scope of this
283 study.

284 To unpick effects of high temperatures on stored sperm from direct effects on females, we
285 then gave a chance for mated females to ‘recover’ fertility after they had used up their
286 viable stored sperm. We found that while the majority of females exposed to all
287 temperatures were able to produce offspring when paired with new males, females heated
288 at 36°C performed worse than controls in *D. virilis*. Therefore, it is likely that 36°C thermal
289 stress results in some permanent damage to females of this species, possibly due to
290 elevated ROS due to thermal stress. There could also be a trade-off between the cost of
291 additional mating and any increased fecundity, if females are in a worsened condition as a

292 result of heat-stress. Measuring additional reproductive traits, such as the number of
293 emerging offspring, could reveal more subtle changes in reproduction that could begin to
294 address these questions. However, the almost complete sterilisation of sperm stored in
295 female *D. virilis* paired with a general capacity to 'recover' fertility suggests that initial
296 sterilisation in this species is likely due to the destruction of stored sperm by high
297 temperatures and not direct effects on females. Mated *Z. indianus* females were equally
298 able to recover fertility when paired with new males, regardless of the heat-stress
299 temperature experienced. While the temperatures required to reduce fertility of mated
300 females were higher in this species, there was no long-term effects of temperature on
301 female recovery when females were presented with new males, suggesting that this initial
302 reduction of fertility in *Z. indianus* is also driven by effects on stored sperm.

303 Sterilisation of mated females could be particularly devastating to species with low remating
304 rates. However, females can use facultative polyandry to improve offspring production
305 when mating with sub-fertile males (Sutter et al., 2019; Vasudeva et al., 2021). For example,
306 heat-shocked males of the flour beetle *Tribolium castaneum* have low numbers of viable
307 sperm after heat-stress (Vasudeva et al., 2021). Here, females increase their remating rate
308 when mated with a heat-shocked male, rescuing fertility to normal levels. However,
309 whether increased polyandry is observed when sperm within the female is sterilised by high
310 temperatures remains an open question. Also, there may be species where facultative
311 polyandry is impossible, for example in seasonally reproducing animals with discrete mating
312 opportunities. Those particularly at risk include species that store sperm for long periods of
313 time, such as hymenopteran insects that have been observed to store sperm for up to 10
314 years (Keller, 1998; Pamilo, 1991). In these cases, sterilisation of mated females may
315 actually be worse for population persistence than sterilisation of males.

316 Understanding how high temperatures affect male fertility has improved our ability to
317 predict the consequences of climate change on species (Parratt et al., 2021; van
318 Heerwaarden and Sgrò, 2021; Walsh et al., 2019b). When these severe long-term effects on
319 male fertility are combined with the immediate sterilisation of mated females like we have
320 demonstrated, the impact of rising temperatures on wild populations may be exacerbated.
321 Further, we find here that the temperatures required to sterilise mated females are not
322 always consistent with the temperatures required to sterilise males. It will be important to
323 determine whether this is true across species and taxa to help forecast vulnerability climate
324 warming effects. Species where sperm in both males and mated females cannot be
325 protected may be particularly vulnerable, whereas species where females can protect sperm
326 effectively may be more resilient to an increasing incidence and severity of heat-waves.

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334 **CRediT authorship contribution statement**

335 **BSW:** conceptualization, methodology, validation, formal analysis, investigation, data
336 curation, writing- original draft, writing- review and editing, visualisation. **SRP:**
337 conceptualization, methodology, formal analysis, writing- review and editing, visualisation.

338 **RRS:** writing- review and editing **AB:** writing- review and editing **DA:** writing- review and
339 editing. **TARP:** conceptualization, resources, writing- original draft, writing- review and
340 editing, supervision, project administration, funding acquisition.

341 **Data and materials availability**

342 All data and analysis R code is available at

343 <https://datadryad.org/stash/share/7wn67Q4UVZBXStL1OKTk87xJ9CzXh-GrQ1H2ZoxC7TA>

344 **Figure captions**

345 **Figure 1: Experimental design outlining the two experiments.** Each treatment designation
346 combines various pre and post-stress mating treatments. **Experiment 1:**
347 **Virgin/Heat/Mated**, where virgin females were heat-stressed and mated following heat-
348 stress. **Experiment 2: Mated/Heat/Isolated**, where mated females are heat-stressed and
349 kept alone for 7 days to produce offspring from previous matings. After 7 days post heat-
350 stress, the experiment was divided into two treatments. For an additional 7 days, females
351 were either kept in isolation (**2a: Mated/Heat/FullyIsolated**), or given new male partners to
352 mate with (**2b: Mated/Heat/Isolated/Remated**). Focal females were exposed to either
353 benign (23°C) or stress (35 & 36°C) temperatures for 4h in water baths. Day 0 in the post-
354 stress treatment represents the time-point when the fertility assay begins (Figure 2 & Figure
355 3).

356

357 **Figure 2: Proportion of fertile *D. virilis* and *Z. indianus* females over time in Experiment 1:**
358 **Virgin/Heat/Mated.** Virgin females were heat-shocked at either benign (23°) or two stress
359 temperatures (35 & 36°C) for 4 hours, and paired with 4 male partners immediately
360 following heat-stress. This mating group was given 3 days to lay eggs, then tipped onto fresh
361 vials twice, giving three recorded time-points where fertility was measured. Error bars
362 represent 95% confidence intervals. Sample sizes for each species are given in Table 1.

363

364 **Figure 3: Proportion of fertile *D. virilis* and *Z. indianus* females over time in Experiment 2:**
365 **Mated/Heat/Isolated.** Mated females were heat-shocked at either benign (23°) or two

366 stress temperatures (35 & 36°C) for 4 hours. Following heat stress, all females were isolated
367 and allowed to lay eggs in fresh vials three times. After 6 days, the experiment was split into
368 two treatments. **2a Mated/Heat/FullyIsolated:** females remained isolated and moved onto
369 three fresh vials to lay any remaining eggs. **2b Mated/Heat/Isolated/Remated:** focal
370 females were paired with new male partners, and the mating group were given 3 fresh vials
371 to produce offspring. Error bars represent 95% confidence intervals. Sample sizes for each
372 species are given in Table 1.

373

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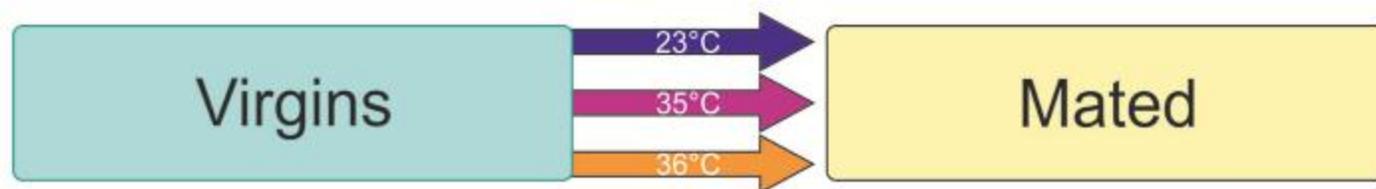
458

Pre-stress
treatment

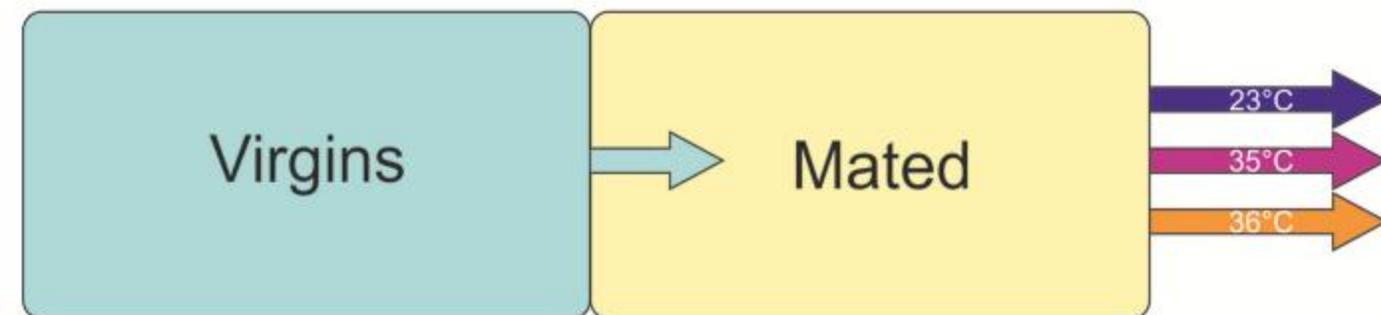
Heat
stress

Post-stress
treatment

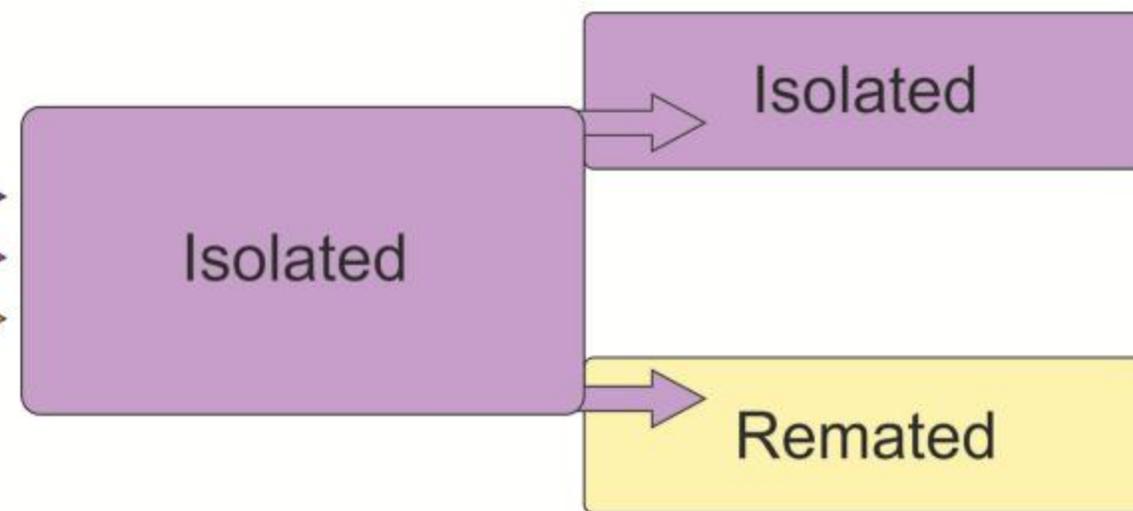
Experiment 1: Virgin/Heat/Mated



Experiment 2: Mated/Heat/Isolated

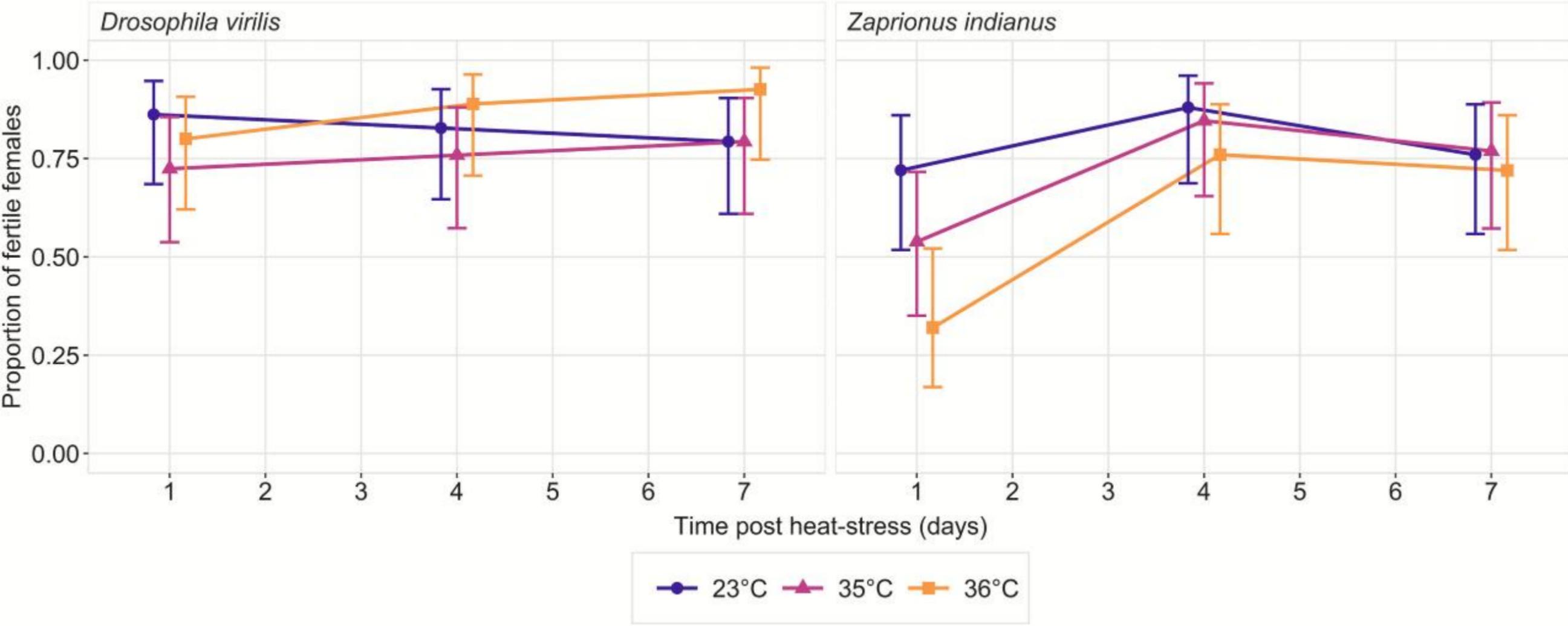


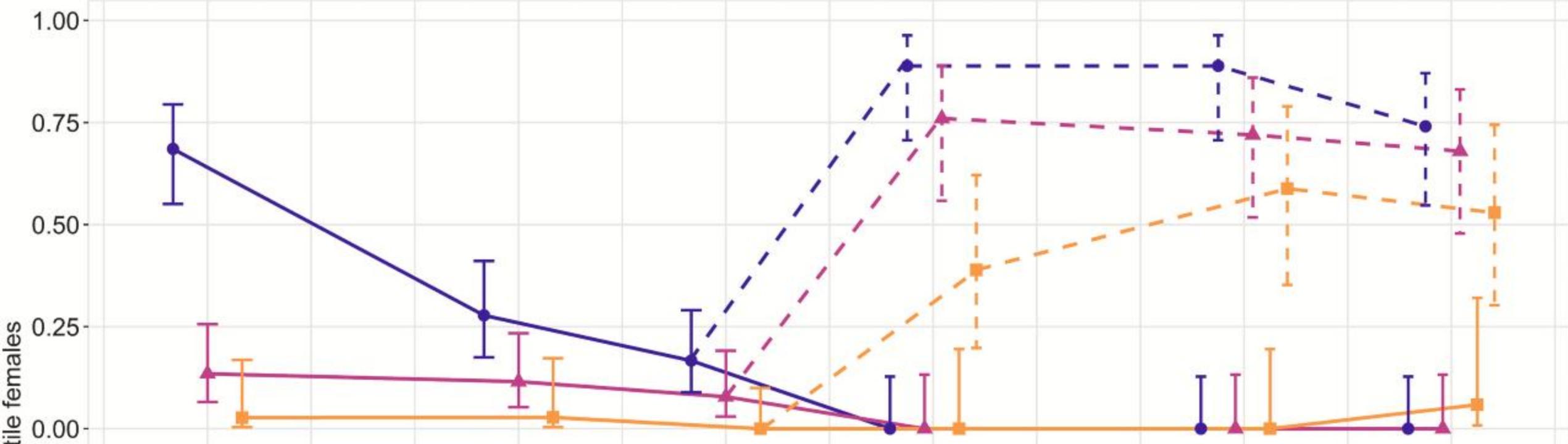
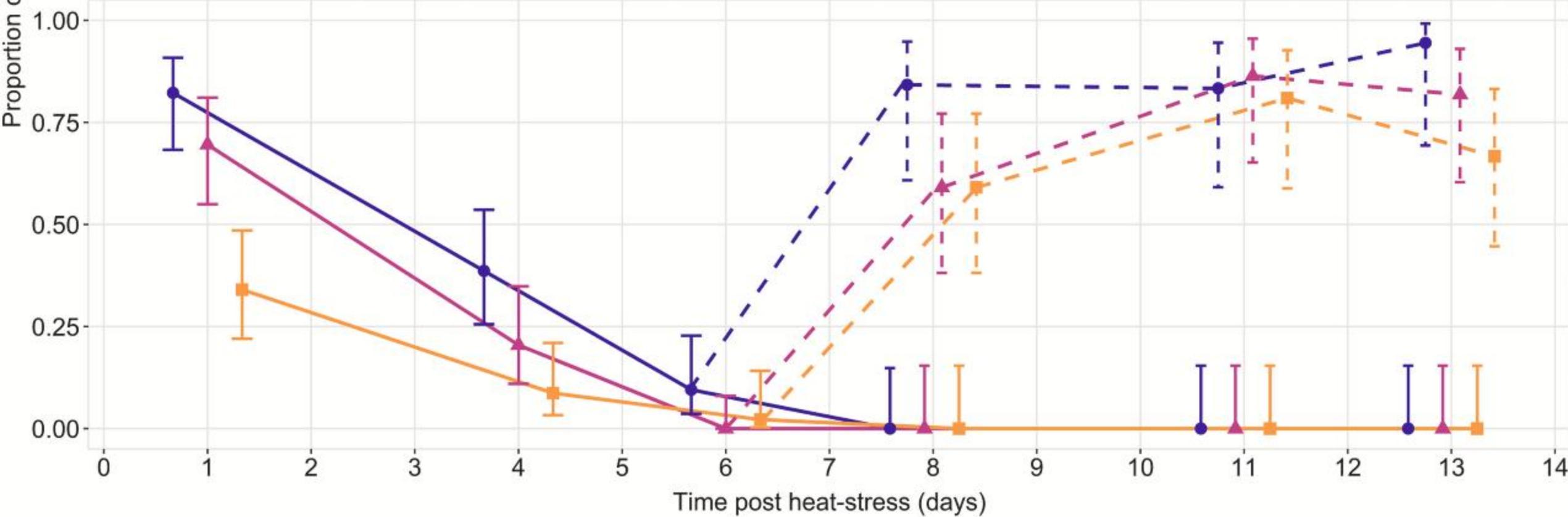
2a) Mated/Heat/Fully Isolated



2b) Mated/Heat/Isolated/Remated





Drosophila virilis*Zaprionus indianus*

● 23°C ▲ 35°C ■ 36°C

— Single mating - - Remated