

1 **Maternal loading of a small heat shock protein increases embryo thermal**
2 **tolerance in *Drosophila melanogaster***

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35 **SUMMARY STATEMENT**

36 A gene-specific maternal effect confers thermal tolerance to offspring embryos in the
37 fruit fly *Drosophila melanogaster*.

38

39 **ABSTRACT**

40 Maternal investment is likely to have direct effects on offspring survival. In oviparous
41 animals whose embryos are exposed to the external environment, maternal provisioning
42 of molecular factors like mRNAs and proteins may help embryos cope with sudden
43 changes in the environment. Here we sought to modify the maternal mRNA contribution
44 to offspring embryos and test for maternal effects on acute thermal tolerance in early
45 embryos of *Drosophila melanogaster*. We drove in vivo overexpression of a small heat
46 shock protein gene (*Hsp23*) in female ovaries and measured the effects of acute
47 thermal stress on offspring embryonic survival and larval development. We report that
48 overexpression of the *Hsp23* gene in female ovaries produced offspring embryos with
49 increased thermal tolerance. We also found that brief heat stress in the early embryonic
50 stage (0 to 1 hour-old) caused decreased larval performance later in life (5 to 10 days-
51 old), as indexed by pupation height, as well as increased development time to pupation.
52 Maternal overexpression of *Hsp23* protected embryos against these heat-induced larval
53 defects. Our data demonstrate that transient products of single genes have large and
54 lasting effects on whole-organism environmental tolerance. Further, our results suggest
55 that maternal effects have a profound impact on offspring survival in the context of
56 thermal variability.

57

58 INTRODUCTION

59 Acute thermal stress is principally felt at the cellular and biochemical levels through the
60 disruption of macromolecular structures (Richter et al., 2010; Somero et al., 2017).
61 These thermal perturbations pose challenges for ectotherms that live in variable thermal
62 environments where sudden changes in temperature are a frequent occurrence (Denny
63 et al., 2011; Terblanche et al., 2011; Dowd et al., 2015; Buckley and Huey, 2016).
64 Thermal stress causes proteins to unfold, which not only leads to the loss of protein
65 function but also to protein aggregation that is toxic to cells (Richter et al., 2010;
66 Somero et al., 2017). To combat these effects, nearly all living organisms possess a
67 conserved set of cellular responses—collectively referred to as the heat shock response
68 or cellular stress response—which are characterized by rapid shifts in the expression of
69 hundreds to thousands of gene loci (Gasch et al., 2000; Leemans et al., 2000; Buckley
70 et al., 2006; Lockwood et al., 2010; Brown et al., 2014). A key component of the heat
71 shock response is the dramatic induction of genes that encode heat shock proteins
72 (HSPs), while the majority of the rest of the proteome ceases to be expressed (Tissi eres
73 et al., 1974; Mirault et al., 1978; Lindquist, 1981; Hofmann and Somero, 1996; Tomanek
74 and Somero, 1999; Tomanek and Zuzow, 2010). HSPs function as molecular
75 chaperones that bind, sequester, and help refold thermally denatured proteins (Richter
76 et al., 2010), providing thermal protection at the molecular level that scales up the whole
77 organism. Indeed, sublethal thermal exposures that induce the heat shock response
78 allow organisms to survive more extreme subsequent thermal exposures that would
79 otherwise be lethal (Arrigo, 1987; Feder et al., 1996). Transgenic overexpression of
80 HSPs confers increased whole-organism thermal tolerance (Welte et al., 1993; Feder et
81 al., 1996), and the expression of HSPs has been shown to be adaptive under conditions
82 of heat stress, as laboratory selection to high temperatures leads to higher expression
83 of HSPs (Rudolph et al., 2010). In addition, many populations and species that live in
84 environments characterized by frequent, acute exposures to extreme heat, have
85 evolved higher expression of HSPs than closely related species that inhabit more
86 benign thermal environments (Hofmann and Somero, 1996; Tomanek and Somero,
87 2000; Dong et al., 2008; Lockwood et al., 2010; Schoville et al., 2012; Dilly et al., 2012).
88 Despite the broad evolutionary conservation of the heat shock response across
89 taxa (K ultz, 2005; Somero et al., 2017), animals in the earliest life stages have vastly
90 reduced heat shock responses (Graziosi et al., 1980; Welte et al., 1993) due to the low
91 transcriptional activity of early zygotes (Tadros and Lipshitz, 2009). This poses a
92 challenge to oviparous species with external embryonic development. Early embryos of
93 these organisms are directly exposed to the thermal environment and may have little
94 opportunity to express protective proteins from their own genomes. Rather, their
95 mechanisms for coping with thermally-induced molecular damage are limited to the
96 molecular factors (i.e., RNAs, protein, and organic osmolytes) that are loaded into eggs
97 by mothers (Wieschaus, 1996). Indeed, previous studies have shown early embryonic
98 stages to be more thermally sensitive than later stages (Walter et al., 1990; Welte et al.,
99 1993).
100 Given that maternal oogenesis establishes the early embryonic transcriptome
101 and proteome (Sch upbach and Wieschaus, 1986; Wieschaus, 1996; Tadros and
102 Lipshitz, 2009), maternal molecular factors are likely to be a major determinant of
103 developmental robustness and survival in the face of variable thermal environments.

104 However, few studies have characterized the molecular roles of maternal effects in the
105 context of embryonic thermal tolerance (Sato et al., 2015). In fruit flies (*Drosophila*
106 *melanogaster*), the early time window of minimal zygotic transcriptional activity spans
107 the first 2 hours of development, after which zygotic transcription begins to predominate
108 over the maternally provided pool of mRNAs (Blythe and Wieschaus, 2015a).
109 Consequently, 4-h-old embryos are more heat tolerant than earlier stages (Walter et al.,
110 1990), and by 14 h post-fertilization (Welte et al., 1993) embryos attain approximately
111 the same degree of thermal tolerance that they possess later on as larvae, pupae, and
112 adults (Huey et al., 1991; Feder et al., 1997).

113 Among the mRNAs that are loaded into eggs by *D. melanogaster* mothers,
114 messages that encode members of the small heat shock protein (sHSP) family are
115 highly abundant, with two sHsps being among the top 1% of most highly abundant
116 transcripts in the early embryo (see Fig. 1)(Pauli et al., 1989; Michaud and Tanguay,
117 2003; Brown et al., 2014; Morrow and Tanguay, 2015). Genes encoding this class of
118 proteins are also among the most highly expressed genes following heat stress in
119 larvae, pupae, and adults (Berger and Woodward, 1983; Ayme and Tissières, 1985;
120 Horwitz, 1992; Brown et al., 2014). sHSPs are a family of molecular chaperones that
121 serve a wide-range of molecular functions, including stabilizing major cellular structural
122 components like the cytoskeleton (Leicht et al., 1986; Horwitz et al., 1992) and the cell
123 membrane (Tsvetkova et al., 2002; Horváth et al., 2008). Thus, the maternal
124 contribution of these proteins may be a critical factor in maintaining embryonic
125 development of offspring in both benign thermal conditions and in the presence of
126 thermal stress.

127 Here, we establish a role for maternal effects in conferring embryonic thermal
128 tolerance in *D. melanogaster* via maternal loading of the sHSP gene *Hsp23*, which is a
129 major component of the heat shock response. We report that among sHSP genes,
130 *Hsp23* is unique in that it is a major component of the adult heat shock response but
131 only present at low abundance in early embryos. Further, by driving overexpression of
132 this gene in female oocytes, we observed marked increases in thermal tolerance in
133 offspring embryos and lasting effects that influenced larval performance—both of which
134 were significant maternal effects. Overall, our results demonstrate that single genes of
135 large effect can contribute significantly to whole-organism phenotypes, such as thermal
136 tolerance, and that maternal loading of mRNAs can influence not only early embryonic
137 development but also larval performance later in life.

138

139 MATERIALS AND METHODS

140

141 modENCODE expression data

142 modENCODE is a collaborative project that generated transcriptomic data from RNA-
143 sequencing (RNA-Seq) across life stages and in response environmental stressors in *D.*
144 *melanogaster* (Brown et al., 2014). Expression data were downloaded from FlyBase
145 (Attrill et al., 2016) and consist of mRNA levels (expressed as reads per kilobase of
146 transcript per million mapped reads: RPKM) of 18,029 unique transcripts. Among these
147 transcripts, we used nonlinear least-squares regression fitting to compare mRNA levels
148 in early embryos (0-2 h post-fertilization) and 4 d-old heat-shocked adults (36°C for 1 h),
149 with Robust regression and Outlier removal (ROUT) analysis (Motulsky and Brown,
150 2006) to identify outliers.

151

152 Fly stocks

153 To assess the effects of targeted overexpression and increased maternal loading of
154 *Hsp23* in early embryos, we used the Gal4-UAS system (Brand and Perrimon, 1993;
155 Duffy, 2002) in a two-step crossing scheme (Fig. 2A). First, we used a female germline
156 Gal4 driver, *MTD-Gal4* (Bloomington Stock—BL#: 31777), crossed with *UAS-Hsp23*
157 (BL#: 30541) to cause *Hsp23* overexpression in female ovaries (*Hsp23*^{OE}). These
158 constructs when brought together in a genetic cross drive overexpression of the target
159 gene (*Hsp23*) in female ovaries and were chosen to modify the levels of *Hsp23* mRNAs
160 that are loaded into eggs. Second, we tested the effects of this overexpression
161 construct in early embryos by comparing the phenotypes of 0-1 h-old offspring embryos
162 from reciprocal crosses between the *Hsp23*^{OE} and the control genotype (*w*¹¹¹⁸) that
163 switched the female and male genotypes, such that embryos from one cross (female
164 *Hsp23*^{OE} x male *w*¹¹¹⁸) had mothers that overexpressed *Hsp23* and embryos from the
165 other cross (female *w*¹¹¹⁸ x male *Hsp23*^{OE}) were genetically similar but had control
166 mothers with wildtype *Hsp23* expression. The control genetic background was *w*¹¹¹⁸
167 (BL#: 5905), which was the original strain used to generate the *UAS-Hsp23* transgenic
168 line. We note that the *MTD-Gal4* strain was generated in the *w*^{*} genetic background.
169 Therefore, F2 offspring of *Hsp23*^{OE} mothers received mitochondria from *w*^{*}, whereas
170 offspring from the reciprocal cross received mitochondria from *w*¹¹¹⁸. While these
171 represent two distinct mtDNA genetic backgrounds, many lab stocks were originally
172 derived from similar mitochondrial lineages and natural populations of *D. melanogaster*
173 harbor relatively low levels of mtDNA polymorphism (Cooper et al., 2015). Thus, we
174 interpret measurable differences in embryonic thermal tolerance among genotypes to
175 be largely the result of differential maternal loading of *Hsp23* mRNAs, and not an artifact
176 of mitochondrial lineage. All stocks were obtained from the Bloomington Drosophila
177 Stock Center (Bloomington, IN) and maintained at 22°C on standard cornmeal, yeast,
178 and agar medium.

179 We focused our experiments on the maternal effects of overexpression and
180 increased loading of *Hsp23* and did not perform a targeted knockdown of this gene for
181 the following reasons. First, *Hsp23* is present in such low abundance in early embryos
182 (Table 1; Fig. 1) that knocking down the expression of this gene is likely to have little
183 effect. Additionally, there is evidence that the more abundant sHSPs, such as *Hsp26*
184 and *Hsp27*, compensate for the absence of *Hsp23* under heat stress conditions

185 (Bettencourt et al. 2008). Second, recent reviews of the literature suggest that, despite
186 the preponderance of targeted gene knockdown experimental designs, many loss-of-
187 function studies across a broad array of species have failed to produce measureable
188 phenotypic outcomes (Gibney et al., 2013; Evans, 2015). This may be due to functional
189 redundancy among genes or lack of assay sensitivity to characterize more subtle
190 physiological effects (Bischof et al., 2013). Whatever the biological significance of these
191 trends in loss-of-function studies, gain-of-function experimental designs are warranted
192 and have led to the recent creation of comprehensive genetic resources for targeted
193 gene over-expression (Bischof et al., 2013). Third, we predicted that overexpression
194 and increased maternal loading of *Hsp23* into early embryos would more closely
195 phenocopy the higher thermal tolerance of later stages of development that possess an
196 enhanced ability, relative to early embryos, to induce the high levels of expression of
197 heat shock genes, including *Hsp23* (Fig. 1).

198

199 **Quantification of *Hsp23* mRNA levels**

200 We extracted total RNA from separate pooled batches of 20 - 100 embryos (0-1 h-old)
201 that constituted our biological replicates for quantitative PCR (qPCR). Embryos were
202 collected from grape juice agar plates after being exposed to 22°C or 34°C for 45 min
203 (see Embryonic thermal tolerance section below), rinsed in 1x PBS, dechorionated in
204 50% bleach for 1 min, and rinsed again in diH₂O. We note that embryos were less than
205 2 h-old, which is prior to the activation of zygotic transcription of the majority of genes
206 (Ali-Murthy et al., 2013; Blythe and Wieschaus, 2015b). The embryos were then
207 transferred to microcentrifuge tubes, frozen on liquid nitrogen, and stored at -80°C for
208 up to one month prior to RNA extraction. We extracted total RNA with TRIzol (Molecular
209 Research Center, Cincinnati, OH, USA) and Phase Lock Gel tubes (Quantabio, Beverly,
210 MA, USA) that are designed to maintain stable separation of aqueous and organic
211 phases. RNA quality was assessed on a NanoDrop spectrophotometer (NanoDrop
212 products, Wilmington, DE, USA). We then removed any residual DNA with the TURBO
213 DNA-FREE kit (Thermo Fisher Scientific, Waltham, MA, USA). We performed reverse
214 transcriptase reactions with the SuperScript III First-Strand Synthesis kit using oligo dT
215 primers (Thermo Fisher). qPCR was conducted with the Agilent Brilliant III Ultra-Fast
216 SYBR Green Master Mix (Agilent Technologies, Santa Clara, CA, USA) on a Bio-Rad
217 CFX Connect Real-Time PCR system (Bio-Rad, Hercules, CA, USA) using primer sets
218 and reaction conditions that have been previously described (Bettencourt et al., 2008).

219 We calculated reaction efficiencies from standard curves for both the target gene
220 (*Hsp23*) and the reference (*Act5c*), and we found the efficiencies to be identical for both
221 genes ($E = 1.87$). We chose *Act5c* as the reference gene based on previous work that
222 has shown it to exhibit stable expression across benign and heat shock conditions
223 (Hoekstra and Montooth, 2013). We used the efficiency value to calculate average fold-
224 differences among experimental groups as previously described (Pfaffl, 2001). We
225 compared relative *Hsp23* mRNA levels among experimental groups using an ANOVA of
226 $-\Delta CT$ values, where $\Delta CT = CT_{Hsp23} - CT_{Act5c}$.

227

228 **Embryonic thermal tolerance**

229 We measured acute thermal tolerance in early embryos (0 to 1 h post-fertilization) from
230 crosses between genotypes designed to generate overexpression or normal expression

231 (see above)(Fig. 2A). At this early stage, embryos coordinate early developmental
232 processes via the molecular factors (i.e. RNA and protein) provided to them by their
233 mothers (Tadros and Lipshitz, 2009; Blythe and Wieschaus, 2015a). Thus, embryonic
234 phenotypic effects that we report on herein are largely the result of maternal effects
235 mediated by changes in the expression of genes in female ovaries, the products of
236 which are subsequently loaded into eggs. We chose this early stage because we sought
237 to characterize the effects of (1) maternal mRNA contributions and (2) targeted gene
238 overexpression in the absence of a fully developed zygotic heat-shock transcriptional
239 response. This allowed us to better isolate and characterize the functional contribution
240 of the transcription of a single gene (i.e. *Hsp23*) for whole-organism acute thermal
241 tolerance, without the potentially confounding effects of large and concomitant changes
242 in the expression of other heat shock genes.

243 We designed our temperature treatments to mimic sudden (acute) changes in
244 temperature that frequently occur in nature where the temperature of necrotic fruit can
245 increase rapidly on a hot day (Feder et al., 1997; Terblanche et al., 2011). 3 to 5 day-old
246 adult flies of the appropriate genotypes were allowed to mate and lay eggs on grape
247 juice agar plates for 1 h at 22°C. Egg plates were then wrapped in Parafilm and
248 submerged in a water bath set to one of a range of temperatures between 22°C and
249 40°C (22°C, 24°C, 26°C, 28°C, 30°C, 32°C, 34°C, 36°C, 38°C, or 40°C) for 45 minutes.
250 Due to the thermal mass of the egg plates, the embryos did not immediately experience
251 the temperature of the water bath upon immersion, but rather were exposed to a
252 thermal ramp that averaged +0.4°C min⁻¹ among all temperatures. While this rate of
253 change is extreme, it is within the range of maximum measured rates of change in the
254 field (Terblanche et al., 2011). After thermal exposure, a section of the agar containing
255 20 eggs was cut and transferred to a food vial where eggs were allowed to recover and
256 develop at 22°C. Hatching, pupation, and eclosion success were scored as the
257 proportion of these 20 eggs that survived to each stage. Hatching success was scored
258 at 48 h, pupation success at 5 to 10 days, and eclosion at 10 to 15 days post-
259 fertilization. We also scored development time as the length of time (days) to successful
260 pupation and eclosion. Temperature treatments and phenotypic measurements were
261 conducted on four to six vials in each of three to four separate generations for each
262 cross type (i.e., N = 4 to 6 vials x 10 temperatures x 3 to 4 generations per genotype) for
263 a total number of 12 to 24 biological replicates per genotype per temperature.

264 We calculated the lethal temperature at which 50% of the embryos failed to hatch,
265 pupate, or eclose (LT₅₀) via a least-squares logistic regression model. We allowed the y-
266 intercept to vary between 0 and 1 and extrapolated the LT₅₀ from the inflection point of
267 the logistic curve fit. This approach allowed us to infer thermal tolerance independently
268 from other confounding factors that reduce hatching success, such as the presence of
269 unfertilized eggs.

270

271 **Pupation height**

272 We scored the average pupation height as a measurement of larval performance
273 (Mueller and Sweet, 1986; Hoekstra et al., 2013). Pupation height was scored
274 subsequent to early embryonic temperature treatments (see above) at 8 to 10 days
275 post-fertilization. Each food vial was divided into four quadrants; quadrant 1 spanned
276 the distance from the bottom of the vial to 3.5 cm in height and quadrants 2 - 4 each

277 comprised a section of 2 cm up the height of the vial. Each pupa was scored a number
278 between 1 and 4, corresponding to the quadrant in which it pupated. All pupae on the
279 food were scored as 1 (quadrant 1). Average pupation height was then calculated
280 separately for each vial.

281 282 **Statistics**

283 LT₅₀ values were compared by assessing the fit of the logistic regression models to
284 each genotype separately vs. all genotypes combined via the extra sum-of-squares F-
285 test and the Akaike's Information Criteria (AICc). The effects of temperature, treatment,
286 and maternal genotype on development time were analyzed via ANOVA followed by
287 Sidak's multiple comparisons test to assess pairwise differences. Pupation height was
288 analyzed in the same manner via ANOVA and Sidak's test. All analyses were
289 conducted in GraphPad Prism version 7 for Mac (GraphPad Software, La Jolla, CA).

290 291 **RESULTS**

292 Among all 18,029 transcripts included in the modENCODE dataset, mRNA levels in
293 early embryos and heat-shocked adults were positively correlated (Fig. 1; Least-
294 squares regression, $R^2 = 0.17$, $y = 10^{(1.002 \cdot \log(x) - 0.1344)}$). Among the 12 sHSP
295 genes, mRNA levels in embryos and heat-shocked adults were also positively
296 correlated (Fig. 1; Robust regression, $R^2 = 0.97$, $y = 10^{(0.5436 \cdot \log(x) + 1.905)}$), even
297 though mRNA levels were higher in heat-shocked adults compared to embryos (Table
298 1; Fig. 1). However, *Hsp23* was a significant outlier in this relationship (Fig. 1; ROUT
299 outlier analysis, $Q = 1\%$). Of sHSP genes, *Hsp23* has the biggest difference in
300 expression level between early embryos and heat-shocked adults and is present at low
301 levels in non-heat-shocked adults, with a heat-shock induction response of >100-fold
302 (Table 1).

303 We sought to test the contribution of maternal *Hsp23* mRNAs to embryonic
304 thermal tolerance by increasing *Hsp23* abundance in early embryos through
305 overexpression in the maternal germline. We focused our functional genetic analyses
306 on *Hsp23* because this gene (1) was the sole significant outlier among the sHSP genes
307 in the relationship between early embryonic vs. heat-shocked adult gene expression
308 (Fig. 1) and (2) showed the greatest induction in response to heat-shock in adults
309 (Table 1). These observations suggest that *Hsp23* plays a unique role among the sHSP
310 genes in the heat-shock response, and thus may be a key factor in conferring acute
311 thermal tolerance.

312 Maternal genotype (*Hsp23*^{OE} versus *w*¹¹¹⁸ control) and embryonic heat stress (45
313 min at 34°C) both had significant effects on *Hsp23* mRNA levels in early embryos
314 (Table 2 and Fig. 2B; ANOVA temperature effect, $F_{1,8} = 16.45$, $P = 0.0037$, maternal
315 genotype effect, $F_{1,8} = 7.572$, $P = 0.025$), and these effects were additive (ANOVA
316 temperature x maternal genotype interaction, $F_{1,8} = 0.2216$, $P = 0.6504$). *Hsp23*-
317 overexpressing females (*Hsp23*^{OE}) laid eggs with 2.12-fold higher baseline levels of
318 *Hsp23* mRNAs at 22°C and a 2.89-fold higher levels of *Hsp23* mRNAs following heat
319 shock at 34°C, relative to embryos that were offspring of mothers of the control genetic
320 background (*w*¹¹¹⁸) (Fig. 2B). In addition, heat shock led to significant increases in the
321 levels of *Hsp23* mRNAs regardless of maternal genotype, increasing by 4.44-fold and

322 3.26-fold (34°C relative to 22°C) in offspring embryos of (female x male) *Hsp23*^{OE} x
323 *w*¹¹¹⁸ and *w*¹¹¹⁸ x *Hsp23*^{OE}, respectively (Fig. 2B).

324 Maternal overexpression of *Hsp23* significantly increased embryonic thermal
325 tolerance by raising the lethal temperature (LT₅₀) by approx. 1°C (Fig. 3; Extra sum of
326 squares F-test, $F_{1,262} = 5.371$, $P = 0.02$). Embryos that successfully hatched also
327 survived to pupation and adulthood, as 95-100% of larvae and pupae survived to
328 pupation and eclosion, respectively, regardless of maternal genotype. Furthermore,
329 there were no significant differences between the LT₅₀ of hatching, pupation, and
330 eclosion successes for a given genotype (Fig. 3B; Extra sum of squares F-test, $P >$
331 0.05), suggesting that effects of early, acute thermal stress on survival were largely
332 localized to embryogenesis.

333 In addition to the positive and protective effect of maternal *Hsp23* overexpression
334 for whole-embryo survival of thermal stress, maternal loading of this gene in early
335 embryos had significant effects on larval performance, as indexed by pupation height.
336 Exposure of 0-1 h-old embryos to the brief (45 min.) episode of thermal exposure
337 resulted in larvae with significantly reduced pupation height at the highest temperatures
338 (Fig. 4A), which explained 22% of the variation in pupation height (Table 3; ANOVA
339 temperature effect, $F_{8,189} = 7.744$, $P < 0.0001$). Maternal *Hsp23* overexpression had no
340 significant effect on pupation height overall (Fig. 4A; Table 3; ANOVA maternal
341 genotype effect, $F_{1,189} = 3.674$, $P = 0.0568$) but conferred protection against the
342 negative effects of heat stress on pupation height, particularly at 34°C (Fig. 4A; Table 3;
343 ANOVA temperature x maternal genotype interaction, $F_{8,189} = 2.822$, $P = 0.0056$,
344 Sidak's test on pairwise difference at 34°C, $P < 0.001$).

345 Embryonic heat stress also caused significant increases in development time, as
346 indexed by the length of time (days) to pupation (Fig. 4B), in offspring of both maternal
347 genotypes, with a trend of maternal overexpression of *Hsp23* attenuating this
348 developmental delay (Table 3; ANOVA temperature effect, $F_{8,169} = 9.605$, $P < 0.0001$,
349 maternal genotype effect, $F_{1,169} = 2.928$, $P = 0.0889$, temperature x maternal genotype
350 interaction, $F_{8,169} = 0.6591$, $P = 0.73$).

351 Embryonic heat stress also significantly affected the developmental time to adult
352 eclosion (Fig. 4C; Table 4); however, the effect sizes were much smaller than the heat-
353 stress-induced delay to pupation, and the pattern was largely driven by a shorter time to
354 eclosion at 22°C (Fig. 4C; ANOVA temperature effect, $F_{8,347} = 11.511$, $P < 0.001$).
355 Maternal *Hsp23* overexpression had no significant effect on time to eclosion (Fig. 4C;
356 Table 4; ANOVA maternal genotype effect, $F_{1,347} = 0.814$, $P = 0.3676$), regardless of
357 temperature (ANOVA temperature x maternal genotype interaction, $F_{8,347} = 2.020$, $P =$
358 0.1561), sex (ANOVA sex x maternal genotype interaction, $F_{1,347} = 0.098$, $P = 0.7544$),
359 or the interaction among all of these effects (ANOVA temperature x sex x maternal
360 genotype interaction, $F_{1,347} = 0.1091$, $P = 0.7414$). There was a significant difference
361 between females and males in time to eclosion across all temperatures, with females
362 eclosing sooner than males, and sex accounted for the greatest variation in time to
363 eclosion (Table 4; Fig. 4C; ANOVA sex effect, $F_{1,347} = 30.263$, $P < 0.00001$). And, while
364 males on average suffered greater developmental delays to eclosion following acute
365 exposure to 38°C (Fig. 4C), this effect was not significant (ANOVA temperature x sex
366 interaction, $F_{8,347} = 0.149$, $P = 0.70$).

367 There was a slight discrepancy between the thermally induced delays in
368 development to pupation vs. eclosion. Specifically, embryonic thermal stress at 34°C
369 and 36°C caused delays in time to pupation but not eclosion (Figs. 4B and 4C). In effect,
370 this means that the pupae that suffered developmental delays to pupation somehow
371 recovered from this delay to be able to eclose on the same schedule as pupae that
372 were exposed to lower embryonic temperatures. This may have occurred due to the
373 entrainment of eclosion behavior by circadian rhythms (Kyriacou et al., 1990; Paranjpe
374 et al., 2005), in which case delayed pupae could catch up to the eclosion schedule of
375 other pupae, as long as the pupation was delayed by less than 24 hours. Alternatively,
376 this pattern may be an artifact of the low sample sizes and high variance in
377 development times that accompanied the more extreme thermal exposures, as far fewer
378 individuals successfully hatched after exposure to the highest temperatures (Fig. 3A).
379 But despite this incongruity between pupation and eclosion times at the highest
380 temperatures of embryonic heat stress, overall our data indicate that overexpression of
381 *Hsp23* in the maternal germline not only increased embryonic hatching success after
382 exposure to heat stress, but also had persisting effects on offspring performance
383 throughout larval development.

384 385 **DISCUSSION**

386 Despite over five decades of research on the heat shock response, there have
387 been relatively few studies to connect genotype to phenotype in the context of heat
388 shock protein expression and organismal performance (Somero et al., 2017). Here we
389 demonstrate the direct effects of maternal loading of *Hsp23* mRNAs for offspring
390 survival and performance following acute heat stress in a common genetic background.
391 We found that increases in the levels of *Hsp23* in early *D. melanogaster* embryos confer
392 significant protection from heat stress during a thermally sensitive life stage.

393 394 ***Hsp23* maternal loading and the embryonic heat shock response**

395 *Hsp23* overexpression in female ovaries resulted in embryos with increased
396 abundance of *Hsp23* mRNA. Thus, the phenotypic effects of maternal genotype on
397 whole-embryo thermal tolerance that we observed were likely the consequence of
398 increased maternal loading that elevated basal levels of *Hsp23* in early embryos. We
399 also observed that embryos induced the expression of *Hsp23* in response to heat shock
400 regardless of maternal genotype, with no significant interaction between maternal
401 genotype and temperature. Previous work has shown that the transgenic manipulation
402 of heat shock protein 70 (*Hsp70*) gene induction in *D. melanogaster* (Welte et al., 1993)
403 causes massive increases in the heat-induced transcription of *Hsp70* by more than 500-
404 fold (Hoekstra and Montooth, 2013). This increase in heat-inducible *Hsp70* mRNAs
405 translated into approximately 2.5-fold higher levels of Hsp70 protein over the time
406 course of two hours in response to heat stress in wandering third-instar larvae, which
407 allowed larvae to survive significantly longer at 39°C (Feder et al., 1996). In comparison,
408 the higher levels of *Hsp23* induced by Gal4/UAS overexpression in female ovaries that
409 we report herein were subtle. These overexpression levels were similar to previous
410 reports of overexpression of other genes in fly ovaries that were driven by similar
411 transgenic constructs (Dominguez et al., 2016). But regardless of the absolute degree
412 of overexpression, the higher maternal loading of *Hsp23* increased embryonic LT₅₀ by

413 approx. 1°C, and this increase in LT₅₀ signified a substantial increase in thermal
414 tolerance. In particular, following 45 minutes of heat stress at 34°C, 87.5% of the
415 embryos with higher *Hsp23* levels successfully hatched, whereas only 46.7% of
416 embryos with normal levels of *Hsp23* survived this heat treatment.

417 Beyond the aforementioned work in *D. melanogaster* (Welte et al., 1993; Feder
418 et al., 1996) and the present study, there have been few studies to directly test the
419 effect of heat shock protein expression on whole-organism thermal tolerance, with one
420 study showing that targeted gene knockdown of *Hsp22* and *Hsp23* in adult *D.*
421 *melanogaster* decreases cold tolerance (Colinet et al., 2010). A much larger body of
422 work has used interspecies and inter-population comparisons to infer the evolutionary
423 history of the heat shock response (Hofmann and Somero, 1996; Tomanek and Somero,
424 2000; Dong et al., 2008; Lockwood et al., 2010; Schoville et al., 2012; Dowd and
425 Somero, 2013; Nguyen et al., 2016) Based on these studies, it is well-established that
426 HSP expression is an adaptive physiological mechanism for coping with acute thermal
427 stress. Accordingly, population-level comparisons have discovered clinal variation in
428 *Hsp23* alleles across environmental thermal gradients in *Drosophila buzzatii* in Australia
429 (Frydenberg et al., 2010), as well as clines in allele frequencies of *Hsp23* and *Hsp26*
430 among *D. melanogaster* populations in Australia (Frydenberg et al., 2003). In addition,
431 laboratory thermal selection studies have found evolved changes in HSP expression to
432 accompany adaptive shifts in upper thermal limits (Rudolph et al., 2010; Kelly et al.,
433 2017). It is important to note, however, that increased levels of HSP expression do not
434 always accompany thermal adaptation to higher temperatures (Zatsepina et al., 2001).
435 In fact, experimental evolution to a higher constant temperature in *D. melanogaster* led
436 to the evolution of lower *Hsp70* expression and concomitant decreases in acute thermal
437 tolerance (Bettencourt et al., 1999). Thus, higher basal and inducible HSP expression
438 may be adaptive primarily in environments that are characterized by sudden and
439 dramatic heat stress events, rather than constant hot environments (Dong et al., 2008;
440 Dilly et al., 2012).

441 Even though our observed genotypic effects on embryo thermal tolerance were
442 the result of differential maternal loading of *Hsp23*, it is interesting to note that we
443 observed zygotic induction of this gene in offspring of both maternal genotypes. At this
444 early stage of development (0-1 h-old), zygotic genomes are predicted to be
445 transcriptionally inactive because embryos have not undergone the maternal-to-zygotic
446 transition (MZT) that occurs in the mid-blastula stage (approx. 2.5 h-old) in *D.*
447 *melanogaster* (Tadros and Lipshitz, 2009; Blythe and Wieschaus, 2015a). However,
448 prior to the canonical MZT, zygotic gene expression appears to be responsive to
449 thermal variability. A previous analysis of protein expression in early *D. melanogaster*
450 embryos using 2-dimensional gel electrophoresis found that heat shock proteins were
451 heat-inducible at 1 to 2-h post-fertilization (Graziosi et al., 1980). Moreover, recent work
452 in *D. melanogaster* has highlighted the developmental role of early zygotic gene
453 transcription that precedes the MZT (Ali-Murthy et al., 2013), but the full extent to which
454 the early zygotic genome responds to thermal variability warrants new investigation. In
455 the present study, *Hsp23* expression was induced in early embryos to a much lesser
456 extent (approx. 4-fold) than what has been previously observed in later stages of
457 development. In fact, Leemans et al. (Leemans et al., 2000) found *Hsp23* to be heat-
458 induced by more than 10-fold in late-stage embryos (18 h-old) and Brown et al. (Brown

459 et al., 2014) reported this gene to be heat-induced by approx. 100-fold in adults (Table
460 1). Therefore, while embryos at the earliest stages of development appear to exhibit a
461 heat shock response, it is at a much-reduced level compared to later stages. This
462 explains why early embryonic stages are more thermally sensitive than later stages
463 (Walter et al., 1990; Welte et al., 1993) and further emphasizes the potentially critical
464 role of maternally-loaded mRNAs and proteins as thermal protectants.

465 The specific mechanism by which the Hsp23 protein confers thermal tolerance
466 remains elusive. This protein exhibits general chaperoning activity by preventing heat
467 denaturation of proteins in vitro (Heikkila et al., 2006), but it has also been shown to be
468 involved in ventral furrow morphogenesis in early fly embryos under benign thermal
469 conditions (Gong et al., 2004). This developmental role may be mediated through the
470 interaction of Hsp23 with elements of the cytoskeleton, such as microtubules (Hughes
471 et al., 2008) and actin microfilaments (Goldstein and Gunawardena, 2000). The
472 cytoskeletal association of Hsp23 is further supported by the observation that this
473 protein was the only small heat shock protein whose overexpression was observed to
474 prevent actin-dependent contractile dysfunction in cardiomyocytes of *D. melanogaster*
475 larvae (Zhang et al., 2011). Indeed, among the sHSPs that are highly induced in
476 response to heat stress (Table 1), Hsp23 is the only one that is both localized to the
477 cytoplasm (Morrow and Tanguay, 2015) and contains an actin-binding domain
478 (sequence data not shown). Whether or not the interaction of Hsp23 with the
479 cytoskeleton provides protection in the context of thermal stress has not been reported,
480 but this is a worthwhile topic of future study.

481

482 **Effects of embryonic heat stress on post-embryonic larval development**

483 We found that the *Hsp23*-mediated maternal effect extended beyond embryonic
484 thermal tolerance (i.e., hatching success) and attenuated heat-induced defects in larval
485 performance (i.e., pupation height) and prevented heat-induced developmental delays
486 (i.e., days to pupation). It is surprising that a brief thermal exposure experienced during
487 the first 2 hours of life has negative consequences that last for days to weeks,
488 throughout larval development. Drops in pupation height and increases in development
489 time have been previously associated with lower energetic performance and fitness
490 (Mueller and Sweet, 1986; Montooth et al., 2010; Hoekstra et al., 2013; Meiklejohn et al.,
491 2013), and may have important ecological consequences in natural populations.

492 The persistent effects of maternal transcript loading on larval development post-
493 heat stress might have important consequences for the evolution of maternal effects. A
494 recent study reported significant maternal effects that determined both acute (i.e., 1 h at
495 27°C) and chronic (i.e., constant exposure to 24°C) thermal tolerance in offspring
496 embryos among wild populations of *Ciona intestinalis* (Sato et al., 2015). This suggests
497 that there is natural genetic variation for maternal effects of thermal traits in some
498 species. However, evolutionary theory predicts that selection is less effective on alleles
499 that confer maternal effects, compared to genes expressed in both sexes, due to a
500 reduced effective population size (Demuth and Wade, 2007; Van Dyken and Wade,
501 2010). Consequently, maternal effect genes can harbor higher levels of standing
502 genetic variation, presumably because deleterious mutations are not removed as
503 frequently from the population and these genes cannot evolve as readily via positive
504 selection (Barker et al., 2005). Nevertheless, if maternal effects not only determine

505 hatching success but also influence larval performance and development, then the
506 fitness consequences associated with thermal stress may lead to a greater strength of
507 selection on maternal effect genes (i.e., greater difference in fitness among maternal
508 effect genotypes) than would otherwise be predicted from the maternal effects of
509 offspring hatching success alone. Because responses to natural selection depend on
510 both the strength and the efficacy of selection, the broad developmental effects of
511 maternal transcript loading may favor the adaptive evolution of maternal effect thermal
512 traits, depending on the thermal environment (Chevin et al., 2010; Chevin and
513 Hoffmann, 2016) and the underlying genetic architecture (Wolf and Wade, 2016). But to
514 our knowledge, there have been no examples of this phenomenon reported in the
515 literature.

516

517 **Conclusions**

518 Overall, our data suggest that maternal effects can have profound impacts on
519 offspring survival and performance in the context of environmental change. The
520 observation that differential maternal loading of mRNAs of a single gene can have
521 lasting consequences throughout larval development, by modifying pupation height and
522 development time, demonstrates that protective maternal effects extend well beyond the
523 maternal-to-zygotic transcriptional transition. The role of maternal effects and the
524 environmental stress physiology of early life stages has largely been ignored in the field
525 of ecological physiology, but see (Sato et al., 2015). Future work is warranted in this
526 realm, because these factors are likely be critical determinants of species responses to
527 environmental variability (Angilletta et al., 2013; Anderson and Podrabsky, 2014;
528 Buckley et al., 2015; Wagner and Podrabsky, 2015; Svetec et al., 2016), particularly if
529 early life stages are most sensitive to environmental stress (Walter et al., 1990; Welte et
530 al., 1993).

531

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535

536 **Competing interests**

537 The authors have no competing interests.

538

539 **Author contributions**

540 B.L.L. and K.L.M. conceived and planned the study. B.L.L. and C.R.J. conducted the
541 experiments. B.L.L. analyzed the data. B.L.L., C.R.J., and K.L.M. wrote the manuscript.

542

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547

548 **Data availability**

549 modENCODE transcript data are publicly available online at flybase.org (Gramates et
550 al., 2017). Phenotypic data reported herein are available on the Dryad Repository,
551 <http://>.
552
553

554 **References**

555

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823 **Tables**

824

825 **Table 1.** mRNA levels of sHSP genes in 5 day-old adults, 0-2 h-old embryos, and in
 826 response to heat-shock (4 day-old adults at 36°C for 1 h). Data are expressed as mean
 827 expression values (RPKM) and are ordered according to expression levels in heat-
 828 shocked adults. Data are from modENCODE (Brown et al. 2014).

829

CG number	Gene Name	Adult Male (5 d)	Adult Female (5 d)	Embryo (0-2 h)	Heat-shock (Adult - 4 d)
CG4183	<i>Hsp26</i>	134	751	1276	3792
CG4466	<i>Hsp27</i>	57	224	515	2689
CG4463	<i>Hsp23</i>	43	13	25	2604
CG4460	<i>Hsp22</i>	18	7	15	635
CG4533	<i>l(2)efl</i>	209	63	2	257
CG14207	-	115	55	50	219
CG7409	-	421	84	0	76
CG4190	<i>Hsp67Bc</i>	3	0	0	48
CG4461	-	304	66	0	43
CG4167	<i>Hsp67Ba</i>	1	0	0	16
CG13133	-	8	1	0	1
CG43851	-	4	0	0	1

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835 **Table 2.** Analysis of variance of relative *Hsp23* mRNA levels ($\Delta CT = CT_{Hsp23} - CT_{Act5c}$)
 836 among maternal genotypes (*Hsp23^{OE}* vs. *w¹¹¹⁸*) and embryonic heat stress
 837 temperatures (22°C vs. 34°C).

838

Source of variation	SS	DF	MS	F (1,8)	% of total variation	P-value
Temperature	11.88	1	11.88	16.45	43.54	0.0037
Maternal genotype	5.467	1	5.467	7.572	20.04	0.025
Temp. x Mat. genotype	0.16	1	0.16	0.2216	0.59	0.65
Residual	5.776	8	0.722			

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Table 3. Percent of total variation explained by the main effects of embryonic heat stress temperature, maternal genotype, and their interaction on pupation height and days to pupation (Two-way ANOVA). *P*-values are indicated in parentheses and significant effects are indicated in italics.

Trait	Temperature	Maternal genotype	Temp. x Mat. gen.
Pupation height	<i>21.63% (< 0.0001)</i>	1.28% (0.0568)	<i>7.88% (0.0056)</i>
Days to pupation	<i>28.97% (< 0.0001)</i>	1.10% (0.0889)	1.99% (0.73)

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Table 4. Percent of total variation explained by the main effects of embryonic heat stress temperature, maternal genotype, sex, and their interaction on days to adult eclosion (3-way ANOVA). *P*-values are indicated in parentheses and significant effects are indicated in italics.

Source of variation	% Variation explained (<i>P</i> -value)
Temperature	<i>2.94% (< 0.001)</i>
Maternal genotype	0.21% (0.3676)
Sex	<i>7.72% (< 0.00001)</i>
Temp. x Mat. genotype	0.52% (0.1561)
Temp. x Sex	0.038% (0.70)
Mat. genotype x Sex	0.025% (0.7544)
Temp x Mat. genotype x Sex	0.028% (0.7414)

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857 **Figure legends**

858

859 **Figure 1. Relationship between transcriptomes and sHSP mRNA levels in early**
860 **embryos vs. heat-shocked adults.** Data represent 18,029 unique transcripts and are
861 expressed as mean expression values (RPKM) on a log₂-scale of 0-2 hour-old embryos
862 and 4 day-old adults (males and females pooled). Transcripts encoding small heat
863 shock proteins (sHSPs) are shown in blue triangles, *Hsp23* highlighted in red, and all
864 other transcripts are shown in grey circles. The solid black line (+/- 95% confidence
865 bands in black dashed lines) represents the least-squares regression fit of all transcripts
866 ($R^2 = 0.17$, $y = 10^{(1.002 \cdot \log(x) - 0.1344)}$) and the solid blue line (+/- 95% confidence
867 bands in blue dashed lines) represents the robust regression fit of sHSP transcripts (R^2
868 = 0.97, $y = 10^{(0.5436 \cdot \log(x) + 1.905)}$), for which *Hsp23* was a significant outlier (ROUT
869 outlier analysis, Q = 1%).

870

871 **Figure 2. *Hsp23* overexpression in female ovaries increased *Hsp23* mRNA levels**
872 **in offspring embryos.** (A) Crossing scheme used to drive overexpression of *Hsp23* in
873 the maternal germline, thus increasing maternal loading of *Hsp23* mRNAs into offspring
874 embryos. The genotype of each sex is indicated by the gender symbols (♀ female x ♂
875 male). The MTD-*Gal4* construct drives overexpression of the UAS target gene (*Hsp23*)
876 in the germline of F1 females (*Hsp23*^{OE}♀) but not males (*Hsp23*^{OE}♂). Reciprocal
877 crosses between *Hsp23*^{OE} and the control genetic background (*w*¹¹¹⁸) produce F2
878 embryos that possess differential levels of maternally loaded *Hsp23*, as shown in part B.
879 (B) Relative *Hsp23* mRNA levels in early embryos, normalized to the *Act5c* reference
880 gene for each sample, expressed as $-\Delta CT$, where $\Delta CT = CT_{Hsp23} - CT_{Act5c}$. Each data
881 point represents a pooled batch of 50 to 100 embryos (0-1 h-old) that were exposed to
882 the indicated temperature for 45 min. Horizontal lines indicate means among separate
883 embryo batches and error bars indicate standard error. * $P < 0.05$, ** $P < 0.01$ (ANOVA
884 temperature effect, $F_{1,8} = 16.45$, $P = 0.0037$, maternal genotype effect, $F_{1,8} = 7.572$, $P =$
885 0.025, temperature x maternal genotype interaction, $F_{1,8} = 0.2216$, $P = 0.6504$).

886

887 **Figure 3. Higher maternal loading of *Hsp23* mRNAs increased thermal tolerance**
888 **of offspring embryos.** (A) Each data point represents the proportion of eggs (N = 20
889 eggs, 0-1 h-old) that hatched following 45 minutes exposure at the indicated
890 temperature. Lines indicate least-squares fit of the logistic equation and shaded regions
891 indicate 95% confidence bands. (B) Mean LT₅₀ values +/- 95% confidence intervals for
892 hatching, pupation, and eclosion success. LT₅₀ values were extrapolated from the
893 inflection points of the logistic survival curve fits (hatching success is shown in part A),
894 and confidence intervals represent the goodness of fit of the logistic regressions.
895 Different letters indicate statistical significance ($P < 0.05$, Extra sum of squares F-test).
896 The genotypes of the parents are indicated in the legends of both figure panels (female
897 x male).

898

899 **Figure 4. Heat stress in early embryos caused larval defects that were**
900 **ameliorated by maternal loading of *Hsp23*.** (A) Mean pupation height among vials (N
901 = 18 vials), +/- 95% confidence intervals, scored at 5 to 10-days-post-fertilization
902 following acute (45 min) early embryonic (0-1 h-post-fertilization) exposure at the

903 indicated temperature. Higher heat stress temperatures caused significant decreases in
904 pupation height (ANOVA temperature effect, $F_{8,189} = 7.744$, $P < 0.0001$), but higher
905 maternal loading of *Hsp23* removed this effect at 34°C (Sidak's test on pairwise
906 difference at 34°C, $***P < 0.001$). (B) Mean time to pupation (days +/- 95% confidence
907 intervals) following early embryonic temperature exposure, as described above in A.
908 Higher embryonic temperature exposure caused increased time to pupation (Table 3;
909 ANOVA temperature effect, $F_{8,169} = 9.605$, $P < 0.0001$), and there was a trend of
910 maternal loading of *Hsp23* attenuating this effect (maternal genotype effect, $F_{1,169} =$
911 2.928 , $P = 0.0889$, temperature x maternal genotype interaction, $F_{8,169} = 0.6591$, P
912 $= 0.73$). (C) Mean time to eclosion (days +/- 95% confidence intervals) following early
913 embryonic temperature exposure, as described above in A. Males took longer to eclose
914 than females at all temperatures (ANOVA, sex effect, $F_{1,347} = 30.263$, $P < 0.00001$), but
915 the effects of heat stress on development time were similar between the sexes (ANOVA,
916 temperature x sex interaction, $F_{8,347} = 0.149$, $P = 0.70$). Data represent values from
917 females (left panel) and males (right panel). The genotypes of the parents are indicated
918 in the legends of each panel (female x male).
919

Figure 1

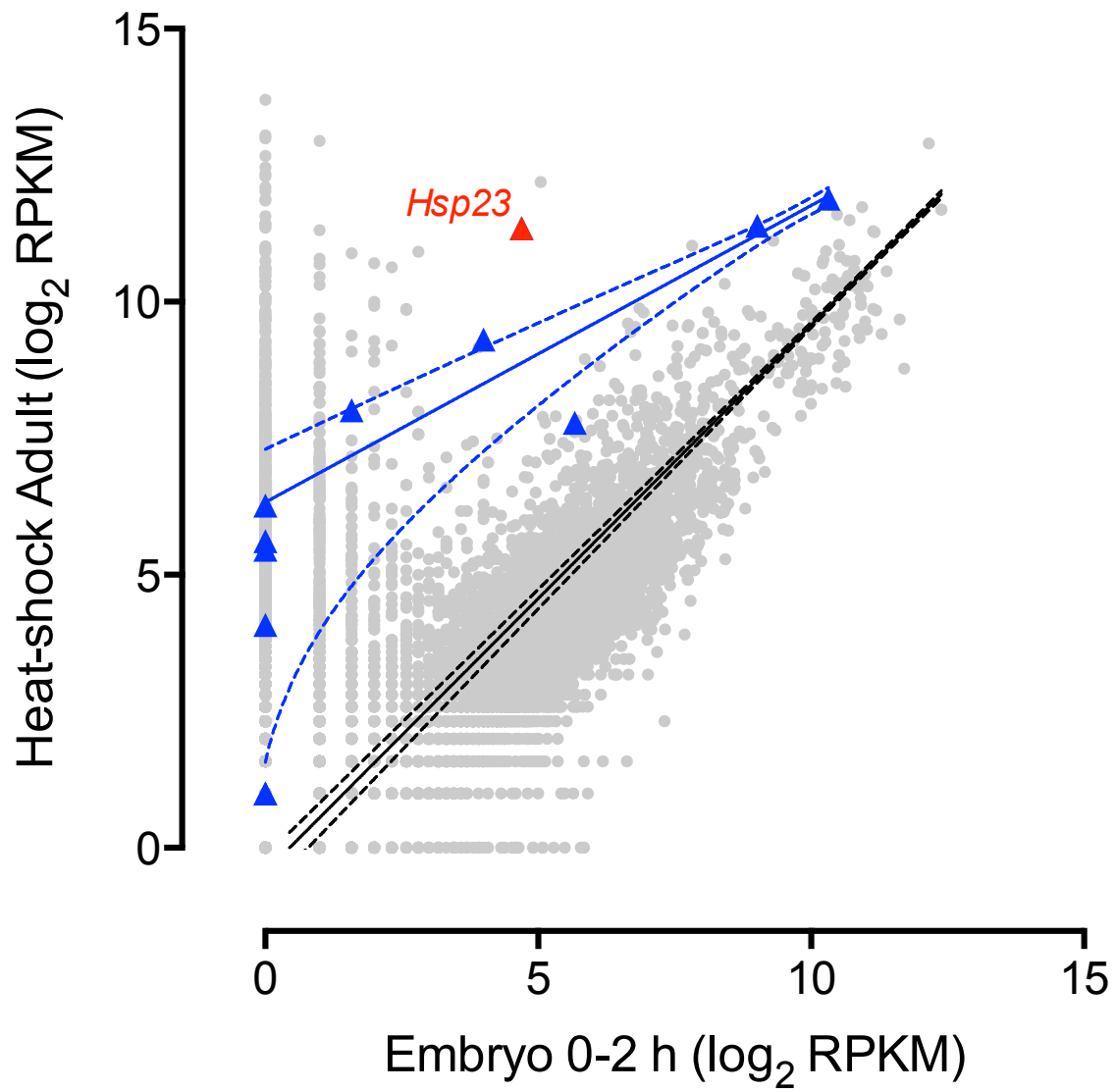


Figure 2

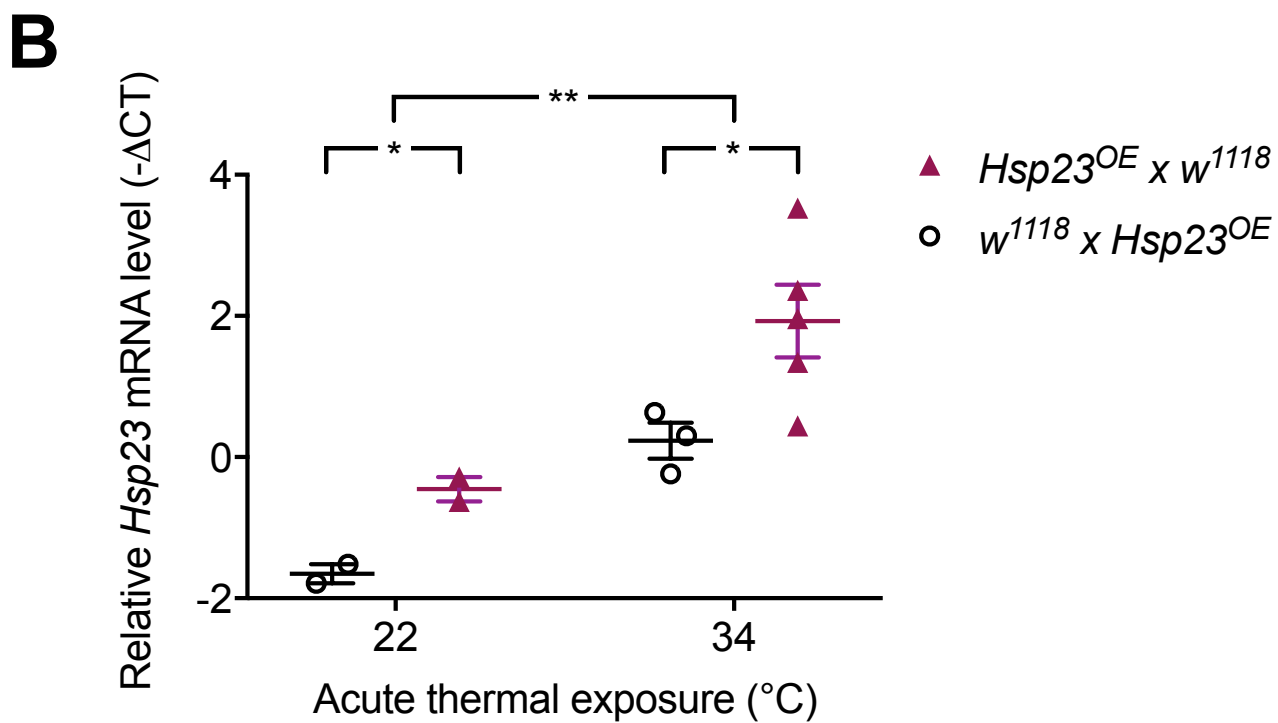
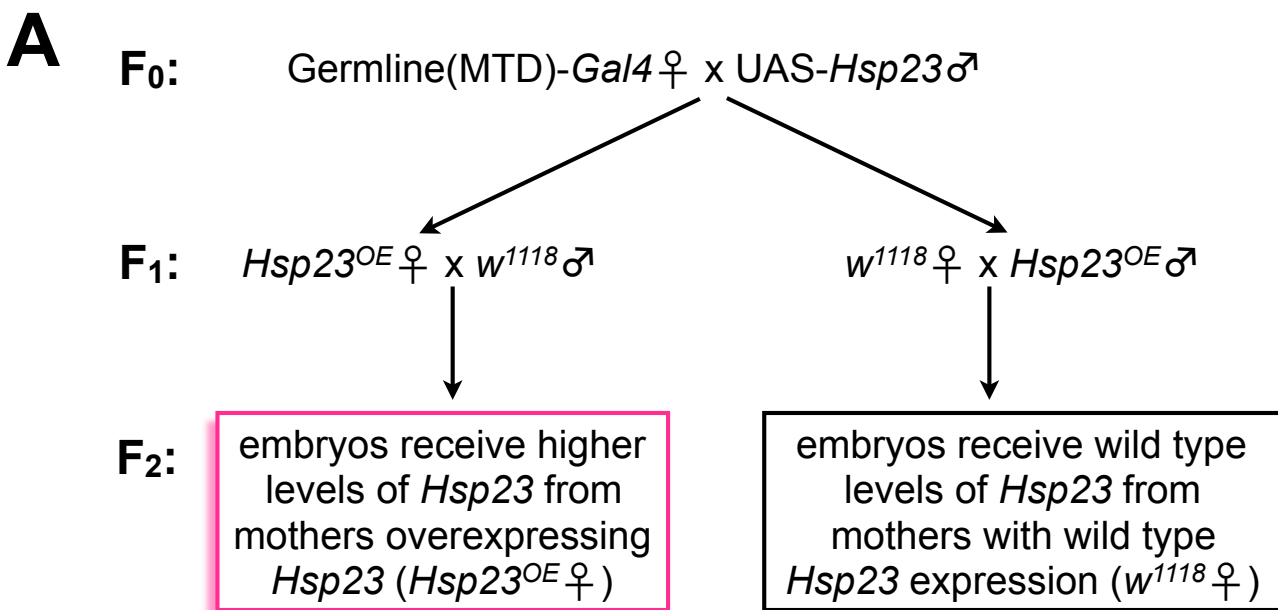


Figure 3

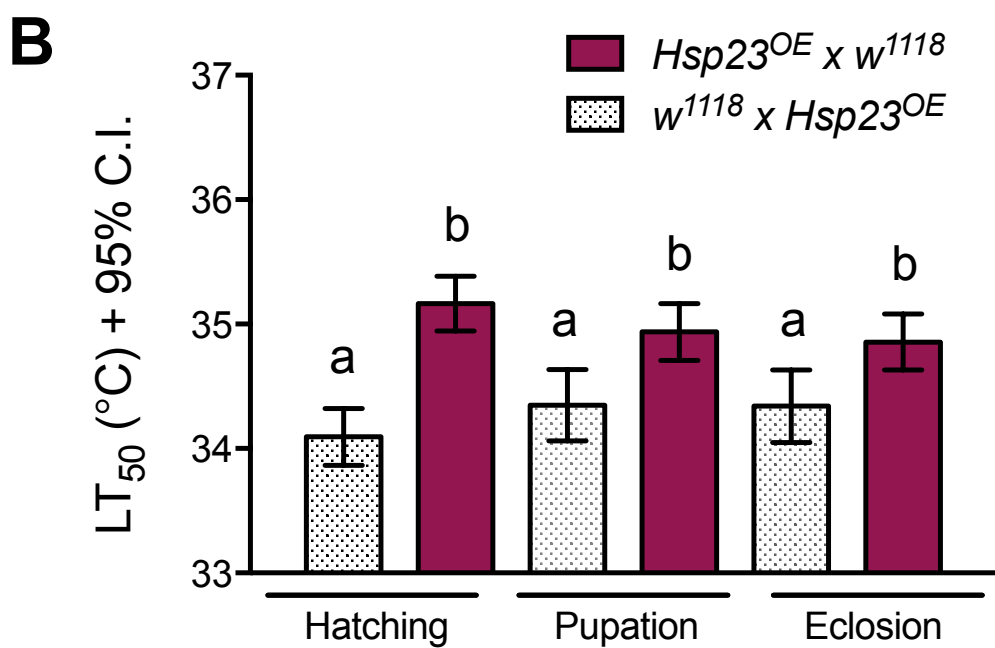
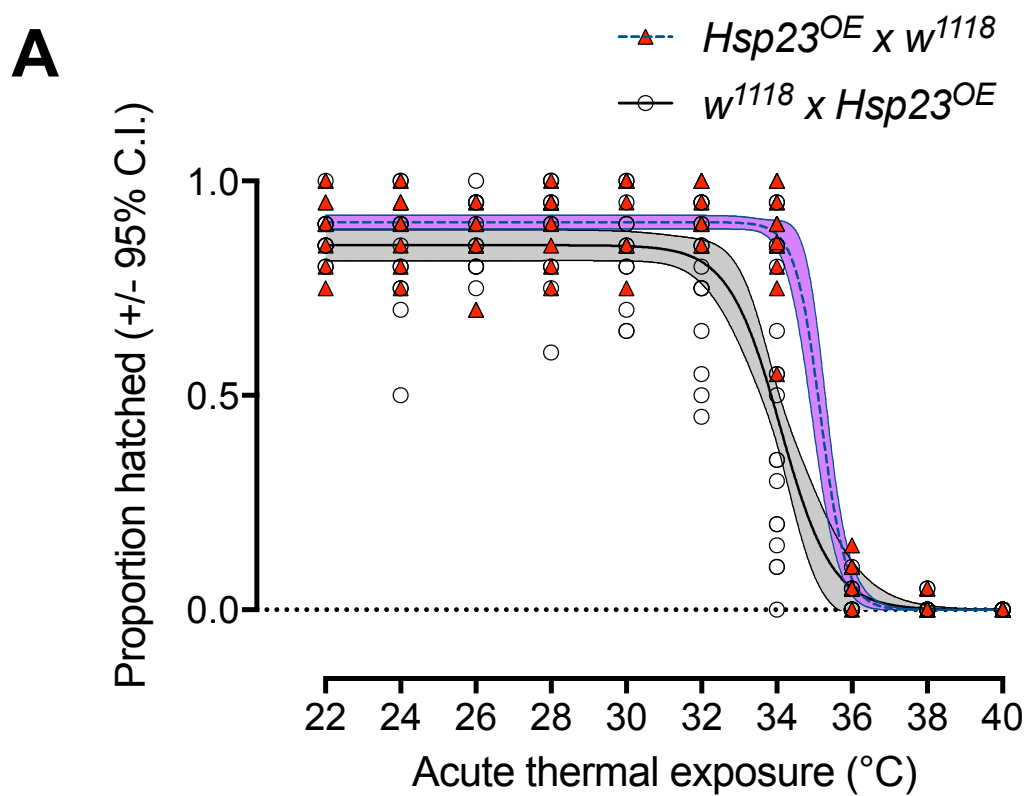
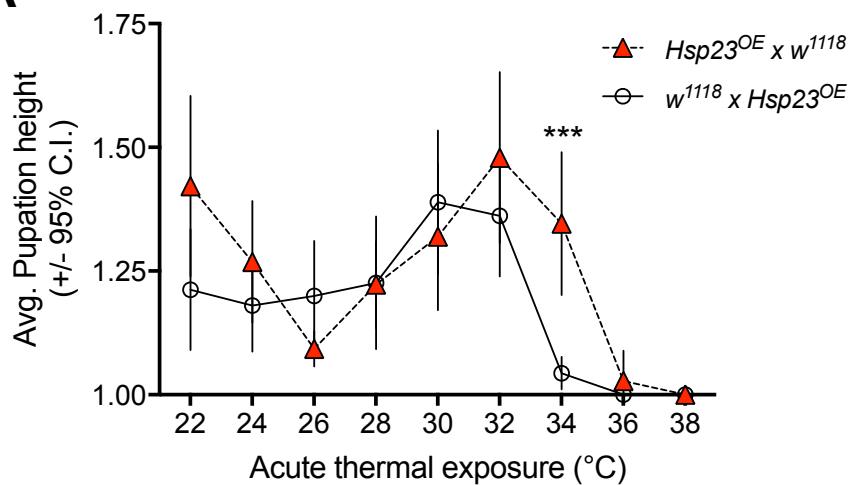
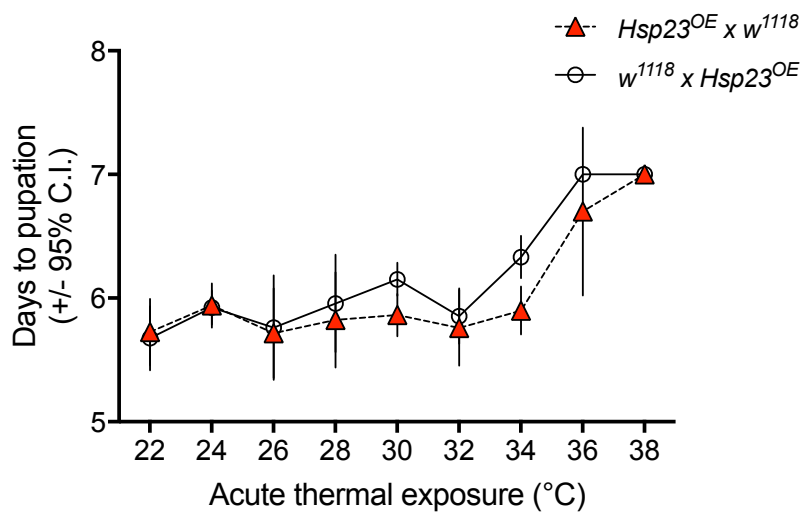


Figure 4

A



B



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