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## TITLE

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Many ways to make darker flies: Intra- and inter-specific variation in *Drosophila* body  
6 pigmentation components

8 AUTHORS

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## ABSTRACT

30

Body pigmentation is an evolutionarily diversified and ecologically relevant trait that  
32 shows variation within and between species, and important roles in animal survival and  
reproduction. Insect pigmentation, in particular, provides some of the most compelling  
34 examples of adaptive evolution and its ecological and genetic bases. Yet, while  
pigmentation includes multiple aspects of color and color pattern that may vary more or  
36 less independently, its study frequently focuses on one single aspect. Here, we develop  
a method to quantify color and color pattern in *Drosophila* body pigmentation,  
38 decomposing thorax and abdominal pigmentation into distinct measurable traits, and we  
quantify different sources of variation in those traits. For each body part, we measured  
40 overall darkness, as well as four other pigmentation properties distinguishing between  
background color and color of the darker pattern elements that decorate the two body  
42 parts. By focusing on two standard *D. melanogaster* laboratory populations, we show  
that pigmentation components vary and co-vary in different manners depending on sex,  
44 genetic background, and developmental temperature. By studying three natural  
populations of *D. melanogaster* along a latitudinal cline and five other *Drosophila*  
46 species, we then show that evolution of lighter or darker bodies can be achieved by  
changing distinct component traits. Our study underscores the value of detailed  
48 phenotyping for a better understanding of phenotypic variation and diversification, and  
the ecological pressures and genetic mechanisms underlying them.

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## KEYWORDS

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quantitative phenotyping; decomposing phenotypes; developmental plasticity; thermal  
54 plasticity; genetic and environmental variance

## 56 INTRODUCTION

58 Diversity in body coloration provides some of the most compelling examples of  
59 adaptive evolution. Insect body coloration, in particular, includes text book cases such  
60 as industrial melanism (e.g. van't Hof et al. 2011; Cook and Saccheri 2013), mimicry  
61 (e.g. Mallet & Joron 1999; Nadeau 2016), and clinal variation (e.g. Bastide et al. 2014;  
62 Endler et al. 2016). Studies in different species have illustrated the ecological  
63 significance of variation in body pigmentation, including visual communication  
64 between individuals of the same (e.g. mate attraction and mate choice; e.g. Wiernasz  
65 1995; Guillermo-Ferreira et al. 2014) or different species (e.g. predator avoidance via  
66 camouflage or aposematism; e.g. Reichstein et al. 1968; Futahashi and Fujiwara 2008;  
67 van Bergen and Beldade 2019), as well as thermoregulation (e.g. Rajpurohit et al. 2008;  
68 Sibilia et al. 2018). Moreover, insect pigmentation is tightly associated with various  
69 other traits that are closely related to fitness (see Wittkopp and Beldade 2009;  
70 Mckinnon and Pierotti 2010). The diversity of insect pigmentation across species,  
71 populations, sexes, and individuals of the same sex has been the focus of many eco-evo-  
72 devo studies, providing key insight into the genetic basis of variation in pigmentation  
73 (e.g. Pool and Aquadro 2007; Futahashi and Fujiwara 2008; Miyagi et al. 2015; Massey  
74 and Wittkopp 2016; Zhang et al. 2017; Orteu and Jiggins 2020) and exploring important  
75 phenomena such as developmental plasticity (e.g. Solensky and Larkin 2009; Shearer et  
76 al. 2016; Monteiro et al. 2020), the origin of novelty (e.g. Shirai et al. 2012; Vargas-  
77 Lowman et al. 2019), and evolutionary constraints (Beldade et al. 2002b; Allen et al.  
78 2008).

80 Variation in body pigmentation can arise from differences in color and/or in the  
81 spatial arrangement of colors into specific patterns. These two aspects rely on largely  
82 distinct classes of genes involved in pigmentation development: those encoding the  
83 enzymes responsible for pigment synthesis, and those encoding the transcription factors  
84 regulating those enzymes' expression at the appropriate time and place (see True 2003;  
85 Wittkopp et al. 2003; Wittkopp and Beldade 2009). Changes in genes associated with  
86 each of these steps can result in changes in pigmentation between individuals and  
87 between body parts (e.g. Wittkopp et al. 2002). In this respect, body pigmentation can  
88 be thought of as a multi-dimensional trait, made up of several components representing  
89 aspects of actual color and of color pattern, and which might develop and evolve more

90 or less independently. This has been explored in studies focusing on specific color  
91 pattern elements, including on butterfly wings (e.g. Nijhout 2001; Monteiro 2015;  
92 Beldade and Peralta 2017), as well as on fly wings and abdomens (e.g. Jeong et al.  
93 2006; Werner et al. 2010). Yet, rarely do studies of body pigmentation variation  
94 combine quantitative analysis of multiple color and color pattern traits.

96 Studies of *Drosophila* body and wing pigmentation have provided very valuable  
97 insight about the genetic and environmental bases of variation between species,  
98 populations of the same species, and individuals of the same population (e.g. Hollocher  
100 et al. 2000; Wittkopp et al. 2003; Gibert et al. 2007; Pool and Aquadro 2007; Massey  
101 and Wittkopp 2016). These studies characterized effects of environmental factors, such  
102 as nutrition (e.g. Shakhmantir et al. 2014) and temperature (e.g. David et al. 1990), as  
103 well as allelic variants of both subtle (e.g. Bastide et al. 2013) and large phenotypic  
104 effect (e.g. Carbone et al. 2005). Variation in *Drosophila* pigmentation has been  
105 associated to clinal and seasonal variation in desiccation resistance, thermo-regulation,  
106 and UV protection (e.g. Rajpurohit et al. 2008; Matute and Harris 2013; Parkash et al.  
107 2014), and shown to correlate with other traits, such as reproductive success, behavior,  
108 and immunity (e.g. Dombeck and Jaenike 2004; Takahashi 2013; Massey et al. 2019).  
109 While studies of *Drosophila* pigmentation have included focus on different body parts  
110 (e.g. trident on thorax, e.g. David et al. 1985; melanic patches on wings, e.g. True et al.  
111 1999; dark bands of abdominal segments, e.g. Dembeck et al. 2015), these studies  
112 typically analyze single and often qualitative properties of pigmentation (but see e.g.  
113 Saleh Ziabari and Shingleton 2017). Indeed, the detail in quantitative phenotyping of  
114 body pigmentation does not match the sophistication of the analysis of its genetic and  
115 developmental bases. This is not unique to *Drosophila* pigmentation; the need for more  
116 attention to be given to phenotyping has been called for repeatedly (Gerlai 2002; Houle  
117 et al. 2010; Kühl and Burghardt 2013; Deans et al. 2015; Laughlin and Messier 2015).

118 Here, we quantify various traits encompassing aspects of both color and color  
119 pattern of abdomen and thorax pigmentation in *Drosophila* adults. We investigate how  
120 each of these pigmentation components (or traits) and the associations between them  
121 differ between genotypes and developmental temperatures, within and across species.  
122 We show that different pigmentation components can vary rather independently, and  
123 that fly bodies can be made lighter or darker by changing different pigmentation

124 components. We discuss our results in the context of the potential for evolutionary  
diversification of pigmentation.

126

128

## RESULTS

130

To investigate patterns and sources of variation in *Drosophila* body pigmentation, we  
132 developed a quantitative method to define five pigmentation traits that include aspects  
134 of color and color pattern (see Figure S1 and Materials and Methods). We focused on  
136 the dorsal surface of thoraxes and abdomens, characterized for having different types of  
138 dark “pattern elements” on a lighter “background” color: a trident at the center of the  
140 thorax and posterior bands on each segment of the abdomen. Flies were imaged under a  
142 binocular scope in controlled light conditions. For each body part, we defined a transect  
144 between an anterior and a posterior landmark and collected color information for each  
146 pixel along these transects (Figure 1A, Figure S1). Using that information, we  
148 quantified a series of traits for each body part: overall darkness (Odk), relative length of  
150 transect occupied by the darker “ornamental” pattern (Pat), actual color of both  
background (Cbk) and “ornamental” pattern elements (Cpa), and the distance in RGB  
space between the darkest and the lightest that corresponds to the range of color  
variation (Ran). We investigated how these pigmentation components vary and co-vary  
between sexes and between rearing temperatures in *D. melanogaster* representing  
152 standard laboratory strains, and natural populations from different geographical  
locations, as well as in five additional *Drosophila* species. For each dataset (*D. melanogaster*  
154 laboratory strains, *D. melanogaster* clinal populations, and *Drosophila*  
species), the multivariate multiple regression analyses showed that pigmentation  
156 differed significantly between strains/genotypes/species, sexes, and temperatures, with  
effects that depended on body part (Table S1).

158

### **Variation in body pigmentation in *D. melanogaster* laboratory populations**

160

We reared flies from two common laboratory genetic backgrounds (or strains) of *D. melanogaster*, Oregon R (OreR) and Canton S (CanS), at either 17°C or 28°C to assess  
162 thermal plasticity and sexual dimorphism in our pigmentation traits (Figure 1, 2, Figure  
S2, Table S2). We confirmed known patterns of thermal plasticity and sexual  
dimorphism for body pigmentation, with flies reared at lower temperature being  
generally darker than those reared at higher temperature, and males being darker than  
females (Figure 1B, 2A, Figure S2). However, we found differences between strains  
and body parts in the extent, and sometimes the direction of both thermal plasticity and

sexual dimorphism for our pigmentation traits (Figure 1B, 2A, Figure S2, Table S2), as

164 well as for the correlations between them (Figure 3A).

166 For overall darkness (Odk; dot plots in Figure 2A), flies reared at 17°C were  
168 generally darker than those from 28°C, with the exception of CanS males (where  
170 differences were not significant in either body part), and OreR females (where  
172 abdomens were darker in flies from 28°C). The abdomens were lighter in females  
174 relative to males (except for CanS from 17°C), but the thoraxes were lighter in males  
176 relative to females (except for CanS from 28°C and OreR from 17°C). We also  
178 observed differences between sexes and temperatures for the other pigmentation traits  
180 (Pat, Ran, Cbk, and Cpa; radar plots in Figure 2A; dot plots in Figure S2, Table S2),  
182 which depended on body part. While for the thorax the most striking differences were  
184 seen in Ran (for females between temperatures), for the abdomen they were seen for Pat  
186 (distinguishing females from 28°C from others) and Ran (extreme for OreR females)  
188 (Figure S2). Variation was only loosely correlated between traits, with few significant  
190 correlations, which differed between genetic backgrounds, sexes, and rearing  
192 temperatures. Overall, correlations between traits were weaker across body parts  
194 relative to within body parts, and in males relative to females (Figure 3A).

182 For those pigmentation traits found to be thermally plastic (i.e. significant

184 differences between individuals reared at different temperatures; cf. Figure S2, Table

186 S2), we investigated which stages of development were thermally responsive. To do so,

188 we compared phenotypes between individuals (specifically, female abdomens) differing

190 in temperature only for specific developmental time windows (Figure 3B, Figure S3,

192 Table S3). We tested nine thermal regimes (i.e. treatments), including three with

194 constant temperatures (whole development at 17°C, 23°C, or 28°C) and six where most

196 of the development took place at 23°C and only one specific stage (either late larval,

198 early pupal, or late pupal) took place at 17°C or at 28°C. Differences between constant

200 temperatures (T17, T23, and T28 treatments), revealed thermal reaction norms, i.e. the

202 representation of phenotype as a function of temperature (see Schlichting and Pigliucci

204 1998), of different shapes for different pigmentation components: T23 phenotype

206 intermediate between T17 and T28 (Ran in OreR; Figure 3B), equal to one of the

208 extreme temperatures (Pat; Figure 3B), or more extreme than both T17 and T28 (Odk;

196 Figure 3B). The period when exposure to a different temperature significantly affected phenotype also differed between traits and genetic backgrounds (Figure 3B, Figure S3).

198

200 **Body pigmentation differences between *D. melanogaster* natural populations and *Drosophila* species**

202 We quantified variation in pigmentation components in wild-caught populations sampled along a latitudinal cline in Europe: Finland, Austria, and Spain (samples from 204 the *DrosEU Consortium*; <http://droseu.net/>). We analyzed pigmentation traits in females from five genotypes (isofemale lines) established from each of the three geographical 206 locations, reared at either 17°C or 28°C. The analysis for each pigmentation component revealed differences between traits in their response to the various explanatory variables 208 and their interactions (Figure 1C, 2B, Table S4). Geographical populations differed in overall darkness (Odk; dot plots in Figure 2B) and in color (both Cbk and Cpa) for the 210 abdomen, but not the thorax (Figure 2B, Figure S5, Table S4). For the thorax, only Ran and Pat differed between locations (Figure 2B, Table S4). Most pigmentation traits 212 (except thorax color; Cpa and Cbk) were thermally plastic, with darker flies for development at 17°C relative to 28°C (Figure 1C, 2B, Figure S4). The Northern- and 214 Southern-most populations (i.e. Finland and Spain, respectively) did not necessarily show the most extreme phenotypes, neither in terms of overall darkness nor in the 216 extent of plasticity therein (Figure 2B, Figure S5). We also found significant differences between isofemale genotypes (and their plasticity) within each geographical location 218 (Figure 2B, Table S4).

220 Finally, we quantified pigmentation traits in flies from five additional *Drosophila* species (two genetic backgrounds for *D. simulans*, and one genetic background for all 222 other species or sub-species: *D. malerkotliana*, *D. repleta*, *D. mojavensis* *baja*, *D. mojavensis* *mojavensis*) reared at either 17°C or 28°C (Figure 1D, 2C). We found 224 differences between species in extent and direction of sexual dimorphism and of thermal plasticity for the different pigmentation traits (Figure 1D, 2C, Figure S2B, 226 Table S5). For instance, for Odk (dot plots in Figure 2C), while *D. malerkotliana* showed no differences between temperatures and clear differences between sexes, *D. simulans* had very high thermal plasticity but reduced sexual dimorphism (no 228 differences between females and males reared at 17°C). For the other pigmentation

230 traits (radar plots in Figure 2C and dot plots in Figure S2B), larger differences between  
231 sexes and/or temperatures were observed for Pat and/or Ran, and less for actual colors  
232 (Cpa and Cbk).

234 **DISCUSSION**

236 We decomposed *Drosophila* body pigmentation into different quantitative traits,  
238 including overall darkness (Odk), and traits reflecting properties of color and color  
240 pattern (Pat, Ran, Cbk, and Cpa) of both thoraxes and abdomens. We showed  
242 differences in trait values, as well as in the extent and direction of thermal plasticity and  
244 of sexual dimorphism for laboratory and natural populations of *D. melanogaster* and  
across *Drosophila* species (Figures 1, 2). Different traits, corresponding to different  
properties of body pigmentation, behaved in a largely independent manner, which was  
also reflected in low levels of correlations between traits and in differences in the period  
of development during which traits are thermally responsive (Figure 3).

246 *Drosophila* pigmentation has been the focus of various studies exploring aspects  
of its ecology, development and evolution (e.g. Kopp et al. 2000; Williams et al. 2008;  
248 Matute and Harris 2013; Shearer et al. 2016; Gibert et al. 2017). This has provided great  
insight about the genetic basis and ecological significance of variation, across  
250 temporally (e.g. seasonal variation) or geographically (e.g. clinal variation) distinct  
populations (e.g. Parkash et al. n.d.; Hollocher et al. 2000b; Rajpurohit et al. 2008), as  
252 well as across species (Hollocher et al. 2000a,b). Many of those studies focused on  
specific pigmentation elements in particular species, and often used qualitative  
254 assessments of pigmentation variation or presence/absence of specific pattern elements  
(e.g. Hollocher et al. 2000; David et al. 2002). In *D. melanogaster* for instance, most  
256 work has focused on abdominal pigmentation, and specifically on the dark bands of the  
posterior-most segments, which is sexually dimorphic (males are generally darker than  
258 females; e.g. Kopp et al. 2000) and thermally plastic (flies from lower developmental  
temperatures are generally darker than flies from higher developmental temperatures;  
260 e.g. David et al. 1990; Gibert et al. 2007, 2009). We extended the analysis of body  
pigmentation to quantifying different properties of both abdomen and thorax  
262 pigmentation in *D. melanogaster* and other *Drosophila* species. This more detailed  
analysis ultimately painted a more complex picture of variation in *Drosophila* body  
264 pigmentation. We did not, for instance, always find that males were darker than  
females, or that flies reared at lower temperatures were darker than those from higher  
266 temperatures, but rather, we found trait specificities in how pigmentation varied  
between sexes and between developmental temperatures. This was true for overall

268 darkness (Odk) of the abdomen, the trait that would presumably be more similar to  
269 previous (largely qualitative) characterizations of abdominal pigmentation (e.g. David et  
270 al. 1990; Hollocher et al. 2000a), but also for other properties of body pigmentation,  
271 including actual color of background and pattern elements (i.e. abdominal bands and  
272 thoracic trident). Moreover, we also showed that pigmentation components, as well as  
273 sexual dimorphism and thermal plasticity therein, vary greatly between species,  
274 genotypes, and body parts. The mechanisms underlying such intra- and inter-specific  
275 variation in different traits, as well as the trait-specific responses to temperature, remain  
276 to be explored and might involve differences in the environmental sensitivities of the  
277 regulatory regions (e.g. enhancers) controlling pigmentation-related genes (e.g. De  
278 Castro et al. 2018).

280 Our results also highlight that the different components vary largely  
281 independently, with only weak correlations between traits (Figure 3A) and differences  
282 between traits in the extent and direction of thermal plasticity and of sexual dimorphism  
283 (Figure 1, 2). Pigmentation components were shown to even differ in the period of  
284 development in which they are responsive to temperature (Figure 3B). Similar  
285 environmental effects on trait associations have been described previously; for instance,  
286 cold temperature triggered a shift in the sign of the correlation between body size and  
287 longevity in *D. melanogaster* (Norry and Loeschke 2002). Differing correlations  
288 between body parts (or regions within a body part) have also been identified for *D. melanogaster*  
289 pigmentation (e.g. Gibert et al. 2000; Bastide et al. 2014), with the extent  
290 of genetic correlations decreasing with increasing distance between body segments  
291 (Gibert et al. 2000). Ultimately, the dependency of trait associations on genetic and  
292 environmental factors has the potential to influence adaptation (e.g. Marquez &  
293 Knowles 2007; Manenti et al. 2016), as evolutionary change can result from both direct  
294 and correlated responses to selection (e.g. Rajpurohit and Gibbs 2012). Altogether, our  
295 results suggest a large degree of developmental and evolutionary independence between  
296 pigmentation components, which could facilitate the diversification of body coloration  
297 in *Drosophila*.

298

299 Studies exploring the ecological conditions driving the evolution of melanism in  
300 *Drosophila* have documented correlations between body pigmentation and several eco-  
301 geographic variables (e.g. latitude, altitude, temperature, humidity) (e.g. Rajpurohit et

302 al. 2008; Gibert et al. 2016; Shearer et al. 2016). Clinal variation in pigmentation, for  
303 instance, has been shown for thoracic trident (e.g. David et al. 1985; Telonis-Scott et al.  
304 2011) and for abdominal pigmentation (e.g. Pool and Aquadro 2007; Das 2009).  
305 Generally, darker phenotypes in colder environments (e.g. at high latitudes or altitudes)  
306 have been hypothesized to allow flies to better absorb solar radiation (c.f. thermal  
307 budget or thermal melanism hypothesis; Trullas et al. 2007; Clusella-Trullas et al.  
308 2008), to increase desiccation resistance (e.g. Parkash et al. 2008), and/or to provide  
309 protection against UV radiation (e.g. Bastide et al. 2014). Plasticity, on the other hand,  
310 is expected to be greater in environments that are more variable (Lande 2014), such as  
311 those with larger seasonal fluctuations, often occurring at higher latitudes. However, our  
312 analysis of the pigmentation patterns from *D. melanogaster* populations collected along  
313 a European latitude cline (Finland, Austria, Spain) did not always revealed darker  
314 pigmentation nor higher plasticity in the Northern-most population (i.e. Finland), which  
315 may indicate that other environmental parameters and ecological conditions not  
316 considered here could account for the differences between populations in the different  
317 pigmentation components. Having only three populations from three latitudes may also  
318 be limiting in terms of assessing latitudinal patterns in pigmentation and plasticity  
319 therein.

320

321 In terms of a function in thermo-regulation favoring darker flies in cooler  
322 environments (David et al. 1985; Hollocher et al. 2000a; Wittkopp et al. 2011; Matute  
323 and Harris 2013; Shearer et al. 2016), we could expect our trait overall darkness (Odk)  
324 to be the most relevant trait. Our analyses revealed that flies can become overall darker  
325 (higher Odk) by changing actual colors of background or of pattern elements (Cbk and  
326 Cpa, respectively) or the proportion of the abdomen/thorax length covered with the  
327 darker bands/trident (Pat). For instance, males of CanS reared at 17°C and 28°C, show  
328 the same overall darkness (Odk), but differ in what pigmentation components make that  
329 up; Odk is mostly determined by color components at 17°C and by color pattern  
330 components at 28°C (i.e. Cpa and Cbk are lower, while Pat and Ran are higher at 17°C  
331 than at 28°C). It is unclear whether these traits are mere components of Odk or are  
332 themselves under direct natural selection.

334

Variation in pigmentation between body parts, individuals, populations, and species can be caused by differences in actual color and/or in how colors are spatially organized to make up color patterns (Wittkopp and Beldade 2009; Nijhout 2010). However, seldom do studies of animal pigmentation consider and quantify distinct pigmentation component traits, and the extent to which they might be differently affected by genetic and/or environmental variation. The increased attention to studying the mechanisms underlying phenotypic variation resulted in great detail and sophistication in the characterization of its genetic underpinnings. However, the detail in describing and quantifying phenotypes has lagged behind. The lack of quantitative methods for phenotyping (see Gerlai 2002; Houle et al. 2010) can result in an oversimplification of complex phenotypes, dismissing that those phenotypes are often made up of distinct component traits that can respond to internal and external factors in different manners (e.g. Vrieling et al. 1994; Mateus et al. 2014). We attempted to provide a better resolution of variation in *Drosophila* body color, a visually compelling example of adaptive evolution. Combining it with existing genetic resources and with access to natural variation can provide a deeper resolution of the patterns and processes underlying phenotypic variation, within and between species.

352

354 **MATERIAL AND METHODS**

356 **Fly stocks**

358 *D. melanogaster* genetic backgrounds CantonS (CanS) and OregonR (OreR) and  
360 *Drosophila* species *D. simulans*, *D. malerkotliana*, *D. repleta*, *D. mojavensis baja* and  
362 *D. mojavensis mojavensis* were obtained from C. Mirth's lab. *D. melanogaster*  
364 populations from Finland (Akaa; 61.1, 23.52; collected in July 2015), Austria  
366 (Mauternbach; 48.38, 15.57; collected in July 2016) and Spain (Tomelloso; 39.16, 3.02;  
368 collected in September 2015) were obtained from E. Sucena's lab and collected by  
370 members of the *European Drosophila Population Genomics Consortium (DrosEu*;  
372 <http://droseu.net>). All stocks were maintained in molasses food (45 gr. molasses, 75 gr.  
374 sugar, 70 gr. cornmeal, 20 gr. Yeast extract, 10 gr. Agar, 1100 ml water and 25 ml of  
376 Niapagin 10%). All stocks were kept at 25°C, 12:12 light-dark cycles. For the  
378 experiments, we performed over-night egg-laying from ~20 females of each stock in  
380 vials with *ad libitum* molasses food. Eggs were then placed at either 17°C or 28°C  
382 throughout development. We controlled the population density by keeping between 20  
and 40 eggs per vial.

372

374 For the experiment of the windows of sensitivity for pigmentation, we exposed  
376 developing flies to 17°C or 28°C during one window of development while they were  
378 kept at 23°C for the remaining stages. We tested four different treatments at 17°C and at  
380 28°C: T (flies always kept at constant temperature), L (late larval development; staging  
done by using tracheal and mouth hook morphology), p (only early pupal period; from  
white pupa to the onset of eye pigmentation), P (only late pupal period; from the onset  
of eye pigmentation until adult eclosion).

380

382 **Phenotyping pigmentation components**

384

386 Adult flies (8-10 days after eclosion) were placed in 2 ml microcentrifuge tubes and  
frozen in liquid nitrogen. The tubes were shaken immediately after submersion in liquid  
nitrogen to remove wings, legs and bristles. Headless bodies of flies were then mounted  
on 3% Agarose in Petri dishes, dorsal side up, and covered with water to avoid specular  
reflection of light upon imaging. Images containing 10 to 20 flies were collected with a

388 LeicaDMLB2 stereoscope and a Nikon E400 camera under controlled conditions of  
389 illumination and white-balance adjustment. Images were later processed with a set of  
390 custom-made interactive Mathematica notebooks (Wolfram Research, Inc.,  
391 Mathematica, Version 10.2, Champaign, IL, 2015) to extract pigmentation  
392 measurements. For this purpose, two transects were defined on each fly, one in the  
393 thorax and one in the abdomen, using morphological landmarks (as shown in Figure  
394 S1). To minimize image noise, for each pixel position along the transect line we  
395 calculated the mean RGB (Red, Green, Blue) values of the closest five pixels located on  
396 a small perpendicular line centered on the transect. For abdominal transects, when  
397 necessary, we removed the sections corresponding to the membranous tissue that  
398 occasionally is visible between abdominal segments. The few transects that were drawn  
399 over debris particles were excluded from the analysis, as pigmentation measurements  
400 could not be accurately extracted.

402 The sequence of averaged RGB pixel values corresponding to each transect was  
403 then used to define each of the five pigmentation components as follows. For each  
404 pixel, we calculated a normalized darkness value as  $D_{max}-D_{bk}$ , where  $D_{max}$  is the  
405 largest possible Euclidean distance between two colors in the RGB color space (in this  
406 case  $D_{max}=\sqrt{3}$ ), and  $D_{bk}$  is the distance of the pixel's color coordinates to the color  
407 black ( $R=0$ ,  $G=0$ ,  $B=0$ ). Overall darkness ( $O_{dk}$ ) was calculated as the sum of the  
408 normalized darkness values for each pixel divided by the number of pixels in the  
409 transect. Taking the sequence of normalized darkness values along a transect, we  
410 estimated its two enveloping lines (blue and green lines in Figure S1A) by calculating  
411 the baselines of the original and negated values using the Statistics-sensitive Non-linear  
412 Iterative Peak-clipping (SNIP) algorithm (Ryan et al. 1988). The median line of this  
413 envelope (red line in Figure S1A) was then used to separate the transect pixels into two  
414 clusters, where the pixels above or below this line correspond, respectively, to the  
415 pattern element (trident in the thorax and darker bands in the abdomen) or to the  
416 background. Pattern ( $Pat$ ) was calculated as the proportion of pixels corresponding to  
417 the pattern element relative to the transect length. Color of the pattern element ( $C_{pa}$ ) is  
418 the angle defined in the RGB color space between the best-fitted line going through the  
419 color coordinates of the pixels in the transect that correspond to the pattern element  
420 (trident and/or darker bands) in the transect and the gray vector (the black to white  
diagonal in the RGB color space). Similarly, color of the background ( $C_{bk}$ ) was

422 calculated as the angle between the best-fitted line that goes through the color  
423 coordinates of the background pixels in the transect and the gray vector. Pixels  
424 corresponding to pattern element and/or background were defined by grouping all RGB  
425 values in the transect into two clusters each containing 95% of the light or dark pixels  
426 respectively. Range (Ran) was calculated as the Euclidean distance between the median  
427 values of the 20 darkest and the 20 lightest pixels along the transects. The colors  
428 represented in Figure 1 correspond to the mean R, mean G and mean B values for each  
429 strain/species, sex, and temperature, which were calculated from Cpa for color of  
430 pattern elements and from Cbk for color of the background, respectively.

#### 432 Statistical analyses

434 All analyses were conducted in R v 3.6.2 (R Core Team 2019), using the following R  
435 packages: *tidyverse* (Wickham and Henry 2020) to arrange datasets, *ggplot2* (Wickham  
436 2009) to produce all plots, *lme4* (Bates et al. 2015) and *lmerTest* (Kuznetsova et al.  
437 2017) to perform linear mixed-effects models, *corrplot* (Taiyun and Viliam 2017) to  
438 compute correlation matrices, and *emmeans* (Lenth et al. 2018) to perform post-hoc  
439 pairwise comparisons between groups. The statistical models described below are given  
440 in package-specific R syntax (shown in italics).

442 Multivariate multiple regression was performed for the data on *D. melanogaster*  
443 laboratory populations to test for the effect of strain, sex, temperature (fixed explanatory  
444 variables), and interaction terms in all pigmentation traits by combining all traits using  
445 the *cbind* function (model  $lm(cbind(Odk, Pat, Ran, Cbk, Cpa) \sim Strain * Sex * Temperature)$ ). A similar analysis was performed for the data on *D. melanogaster* clinal  
446 populations testing for the fixed effects and interactions of location, genotype (i.e.  
447 isogenic line; nested within location), and temperature (model  $lm(cbind(Odk, Pat, Ran, Cbk, Cpa) \sim Location * Genotype * Temperature)$ ), and for the *Drosophila* species,  
448 testing for the fixed effects and interactions of species, strain (nested within species),  
449 sex, and temperature (model  $lm(cbind(Odk, Pat, Ran, Cbk, Cpa) \sim Species * Species/Strain * Sex * Temperature)$ ), where *Strain* corresponds to the different genetic  
450 backgrounds analyzed in *D. melanogaster* (CanS and OreR) and in *D. simulans* (*D. sim*  
451 *A* and *D. sim B*).

456 Linear mixed effect models were then used to test for the (fixed) effects of  
457 different explanatory fixed variables (strains, genotypes or species, sex and  
458 temperature) and their interactions on each of the pigmentation traits (noted as *trait* in  
459 the model notations below). *Replicate* was included as random effect in the models  
460 (corresponding to the  $(I|Replicate)$  factor in the R syntax below). For *D. melanogaster*  
461 laboratory strains: model  $\text{lm}(\text{Trait} \sim \text{Sex} * \text{Temperature} + (I|Replicate))$ . The same  
462 model was used for all *Drosophila* species, except for *D. simulans*, where we also  
463 included the factor *Strain* which corresponds to the different genetic backgrounds  
464 studied in this species (*D. sim A* and *D. sim B*) (model:  $\text{lm}(\text{Trait} \sim \text{Strain} * \text{Sex} * \text{Temperature} + (I|Replicate))$ ). For the clinal populations: model:  $\text{lm}(\text{Trait} \sim \text{Location} * \text{Location/Genotype} * \text{Temperature} + (I|Replicate))$ . For all the aforementioned mixed  
465 models, we used Satterthwaite's method (via *anova* function in *lmerTest* package;  
466 Kuznetsova et al. 2017) for approximating degrees of freedom and estimating F-  
467 statistics and P-values. For the data on the sensitive stages of development, we used  
468 linear effect models to test for the effect and interaction of strain and thermal regime  
469 (model:  $\text{lm}(\text{Trait} \sim \text{Strain} * \text{Regime})$ ).

472

473 We used *post-hoc* pairwise comparisons (Tukey's honest significant differences)  
474 to identify differences between strains, sexes, temperatures and/or thermal regimes.  
475 Pearson's correlations were used to check correlations between traits and across  
476 temperatures.

478

## DATA ACCESIBILITY

480

482 All data will be made publicly available in Dryad Digital Repository upon acceptance of  
the manuscript.

## 484 AUTHOR CONTRIBUTIONS

486 E.L. and P.B. conceived and designed the study. E.L., J.G.K., and C.M.P. performed the  
experiments. F.A. developed the quantitative method for color pattern analysis and the  
488 respective computational tools. E.L. analyzed the data. E.L. and P.B. wrote the  
manuscript.

490

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500

## 502 CONFLICT OF INTEREST STATEMENT

504

506 We declare that no conflict of interest exists. The funders had no role in study design,  
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782

## FIGURE LEGENDS

784

### Figure 1. Intra- and inter- specific variation in *Drosophila* pigmentation

786 **A.** Example of a mounted *D. melanogaster* headless-body showing the dorsal side of  
788 the thorax and abdomen with transects, and the scheme we used to represent  
790 pigmentation traits for thorax (top rounded rectangle) and abdomen (bottom rounded  
792 rectangle). For each of these, the horizontal dashed line separates the color of pattern  
794 element (Cpa) and the color of background (Cbk). These are shown in mean color (RGB  
796 values) for same-group individuals, and the height of the dashed line represents the  
798 proportion of the transect that is occupied by pattern versus background (Pat). See more  
details in Figure S1. **B.** Pigmentation schemes per strain, sex, and temperature in *D.*  
794 *melanogaster* laboratory populations. **C.** Pigmentation schemes in *D. melanogaster*  
796 clinal populations, showing mean values from the five genotypes (i.e. isogenic lines)  
798 per location. **D.** Pigmentation schemes in five *Drosophila* species with one genetic  
background per species except *D. simulans* where two genetic backgrounds (*D. sim* A  
and *D. sim* B) were studied.

### 800 **Figure 2. Quantitative phenotyping of *Drosophila* pigmentation component traits**

802 For each population, temperature, sex, and body part, dot plots represent variation for  
804 Odk (individual data points and means) and radar plots represent variation for Pat, Ran,  
806 Cpa, and Cbk (means; dotplots in Figure S2). Females/males are shown as closed/empty  
808 circles (dot plots) or solid/dashed lines (radar plots), and flies reared at 17°C/28°C are  
810 shown in blue/red. **A.** *D. melanogaster* laboratory populations. Results of statistical test  
812 for the effect of sex, temperature, and their interaction on each of the traits are shown in  
814 Table S2. Letters in dot plots indicate results of post-hoc pairwise comparisons between  
816 groups: different letters when significantly different (p-value<0.05 for Tukey's honest  
significance test). **B.** *D. melanogaster* clinal populations. For each geographical  
818 population, we phenotyped females from five genotypes (i.e. isogenic lines). Results for  
820 the effect of location, genotype, and temperature (and interactions) on the different  
822 pigmentation traits are in Table S4. Results of the statistical test (p-value) for the effect  
824 of temperature on each of the traits are shown in plots. **C.** *Drosophila* species. Results  
826 of the statistical test for effect of sex, temperature and their interaction are in Table S5.  
828 Letters in dot plots indicate results of post-hoc pairwise comparisons between groups:

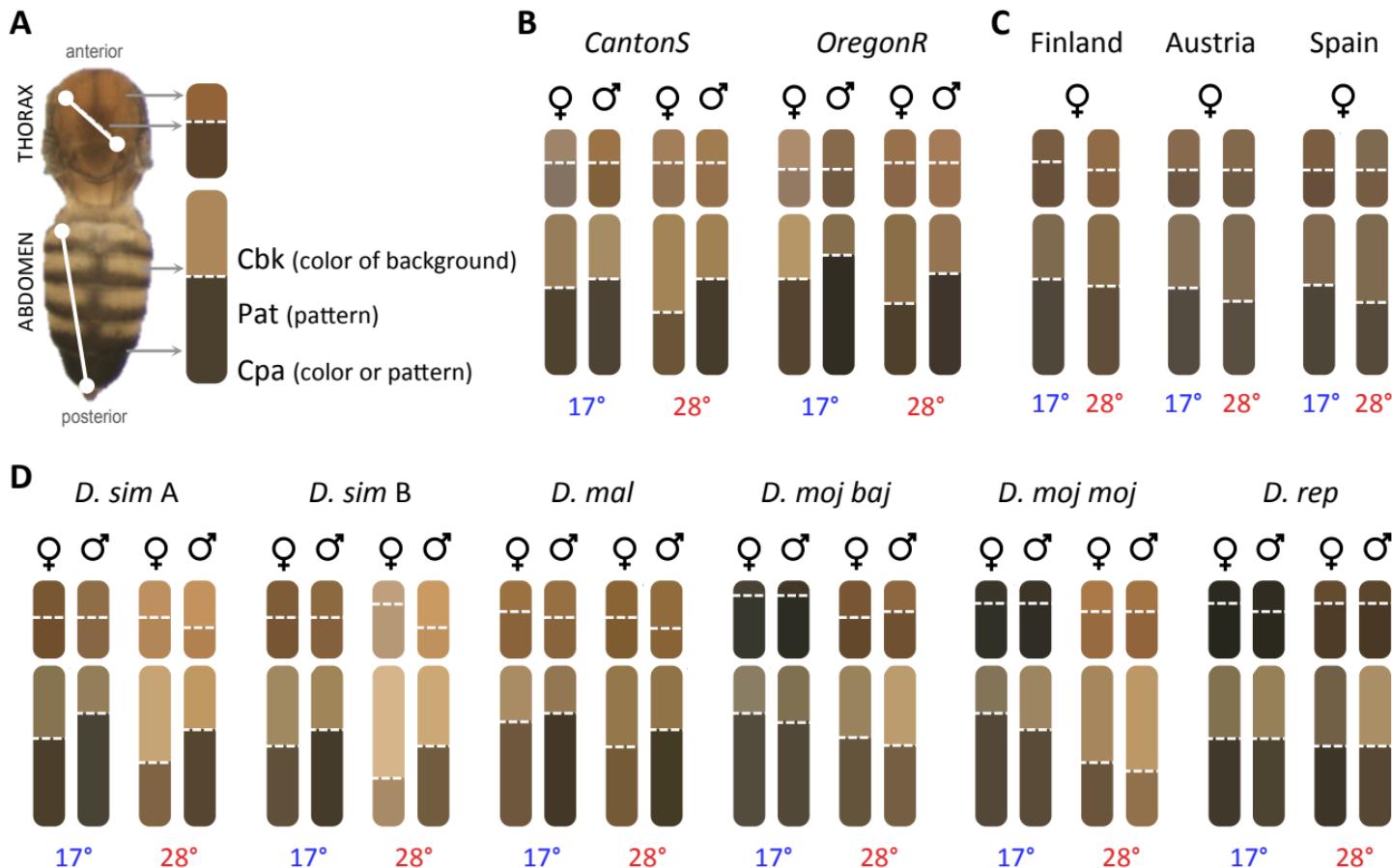
816 different letters when significantly different (p-value<0.05 for Tukey's honest  
significance test).

818

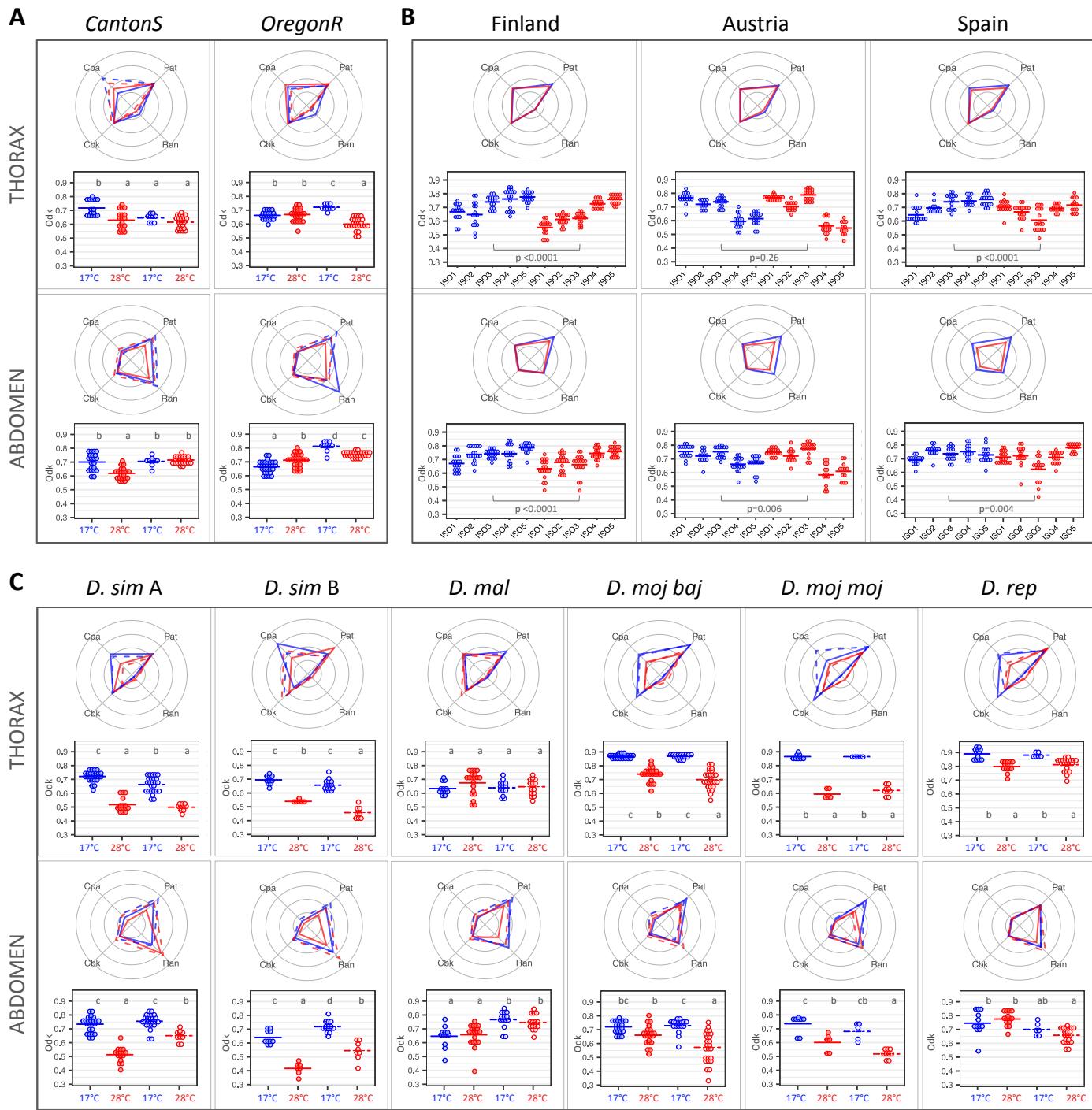
820 **Figure 3. Co-variation and thermal sensitivity of *D. melanogaster* pigmentation  
components**

822 A. Heat map of Pearson's correlation coefficients for all pigmentation traits in  
abdomens and thoraxes of CanS (left panels) and OreR (right panels) of flies reared at  
17°C or 28°C. For each matrix, females are in the left corner and males in the right.  
824 Positive correlations are shown in purple and negative correlations in orange.  
Correlations not statistically significantly different from zero (p-value>0.05) are  
826 indicated with a cross. B. Pigmentation traits (Y axis) in females of two *D.  
melanogaster* genetic backgrounds (CanS and OreR) exposed to each of the temperature  
828 regimes during development (X axis). The thermal regimes codes and corresponding  
stages that were exposed to either 17°C or 28°C (instead of the basal temperature of  
830 23°C) were: T (constant temperature), L (late larval development), p (early pupal  
period) and, P (late pupal period). In each graph, dots represent phenotypes of single  
832 individual females, and the horizontal bar is the mean of those values. The results of the  
test for differences between strains and thermal regimes on the different plastic traits are  
834 shown in Table S3. Letters indicate results of post-hoc pairwise comparisons between  
836 groups: different letters when significantly different (p-value<0.05 for Tukey's honest  
significance test).

Figure 1.



**Figure 2.**



**Figure 3.**

**A**

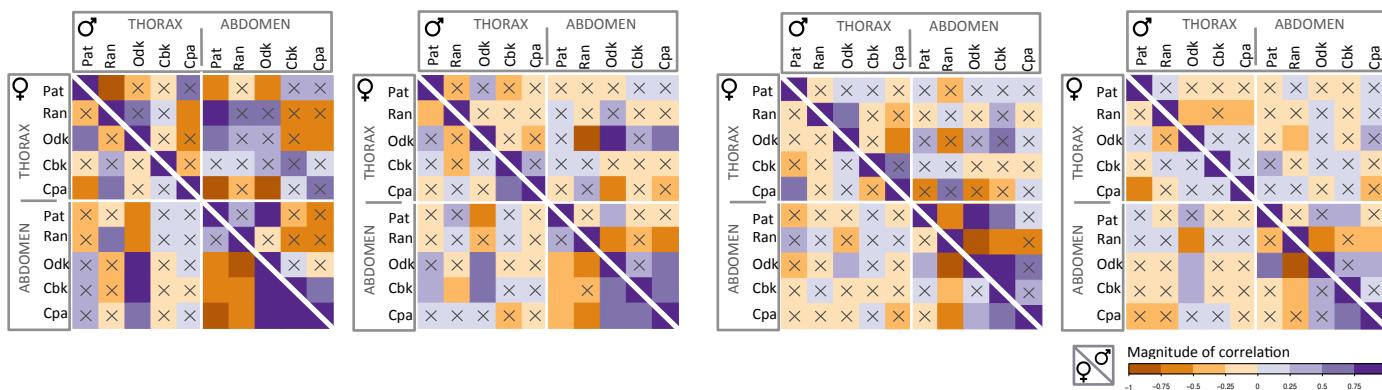
*CantonS*

17°

28°

*OregonR*

28°



**B**

*CantonS*

*OregonR*

