Evolution and regulation of developmental plasticity: body size and pigmentation in *Drosophila*

Elvira Lafuente

Dissertation presented to obtain the Ph.D degree in Evolutionary Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
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It is only by pursuing a Ph.D. in Biology, that Einstein's remarks on time being an illusion have gained value to me. In fact, the years during which I worked on the content of this thesis have meant many things that have little to do with the usual metrics applied to temporality. It could probably be measured more accurately by the challenges that I have faced and by the lessons that I have learned. Or, alternatively, by the places that I have visited or the numerous secret corners of the city, that have made me fall in love with Lisbon. I could certainly quantify it by the thousands and thousands of flies that I have transected (according to my latest calculations I could walk on top of those from Lisbon to Madrid). However, if I had to choose a unit of measure, I would go for the number of great people that have walked alongside me on this journey, many of which have contributed enormously to my work. I honestly don't believe that I would have got here without all the advice, all the discussions, the criticism, the ideas and the support I received and even if I would have made it, it certainly wouldn't have been so enjoyable!

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TABLE OF CONTENTS

SUMMARY

Phenotypic variation is a universal property of biological organisms and is the raw material for evolution by natural selection. Patterns of phenotypic variation in natural populations are greatly dependent on the external environment. Beyond filtering phenotypic variation in the process of natural selection, the environment can also play an instructive role leading to the production of phenotypic variants during development. Environmental cues, such as temperature or nutrition, can influence developmental rates and trajectories and lead to the production of different phenotypes from the same genotype; a phenomenon called developmental plasticity. In the wild, organisms are exposed to a variety of environmental cues that might affect the development of several traits in different manners.

A good match between phenotype and ecological conditions is achieved when the environmental cue that triggers changes in development is a reliable predictor of the future selective environment. In this context, plasticity that results in the production of phenotypes well suited to their selective environment can help organisms cope with environmental heterogeneity. This can have important implications for population persistence, and is a topic of debate in the context of assessing the impact of climate change on natural populations.

Traditional models of adaptive evolution have often neglected a major role for plasticity, mainly because the alternative phenotypes resulting from plastic development typically correspond to non-heritable variation. More recently, some attention has been given to developmental plasticity in the context of whether it facilitates or hinders adaptive evolution, as well as both phenotypic and phylogenetic diversification. Beyond the contribution of plasticity to evolutionary change, researchers have also explicitly addressed the evolution of plasticity. Studies in the field and in the laboratory have shown that plasticity can be heritable, subject to selection and therefore, can evolve. A number of different models have explicitly addressed the conditions that favor the evolution of developmental plasticity, including

periodicity of environmental fluctuations and the costs of plasticity. Experimental studies have started to provide insight into the genetic basis of the evolution of plastic versus robust development. Genetic variation for plasticity, which provides the raw material for its evolution, can affect different aspects of the relation between phenotype and environment. These include the extent of phenotypic change due to changes in environmental conditions, and the environmental threshold after which the phenotype is no longer robust to environmental change. These types of differences can be studied by comparing reaction norms between genotypes.

Despite the prevalence of plasticity in nature and its potential contribution to the ecology and evolution of populations, we know little about the genetic mechanisms whereby the external environment regulates development, and about the loci that harbor allelic variation leading to differences in plasticity. The aim of my project was to contribute to filling some of these gaps. To explore the mechanisms underlying environmentally-induced phenotypic variation, and the genetic basis of variation in plasticity, I focused on two iconic plastic phenotypes of *Drosophila*, body size and pigmentation. These are well studied phenotypes that are closely related to fitness and represent compelling examples of the environmental regulation of development. I made use of available analytical tools and resources (for genetic analysis) and also developed a new method (for quantitative phenotyping of body size and pigmentation; *Chapter 2*) to address a number of open questions about the mechanisms for plasticity and variation in plasticity.

In *Chapter 1,* I lay down the background and identify which outstanding questions this thesis sought out to address. I also explain the choice of model system and provide an overview of the thesis contents.

In *Chapter 2,* we explore the effects of genetic, environmental and genotype-by-environment interactions on different components of body pigmentation, related with color and color pattern. We showed that those traits (and trait-correlations) differ across *Drosophila* species and standard

D. melanogaster "wild-type" genetic backgrounds, as well as between sexes and between developmental temperatures. We also demonstrate that the window of development during which temperature affects end pigmentation phenotype is not the same for different properties of body pigmentation and not the same for different genotypes. Finally, we investigate the pigmentation traits we defined in natural populations of *D. melanogaster* collected across a European cline, and in other *Drosophila* species. We then discuss our results in the context of how independence (or integration) between traits might affect developmental and evolutionary trajectories.

In *Chapters 3* and *4*, we explore the genetic basis of inter-genotype variation in the slope of thermal reaction norms for body pigmentation and for body size, respectively. We took a genetic mapping approach using a panel of community-available *D. melanogaster* genotypes representing naturally segregating alleles from one wild-caught source population. Each of ca. 200 fully-sequenced isogenic lines was reared at either of two temperatures and female adults were phenotyped for size and for color and color pattern traits on both thorax and abdomen. The data were used to: i) quantify genetic and environmental components of phenotypic variation, and correlations between traits, and ii) identify which loci contribute to inter-line differences in thermal plasticity. We found (and validated a selection of) genes associated to variation in thermal plasticity in different properties of the two body parts, with very little overlap between traits. There was also little overlap between loci contributing to variation in plasticity and those contributing to trait variation within one single environment. In terms of putative functions of those genes, they seemed to span the whole process from sensing the external environment, to conveying information about it to developing tissues, to executing functions in accordance to that information. The genes identified contribute to inter-genotype variation in plasticity in the target *D. melanogaster* and are putative targets of selection in the evolution of plasticity in that and possibly other populations.

In *Chapter* 5, I provide an overview of the main results, discuss limitations of our approach, and identify possible future research avenues. I also report on additional preliminary data addressing related open questions in the study of developmental plasticity: i) quantifying combined effects of changes in two environmental cues, which, in isolation are known to impact *D. melanogaster* body size and pigmentation, and ii) testing the hypothesis that the mechanism of RNA editing, by which the same mRNA molecule can result in the production of alternative peptide products, plays a role in the environmental-regulation of developmental outcomes.

Altogether, this thesis explores the molecular underpinnings and evolution of environmentally-induced phenotypic variation. It uncovers the genetic basis of plasticity and explores the mechanisms by which environmental inputs are integrated during development. By using *Drosophila* body size and body pigmentation as a model, we provide insight into some of the outstanding questions in the field of development plasticity.

SUMÁRIO

A variação fenotípica é uma propriedade universal dos organismos vivos e é também a matéria prima para a evolução por selecção natural. Os padrões de variação fenotípica que existem na natureza estão intrinsecamente dependentes do ambiente externo. Esse ambiente não só afecta quais as variantes fenotípicas que aumentam ou diminuem de frequência durante o processo de selecção natural, como pode afectar que variantes fenotípicas são produzidas durante o processo do desenvolvimento que traduz genótipos em fenótipos. Factores ambientais tais como a temperatura ou a nutrição podem influenciar as taxas e/ou trajectórias do desenvolvimento e levar à produção de fenótipos diferentes a partir dum mesmo genótipo. A este fenómeno dá-se o nome de plasticidade fenotípica. Na natureza, os organismos estão expostos a inúmeros factores ambientais, cada um dos quais podendo afectar o desenvolvimento de diferentes características fenotípicas de forma distinta.

A plasticidade do desenvolvimento pode levar a um melhor ajuste entre o fenótipo dos adultos e as condições ambientais a que este vai estar exposto. Este ajuste entre o fenótipo e as condições ecológicas é possível quando o factor ambiental que provoca alterações no desenvolvimento é um predictor fiável onde está inserido. A plasticidade que resulta na produção de fenótipos melhor ajustados ao seu ambiente selectivo diz-se adaptativa. Este tipo de plasticidade é uma forma dos organismos poderem lidar com heterogeneidade ambiental, sem alterações no seu material genético, e pode ter um grande impacto para a persistência das populações sujeitas a alterações ambientais. Como tal, a plasticidade do desenvolvimento face às condições ambientais tornou-se um tópico de interesse, também no contexto do estudo do impacto das alterações climáticas em populações naturais.

Os modelos tradicionais de evolução adaptativa tipicamente não consideram a plasticidade fenotípica como um factor importante, uma vez que os fenótipos alternativos que resultam da plasticidade do desenvolvimento geralmente correspondem a variantes não-hereditários.

Porém, mais recentemente, vários estudos têm abordado a discussão de se a plasticidade do desenvolvimento poderá facilitar ou dificultar a evolução adaptativa e poderá contribuir para a diversificação fenotípica e mesmo filogenética. Para além do potencial impacto da plasticidade na evolução, vários estudos têm também abordado a questão da evolução da plasticidade. Estudos realizados tanto no campo como no laboratório mostram que a plasticidade é ela própria uma característica hereditária que pode ser alvo de seleção natural. Consequentemente, a plasticidade também evolui. Vários estudos teóricos analisaram os tipo de condições que podem favorecer a evolução da plasticidade no desenvolvimento, incluído factores como a periodicidade das flutuações ambientais e os custos da plasticidade.

Por outro lado, estudos experimentais em diferentes organismos têm elucidado a base genética da evolução da plasticidade ou da robustez do processo do desenvolvimento em relação a factores ambientais externos. A variação genética para a capacidade e forma como o desenvolvimento responde a factores externos (plasticidade) proporciona a matéria prima para a evolução da plasticidade. Esta variação genética pode afectar vários aspectos da relação entre o fenótipo e o ambiente, incluindo quanto e de que forma os diferentes factores ambientais afectam o fenótipo. Para estudar estas diferenças entre genótipos são utilizadas tipicamente normas de reação, que correspondem a uma linha (ou curva), que descreve a variação de um dado fenótipo em função de diferentes contextos ambientais.

Apesar da prevalência da plasticidade na natureza e a sua potencial contribuição para a ecologia e evolução das populações, sabemos pouco acerca dos mecanismos genéticos através dos quais o ambiente externo regula o desenvolvimento e sobre os genes que contém variação alélica que contribui para variação nos níveis ou propriedades da plasticidade. O objectivo do meu projecto foi contribuir para preencher algumas destas lacunas no nosso conhecimento da plasticidade fenótipica. Para explorar os mecanismos por detrás da variação fenotípica induzida pelo ambiente e a

base genética da variação na plasticidade do desenvolvimento, foquei-me em dois fenótipos plásticos no modelo na mosca-do-vinagre, *Drosophila* – o tamanho e pigmentação do corpo. Quer o tamanho quer a pigmentação dos adultos das moscas *Drosophila* são características sobejamente estudadas, quer em termos da sua importância para a ecologia das espécies, quer em termos da sua evolução e desenvolvimento. Ambas são características cujo valor adaptativo está bem estabelecido e cuja expressão depende também de factores ambientais, como a temperatura em que as larvas e pupas se desenvolvem. Usando este modelo para estudar abordar várias questões abertas sobre os mecanismos para a plasticidade e para a variação da plasticidade, recorri a ferramentas analíticas e recursos (para analise genética) e também desenvolvi um novo método (para análise quantitativa dos fenótipos escolhidos; *Capítulo 2*).

No *Capítulo 1*, discuto o contexto e revejo a literatura disponível sobre a plasticidade no desenvolvimento, incluíndo aspectos da sua evolução e regulação. Também explico quais as questões pertinentes que esta tese tem como objectivo resolver e a escolha do organismo modelo. No final do capítulo dou ainda uma visão geral do conteúdo desta tese.

No *Capítulo 2,* exploramos a contribuição de factores genéticos e ambientais, bem como da interacção entre os dois, para as diferenças em diferentes componentes da pigmentação do corpo, incluindo cor e padrões de cor. Demonstramos que estas características (e as correlações entre elas) diferem entre genótipos de *Drosophila melanogaster* frequentemente usados em estudos experimentais como representando o “tipo selvagem”. Também caracterizamos diferenças entre sexos e diferenças devidas à temperatura a que os organismos foram expostos durante o desenvolvimento. Neste capítulo mostramos também que a janela temporal durante o desenvolvimento em que a temperatura pode afectar a pigmentação do adulto não é o mesmo nem para todas as componentes da pigmentação nem para todos os genótipos estudados. Finalmente, investigamos as mesmas componentes da pigmentação em populações

naturais de *D. melanogaster* e em diferentes espécies de *Drosophila*. Por último, discuto os resultados no contexto de como a independência (ou integração) entre as diferentes componentes da pigmentação do corpo pode afectar as trajetórias evolutivas desta importante característica adaptativa.

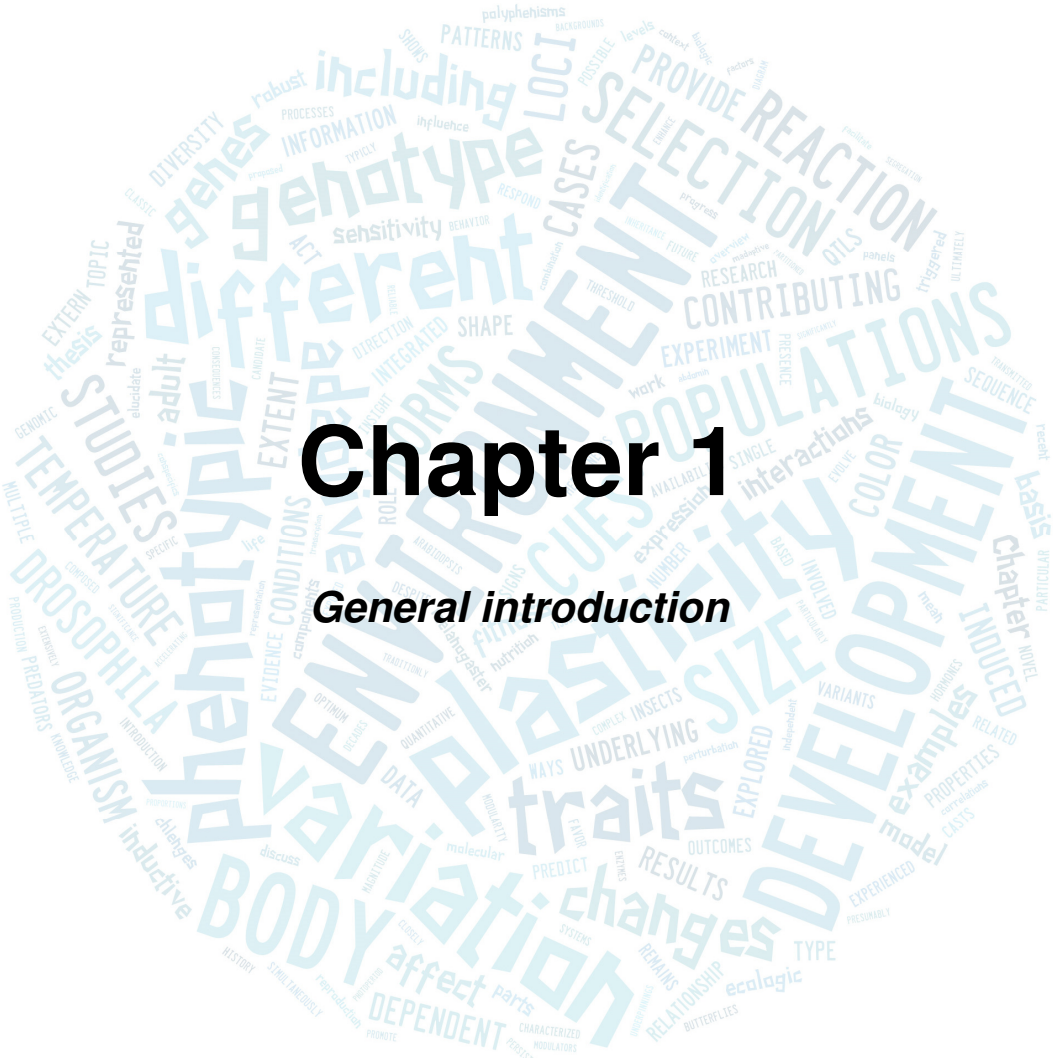
Nos *Capítulos 3* e *4*, exploramos a base genética da variação entre genótipos no declive das normas de reacção térmicas para a pigmentação corporal e tamanho do corpo, respectivamente. O declive das normas de reacção descreve quão plásticas essas características são em relação à temperatura a que as moscas se desenvolvem e permite-nos comparar níveis de plasticidade entre genótipos. Utilizámos uma técnica de mapeamento genético, fazendo uso dum painel de genótipos de mosca-do-vinagre disponível para toda a comunidade científica. Este painel foi criado a partir duma população natural e contém alelos que segregam nessa população. As moscas capturadas na natureza foram usadas para estabelecer aproximadamente 200 linhas isogénicas cujos genomas foram sequenciados. Para estudar plasticidade térmica, moscas das diferentes linhas foram submetidas a duas temperaturas alternativas. As fêmeas adultas resultantes foram caraterizadas em relação ao tamanho, cor e padrões de cor do tórax e abdómen. Estes dados foram usados para: i) quantificar componentes genéticos e ambientais da variação fenotípica, e correlações entre diferentes características e ii) identificar que diferenças genéticas contribuem para as diferenças entre linhas isogénicas na plasticidade térmica. Descobrímos e validámos uma série de genes associados à variação na plasticidade térmica em diferentes propriedades das duas partes do corpo e constatámos que havia pouca sobreposição nos genes relativos aos diferentes fenótipos estudados. Havia também pouca sobreposição entre genes que contribuem para a variação da plasticidade e aqueles que contribuem para a variação de cada característica dentro de um único ambiente. No que toca às possíveis funções desses genes, verificámos que estas poderão estar relacionadas com todas as fases do processo, desde a percepção do ambiente externo, à transmissão dessa

mesma informação aos tecidos em desenvolvimento e finalmente à execução de funções de acordo com a informação processada. Os genes que identificámos contribuem para variação entre genótipos na plasticidade da mosca-do-vinagre e são alvos de selecção putativos para evolução da plasticidade na população de que o painel de mapeamento for derivado, e potencialmente noutras populações.

No *Capitulo 5*, dou uma visão geral dos resultados desta tese, discuto limitações da nossa abordagem e refiro possíveis experiências a realizar no futuro, para ampliar ou consolidar o nosso conhecimento acerca do tema da regulação e evolução da plasticidade fenotípica adaptativa. Neste capítulo, apresento também dados preliminares que obtivemos para explorar duas questões complementares sobre a plasticidade no desenvolvimento, através: i) da quantificação dos efeitos da combinação de diferentes factores ambientais (temperatura e nutrição) cujos efeitos individuais no tamanho e pigmentação da mosca-do-vinagre são conhecidos mas cujas possíveis interacções (também com factores genéticos) estão por documentar, e ii) do teste da hipótese de que o mecanismo de edição do RNA, que pode levar à produção de diferentes proteínas a partir da mesma molécula de RNA mensageiro, afectar a regulação ambiental do desenvolvimento (ou, plasticidade).

Em suma, esta tese investiga a base molecular e a evolução da variação fenotípica induzida pelo ambiente. Revela também a base genética da plasticidade e explora os mecanismos pelos quais os factores ambientais são integrados pelo organismo durante o seu desenvolvimento. Utilizando o tamanho do corpo e pigmentação da *Drosophila* como modelo, obtivemos conhecimento que permitirá responder a questões pendentes acerca da plasticidade do desenvolvimento.

“But all evolutionary biologists know that variation itself is nature's only irreducible essence.” Stephen Jay Gould



Chapter 1

General introduction

ABSTRACT

Phenotypic variation is the result of the effects of genotype and environment that are integrated by developmental and evolutionary processes. Despite the impact that phenotypic plasticity may have on evolutionary outcomes, current paradigms in biology often rule out features of the environmental dependency of development whereby phenotypes can be altered. Moreover, little is known about the loci contributing to variation in plasticity and about the mechanisms by which the environment regulates development. The aim of this introduction is to provide an overview of the key conceptual and empirical knowledge about plasticity. More detailed and targeted information is then given in the introductions of each individual chapter. In the following four sections, I discuss the contribution of the external environment to phenotypic variation, the role of plasticity on adaptive evolution and its genetic underpinnings. Then, I introduce why *Drosophila* body color and body size are suitable systems to study the molecular mechanisms and evolution of developmental plasticity. Finally, I give a brief overview of the contents of the chapters presented in this thesis.

PHENOTYPIC VARIATION AND EVOLUTION

Understanding the diversity in colors, shapes and other phenotypes in the natural world is among the most fascinating and old challenges of biology. It is a considerable challenge given that nature seems to have explored endless possibilities on how to become different or alike. Heritable phenotypic variation is the fuel for evolutionary change, as postulated by Darwin and Wallace in the theory of evolution by natural selection (Darwin & Wallace 1858; Darwin 1859). This theory became widely accepted after the discovery of genes as the fundamental source of inheritance (Mendel 1865), which established the pillars upon which evolutionary biologists have been exploring the mechanistic basis of natural diversity. Our understanding of the proximate and ultimate mechanisms that generate phenotypic variation (Mayr 1963; Tinbergen 1963) has progressed immensely in the last decades and remains a central topic in current research. This progress has benefited greatly from the development and availability of sophisticated technological tools and from multidisciplinary integrative efforts, such as global collaboration initiatives (e.g. DrosEU consortium; www.droseu.net), and even efforts beyond the restricted community of "professional" researchers, such as those of citizen-science projects (e.g. Silvertown *et al.* 2011), and amateur scientists (Lafuente & Alonso 2010).

Eco-evo-devo (i.e. ecological evolutionary developmental biology) has played an instrumental role in our understanding of the living world, by placing biological phenomena at the intersection of different disciplines. This field of research studies the interactions between an organisms' environment, genotype, and development and incorporates these into evolutionary theory (Laubichler & Maienschein 2007; Abouheif *et al.* 2014). Researchers have recently proposed to incorprate knowledge derived from main topics of eco-evo-devo studies, such as modularity or plasticity, into other scientific areas, namely climate change and conservation biology (Campbell *et al.* 2017). Some of that interest relies on the possibility of being able to predict evolutionary paths, particularly in the context of environmental

threats induced by human activity. Despite great progress and an astounding accumulation of both sequence data and sophisticated analytical tools, understanding the relationship between genotype and phenotype has remained much more elusive than anticipated in the early days of whole genome sequencing. Predicting phenotypes based on genomic and environmental data is hard given that genotype-phenotype maps (and evolutionary outcomes) in nature are far more complicated than the usually simplistic set-ups used in the laboratory. In reality, genes are not isolated independent entities, and phenotypes are composed of many traits with complex associations. Moreover, multiple environmental cues act (and interact) in combination to influence phenotypic outcomes during development, and, ecological challenges are highly variable in time and in space. At any of these different levels of biological organization we are faced with the premise that "the whole is different than the sum of its parts".

Environmentally-induced phenotypic variation

Many studies in the last decades have provided insight into the mechanisms that shape patterns of variation within and among species, using a variety of taxa and traits (e.g. Kato *et al.* 1993; Stern 2000; Kichenin *et al.* 2013). It has become evident that the environment, beyond filtering phenotypic variation during evolution, can also produce new phenotypic variants from a single genotype; a phenomenon called phenotypic plasticity (Bradshaw 1965). Plasticity is widespread in nature, occurring in virtually all biological kingdoms, from bacteria to mammals, and at all levels of biological organization, from regulation of gene expression in the cell-compartment, to overall physiological changes in the organism (Sultan 2000; Nijhout 2003a; Beldade, Mateus & Keller 2011). The study of plasticity and of its potential consequences for the ecology and evolution of organisms are central to our understanding of the genotype-phenotype-fitness interplay. Moreover, as portrayed by the controversial 'nurture vs. nature' dichotomy (Moore 2003), the topic has been at the boundary between scientific and sociological

concerns. This is, at least partly, because plasticity exemplifies the complexity of genotype-phenotype maps and sustains the need for a revision of the traditional genetic deterministic view of development (i.e. the unfolding of an already set sequence of events), giving way to a scenario where development (and phenotypic variation) in itself is a set of integrated responses to genotype-by-environment interactions (see Lewontin 2000). The study of plasticity may also be relevant in the context of human health. Indeed, an increasing number of epidemiological studies associate external effects during early life to the risk of developing some modern diseases, such as cardiovascular disorders, type 2 diabetes, obesity and osteoporosis (see Gluckman & Hanson 2007).

Plastic responses in nature are incredibly diverse. This is illustrated by the variety of cues, traits, and ecological scenarios that have been characterized to date in different species (see Schlichting & Pigliucci 1998; Figure 1.1). During adulthood, environmentally-induced phenotypic variation usually leads to changes that are reversible, such as rapid metabolic, physiological or behavioral alterations. In the case of developmental plasticity, external environmental cues influence developmental rates and trajectories during pre-adult stages, typically leading to changes in adult phenotypes that are often, irreversible (Figure 1.2). Iconic examples of developmental plasticity include temperature-induced polyphenisms in butterflies (Shapiro 1984), the nutritional determination of casts in social insects (Maleszka 2008; Smith *et al.* 2008), and the changes in body morphology upon predators' presence in water fleas (Eads, Andrews & Colbourne 2008).

Plastic responses can be triggered by different types of environmental cues that can be biotic (e.g. presence of conspecifics) or abiotic (e.g. temperature) and that can reflect heterogeneity in environment, in space and/or in time. While in some cases there is a substantial degree of cue-to-trait specificity in determining how development will be altered, in others, a single cue can simultaneously affect different traits and the same trait can

simultaneously be affected by several cues. For instance, temperature (e.g. Bochdanovits, van der Klis & de Jong 2003), nutrition (Brian 1975) and presence of predators (e.g. Beckerman, Rodgers & Dennis 2010), often affect development time, body size and other correlated life-history traits. Traditionally, most work on developmental plasticity has concentrated on studying the effect of a single environmental cue on a particular trait but more recent work is starting to elucidate the way in which multiple environmental cues are integrated during development (Braendle & Félix 2008; Rodrigues *et al.* 2017). In fact, in natural habitats organisms are exposed to a multitude of environmental influences, that might be redundant, additive or interact in some form (e.g. synergistically or antagonistically), and may impact the development of multiple traits in different manners (Chevin 2013; Piggott, Townsend & Matthaei 2015). For instance, in a cooperatively breeding cyclid fish, the combination of social environment and predation risk experienced during pre-adult phases determines whether the adult will follow a 'dispersal' or a 'philanthropic' strategy later in life. These different strategies involve changes in several behavioral traits (Fischer *et al.* 2017). More research on this direction is likely to follow trying to elucidate the extent to which environment-by-environment interactions may impact developmental and evolutionary processes.

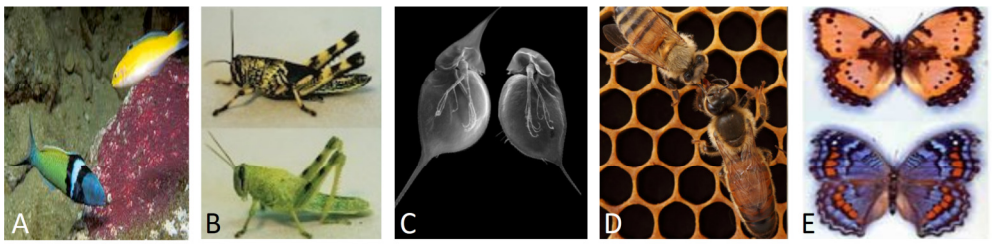


Figure 1.1. Environmentally-induced phenotypic variation. Textbook examples of plasticity in various taxa. **A.** Females of the bluehead wrasse (yellow morph) can become males (blue morph), in the absence of a male in the harem. **B.** In the grasshopper *Schistocerca lineata*, adult body color and wing development is density-dependent and determined by tactile stimulation from conspecifics during development. **C.** In response to predators, the water flea, *Daphnia longicephala*, develops protective crests and tail spines. **D.** Larval nutrition determines casts during development in honeybees. **E.** Seasonal dimorphism in the pigmentation patterns of the wings of the gaudy commodore, *Precis octavis*.

Environmental regulation of development

Development can either respond to or resist environmental perturbation and the balance between these two processes is crucial for organismal survival and fitness. This corresponds to the phenomena of plasticity and robustness, respectively. Even though plasticity and robustness are at the two ends of the spectrum, neither of them are absolute properties of an organism or of a developmental program. Different traits of an individual can have different responses to the same cue, as shown for example in *Mycalesis* butterflies where the size of the eyespots on ventral and dorsal surfaces of the wing respond in opposite directions to thermal variation (van Bergen *et al.* 2017). Moreover, particular traits may only show environmental sensitivity during a time window while being highly robust throughout the rest of development (e.g. Koyama, Mendes & Mirth 2013; Mendes & Mirth 2016).

The environmental regulation of development can be mechanistically divided into a series of steps whereby i) the environmental cue needs to be sensed, ii) those signals need to be transmitted by a modulating system that conveys information about the external environment to the developing tissues, and iii) effector mechanisms, such as changes in gene expression, will result in different developmental outcomes based on the signals received (Figure 1.2). Several molecular players have been identified as candidates for mediating the environmental effects on development. In most, if not all, cases that are well described, hormones work as modulators that instruct developmental changes based on environmental information (see Nijhout 1998). Examples of this physiological mediation of plasticity include the case of several amphibian species where conditions of desiccation trigger changes in the endocrine system capable of accelerating metamorphosis (Denver 1997), and the case of dung beetles where ecdysteroid titers underlie the nutrition-dependent horn size in males (Emlen & Nijhout 1999).

Differences in gene expression due to alternative developmental environments have also been identified for a number of species and in relation to several environmental cues. Using a variety of methods, from

candidate-gene approaches (Abouheif & Wray 2002; Shoemaker-Daly *et al.* 2010; Miyakawa *et al.* 2010) to more unbiased transcriptomic analysis (Levine, Eckert & Begun 2011; Zhou *et al.* 2012), studies in this area have provided insights onto the magnitude and the identity of the genes affected by environmental changes. Underlying some these are a number of mechanisms that extend genetics (and inheritance) beyond the classical DNA coding sequence. These include DNA methylation and/or post-transcriptional modifications that are known to contribute to inter-individual variation in many traits, including those representing cases of plasticity (see Bossdorf *et al.* 2008). For instance, diet-dependent methylation patterns regulate the induction of queen and worker castes in honeybees (Lyko *et al.* 2010), and experimental demethylation leads to increased plasticity of flowering traits in *Arabidopsis* (Bossdorf *et al.* 2010). Future research in this direction will reveal the prevalence of such mechanisms and how they interact with the hormones that mediate plasticity.

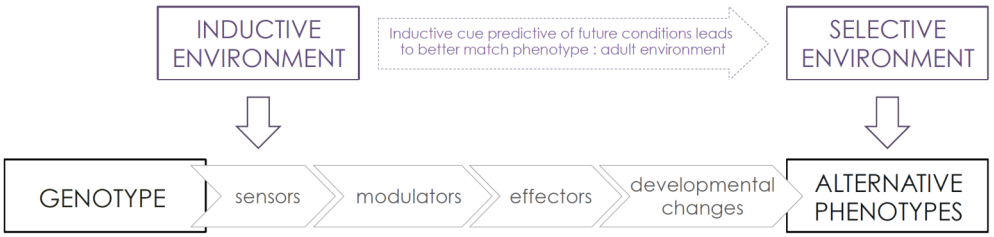


Figure 1.2. Sequence of steps from the inductive to the selective environmental cues. For the environment to affect development, environmental cues need to be sensed and this external information has to be transmitted to the developing tissues by internal signals. Upon receiving such signals, changes in gene expression will modify development and give rise to alternative adult phenotypes. In cases of adaptive plasticity, when the inductive cue is a reliable predictor of the future conditions, plasticity leads to adult phenotypes better suited to their environment. Adapted from P. Beldade.

Reaction norms

Developmental plasticity is commonly studied using reaction norms (Figure 1.3) that are graphical representations in which variation in phenotype is represented as a function of variation environment (Woltereck, 1909; Schmalhausen, 1949; Sultan and Bazzaz, 1993). Reaction norms will be of different shapes depending on the type of plastic response (Figure 1.3B). When development is robust to environmental perturbation, reaction norms are flat. A gradual relationship between environment and phenotype, such as the temperature-induced differences in body size in many insects (Nijhout 2003b; Mirth & Shingleton 2012), leads to continuous reaction norms. More dramatic responses, with an environmental threshold upon which the phenotype changes, are depicted by switch-type reaction norms, such as the casts in social insects or the polyphenisms in Lepidoptera (Figure 1.3B).

In natural and experimental populations, there is variation in the way different genotypes respond to the environment. This variation is reflected in the genotype-by-environment (G × E) interaction component of the partition of phenotypic variance (Figure 1.3A) and can be assessed by comparing reaction norms for different genetic backgrounds (Figure 1.3C-D). These comparisons can provide information about the extent of plasticity, the form of the reaction norms (e.g. linear, quadratic), and the extent of genetic variation for plasticity. Differences in plasticity between genotypes can affect different aspects of the plastic response, including properties of reaction norms such as the intercept, shape and the slope (Figure 1.3C). The genes underlying those differences presumably provide the raw material for natural selection to act upon during the evolution of plasticity under heterogeneous environments. This will be a central topic of this thesis.

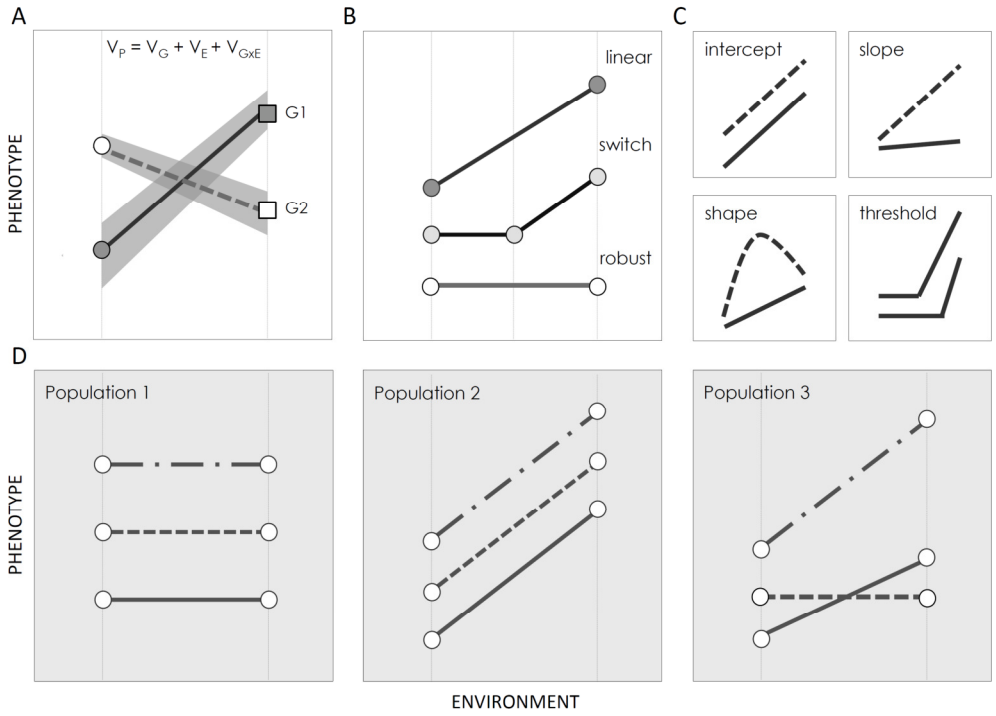


Figure 1.3. Genetic, environmental and genotype-by-environment effects on phenotypic variation. All the plots illustrate reaction norms where variation in phenotype (Y axis) is represented as a function of environmental variation (X axis). **A.** Schematic representation of phenotypic values for two genetic backgrounds (G1 and G2) measured at two environmental conditions. Total phenotypic variation (V_P) in a population can be partitioned into genetic variation (V_G; difference between filled and empty symbols), environmental variation (V_E; difference between circles and squares) and genotype-by-environment variation (V_{GxE}; difference between solid and dashed lines). There is also an intra-genotype, intra-environment component of variation, which is represented in the diagram by the shadow. **B.** Reaction norms can be different depending on the type of environmental dependence of the phenotype and the plot illustrates three types: a continuous response (linear reaction norm), a nonlinear relationship with discrete alternative phenotypes (switch-type reaction norm), and a case in which development is robust to environmental perturbation (flat reaction norm). **C.** Genetic variation for plasticity can affect different properties of reactions norms such as the intercept, slope, shape and/or threshold at which the phenotype responds to environmental variation. **D.** Populations can differ in the extent of genetic variation for plasticity. In population 1, there is no plasticity. In population 2, there is plasticity (i.e. genotypes show different phenotypes depending on the environment), but there is no genetic variation for the magnitude of the environmental effect on phenotype (i.e. all genotypes show parallel reaction norms). Population 3 demonstrates genetic variation for the extent of plasticity in that different genotypes have reaction norms of different slopes.

THE ROLE OF PLASTCITY IN ADAPTIVE EVOLUTION

Plasticity is, by definition, a property of a genotype under a given environment and can be adaptive, maladaptive, or have no major implications for organismal fitness (Ghalambor *et al.* 2007). In many cases of adaptive developmental plasticity, an inductive environmental cue predicts the selective environment that the individual will experience as an adult and this triggers the development of phenotypes better suited for that environment (Figure 1.2) (Scheiner 1993; West-Eberhard 2005). Even though most attention has been paid to the study of adaptive plasticity, conceptual and empirical work also provide examples in which plasticity is neutral or even maladaptive, for instance when the inductive cue becomes an reliable predictor of the forthcoming selective environment (e.g. Langerhans & Dewitt 2002).

Whether plasticity hinders or promotes adaptive evolution has been the topic of intense debate in the last decades, with observations from the field and the laboratory providing evidence for both. These data suggest that plasticity has the potential to hinder or facilitate adaptation, and that this ultimately depends on the relationship between the available genetic variation, the environmentally-induced phenotypes, and the selection pressures at play (see Ghalambor *et al.* 2007). When populations are faced with a novel environment, plasticity can affect adaptive evolution in different ways (Figure 1.4). If the environmentally-induced changes in development produce phenotypes that are better adjusted to the novel conditions experienced (i.e. closer to the local fitness optimum), then plasticity can have an immediate benefit allowing the population to persist as genetic changes still occur (Figure 1.4A). Conversely, if the induced phenotypes are maladaptive in the new environment (i.e. further away from the local fitness optimum), then plasticity can reduce the chances of the population persisting (Figure 1.4B). Novel conditions, outside the original realm of conditions experienced by the population, can also release cryptic genetic variation that might provide new raw material for adaptive evolution (Figure 1.4C). On the

other hand, plasticity can also hide genetic variation in cases where particular genes are not expressed under the novel developmental conditions.

Even though traditional models of adaptive evolution have often neglected any major contribution of phenotypic plasticity, more recent work proposed that plasticity can promote phenotypic diversification and speciation (see West-Eberhard 2005; Susoy *et al.* 2015) and can accelerate adaptive evolution (Moczek & Nijhout 2003; Wund *et al.* 2008). The role of plasticity in adaptive evolution remains contentious, however, it is generally recognized that adaptive plasticity can affect the immediate survival of populations exposed to change by providing the means to cope with environmental heterogeneity (Gotthard and Nylin 1995, Schlichting and Pigliucci 1998, West-Eberhard 2003, Nylin *et al.* 2005, Pfennig *et al.* 2010, Ghalambor *et al.* 2015). This has been illustrated for example, in a study on populations of *Daphnia* where plasticity has repeatedly been co-opted to facilitate rapid adaptation to the presence of predators (Scoville & Pfrender 2010). This and other examples (Pfennig *et al.* 2010), provide evidence that plasticity may represent a solution to challenges posed by changing environments (De Jong 2005), including those triggered by climate change.

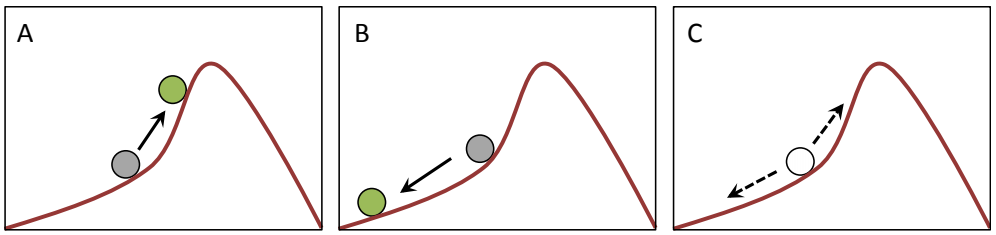


Figure 1.4. Plasticity can hider or promote adaptive evolution. The grey symbol represents the mean phenotype of a population without the effect of plasticity while the green symbol shows the mean phenotype generated via plasticity. **A.** When plasticity places the population closer to the fitness optimum, the population can persist and still be subjected to directional selection, facilitating adaptation. **B.** Plasticity can be deleterious for adaptation if it places the population far away from the fitness optimum. **C.** Plasticity can lead to a release of cryptic genetic variation and this would turn adaptation dependent on the new genetic variation.

EVOLUTION OF PLASTICITY

Theoretical models have begun to outline the factors that could favor the evolution of plasticity, such as the predictability of the environmental fluctuation (Leimar, Hammerstein & van Dooren 2006; Reed *et al.* 2010), the reliability of inductive cues and the potential costs of plasticity (Callahan, Maughan & Steiner 2008; Snell-Rood 2012; Murren *et al.* 2015). The inductive cue should accurately predict the future selective environment that adults will experience. Inductive and selective environments can, but do not need to be the same. In several cases of seasonal polyphenism in butterflies, photoperiod and temperature experienced during larval stages act as inductive cues while the selective pressures are related to predation risk and opportunities for reproduction (Brakefield & Reitsma 1991). The optimal response to a heterogeneous set of environmental challenges would be to always evolve plasticity, allowing organisms to exhibit the best phenotype in every environment without the need for genetic change (see Moran 1992). There are, however, both limits and costs associated with plasticity, such as for instance energetic costs of producing and maintaining sensory or regulatory mechanisms, that may constraint the evolution of plasticity (see Dewitt, Sih & Wilson 1998; Murren *et al.* 2015).

Transitions from environmentally-sensitive to robust development, and vice versa, have been documented for a variety of species. Examples include the evolution of different degrees of genetic caste determination in ants (Schwander *et al.* 2010) or the erosion of head size plasticity in snakes (Aubret & Shine 2009). Beside studies from natural populations, there are also precedents of changes in plasticity resulting from artificial selection experiments. For instance, in *Manduca sexta* a single mutation in the juvenile hormone-regulatory pathway conferred environmental sensitivity and preceded the evolution of a larval color polyphenism (Suzuki & Nijhout 2006).

Environmental-sensitivity of development is likely to be the ancestral state in most cases, with selection then favoring the ability to buffer

environmental effects (Newman & Müller 2000; Nijhout 2003a). In recent years, sophisticated analyses have highlighted specific mechanisms that may enhance robustness (see Nijhout *et al.* 2017) such as redundancy in cell precursors (Braendle & Félix 2008), gene enhancers (Frankel *et al.* 2010), and/or regulatory microRNAs (Brenner *et al.* 2010). Conversely, modularity in molecular networks (Snell-Rood *et al.* 2010) may enable plasticity. By acting on those and other mechanisms selection can adjust the extent of plasticity in trait development. When the ecological conditions favor the evolution plasticity, selection can act on the regulation of environmentally sensitive phenotypes by genetic accommodation (see West-Eberhard 2003; Braendle & Flatt 2006; Crispo 2007) and enhance the precision plastic responses.

THE GENETIC BASIS OF PLASTICITY

Despite the prevalence of plasticity in nature and its potential consequences for the ecology and evolution of organisms, we still know very little about the genetic bases underlying plasticity and how it varies in natural populations. The genetic basis of plasticity includes loci involved in environmental-responsiveness (e.g. hormones that regulate plastic responses) as well as loci responsible for variation in plasticity (e.g. loci underlying inter-genotype differences in plasticity). Traditionally, studies have mostly focused on the former, unraveling the effects of candidate genes putatively involved in the environmental-regulation of development (Gibert, Peronnet & Schlötterer 2007; Gibert, Mouchel-Vielh & Peronnet 2017; Mendes & Mirth 2016) and identifying quantitative trait loci (QTLs) whose effects vary across environments (QTL-by-environment interactions) (Fry *et al.* 1998; Gurganus *et al.* 1998; Vieira *et al.* 2000; Leips & Mackay 2000; Bergland *et al.* 2008). Much less attention has been given to identifying the genes that carry natural allelic variants that contribute to inter-genotype variation in plasticity itself, which are those that alter the properties of reaction norms (e.g. genes leading to flat vs. steep reaction norms). These can presumably be a subset

of the genes involved in environmentally-sensitive development, and will be main focus of study in this thesis.

Studies in different systems have shown that plasticity is heritable, subject to selection, and, therefore, can evolve (Bradshaw 1965; Via & Lande 1985; Scheiner 1993; de Jong 2005; Ghalambor *et al.* 2007; Aubret & Shine 2009). Overwhelming evidence from natural and experimental populations from different species shows that individuals can differ significantly in the extent of sensitivity and responsiveness to environmental cues (Scheiner & Goodnight 1984; Newman 1994; Robinson & Wilson 1996; Smekens & van Tienderen 2001; Gockel *et al.* 2002; Nussey *et al.* 2005; Lardies 2008). We also know of the polygenic nature of changes in reaction norms, including the extent of phenotypic change resulting from environmental conditions (Lind & Johansson 2007) and the environmental threshold at which the phenotype changes (Moczek & Nijhout 2003). Genetic variation for plasticity can affect different aspects of the relationship between phenotype and environment, and selection on this genetic variation can lead to different outcomes, being at the two extremes, the evolution of canalized, environmentally insensitive phenotypes and the dramatic, threshold polyphenisms. Properties of reaction norms are likely to be quantitative traits for which variation results from the simultaneous segregation of alleles at multiple QTLs and thus, can be studied within a classical evolutionary genetics framework. Successful attempts to map the genetic basis for variation in plasticity include the identification of QTLs associated with thermal regulation of life-history traits in *Caenorhabditis elegans* (Gutteling *et al.* 2007) and for photoperiod-induced flowering in *Arabidopsis thaliana* (Ungerer *et al.* 2003).

Whether reaction norms emerge as direct targets of selection, an idea that favors the existence of the so-called “plasticity genes”, or as by-products of selection acting on the traits themselves, has been a highly disputed topic. In this context, different theoretical models have been proposed accounting for the genetic basis of variation in plasticity (all discussed in Weber &

Scheiner 1992). These include the “pleiotropy model” that proposes that plasticity is the product of a differential expression of alleles in different environments (Via & Lande 1985) and the “epistasis model” whereby plasticity is itself a trait that can evolve independently (Scheiner & Lyman 1991). Even though experimental evidence about this is still scarce, some examples illustrate that selection on plasticity can be independent of selection on the trait mean, as, for example, shown for inflorescence height in *Arabidopsis thaliana* under different levels of shading (Scheiner & Callahan 1999) and for thermal plasticity in the timing of egg-laying in collared flycatchers (Brommer *et al.* 2005).

A more coherent understanding of plasticity and its potential contribution to evolution requires expanding our knowledge about the natural allelic variants that cause inter-individual variation in plasticity, which would represent the raw material upon which selection can act or drive the evolution of plasticity. Identifying these loci will help elucidating what is the nature and the magnitude of allelic effects contributing to variation in plasticity as well as what is the identity of the genes involved. For instance, what are the loci that underlie variation in plasticity? At which level of the environmental regulation of development do these genes act? Are those loci common between traits, body parts, and between variants that determine variation in mean trait values?

OUR MODEL: BODY SIZE AND PIGMENTATION IN DROSOPHILA

Insect body size and body pigmentation are emblematic examples of how the interactions between genotype and environment can determine phenotypes and affect fitness. Both traits play fundamental roles in reproduction and survival by influencing organismal performance under ecological challenges such as predation risk (e.g. Barnes *et al.* 2010; Ahlgren *et al.* 2013), thermo-regulation (e.g. Gibert & DeLong 2014; Kingsolver & Wiernasz 1991) and competition for mates (Badyaev & Young 2004; Head, Kozak & Boughman 2013). Body size is a key trait that is

closely associated with many other life history traits (including lifespan and reproductive output) and has been shown to be under strong selection in natural populations (Lafferty & Kuris 2002; Barnes *et al.* 2010). Body pigmentation represents a classic example of adaptive evolution, including mimicry (Nadeau 2016), industrial melanism (Cook & Saccheri 2013), modularity (Beldade & Brakefield 2003) and novel traits (Brakefield, Beldade & Zwaan 2009). It is too, often closely associated with a number of fitness-related traits, including behavior and immunity (see Wittkopp & Beldade 2009).

Body size and body pigmentation vary between species, populations and environments (including clinal and seasonal). Moreover, both traits are typically sexually dimorphic and differ significantly between the body parts of an individual (e.g. Ng *et al.* 2008; Signor *et al.* 2016). This great diversity has triggered a vast number of ecological and evolutionary studies that have shed light onto the mechanisms that shape inter- and intra-specific variation in body size (Honěk & Honek 1993; Scharf, Juanes & Rountree 2000; Yom-Tov & Geffen 2006) and in body pigmentation (e.g. Cloney 2017; Steiner *et al.* 2007; Pool & Aquadro 2007) in different species.

Both traits are determined by genetic, environmental and genotype-by-environment components, and are plastic in response to different environmental cues, such as nutrition and temperature (Nijhout 2003b; Shingleton *et al.* 2007; Wittkopp & Beldade 2009; Beldade *et al.* 2011). Thermal plasticity is, indeed, quite common in ectothermic animals, most notably in insects, where the temperature experienced during development has a strong effect on several traits, including adult body size and coloration (e.g. Sutcliffe, Carrick & Willoughby 1981; Solensky & Larkin 2009).

Work with *Drosophila* has provided much insight into the genetic and physiological mechanisms underlying the development of body size and body color. Body size and body size proportions are mostly regulated by insulin, juvenile and ecdysone hormones (Figure 1.5B) (see Mirth & Shingleton 2012) and pigmentation development results from the enzymes

that synthesize pigments (effector genes) and the transcription factors that regulate the spatial and temporal expression of those enzymes (patterning genes) (see Massey & Wittkopp 2016) (Figure 1.5A). A great number of studies have also explored the genetic variants and respective associations with environmental factors that might shape the patterns of variation in body size and body color (e.g. French, Feast & Partridge 1998; Loeschcke, Bundgaard & Barker 2000; Pool & Aquadro 2007b) (Figure 1.6). This has led to the development of very sophisticated methods to analyze genomic data, in contrast to the rather coarse analytical tools to quantify phenotypes.

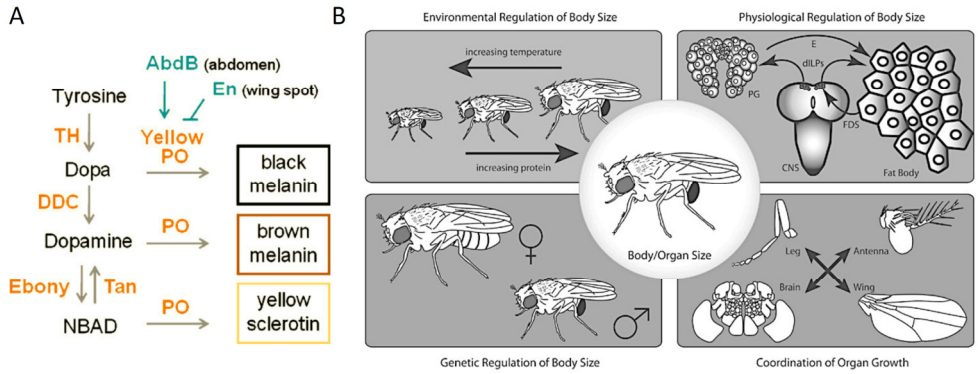


Figure 1.5. Regulation of body size and body pigmentation in *Drosophila*
A. Melanin synthesis pathway in *Drosophila* and its central components with enzymes (in orange), pigments (in black) and regulators (in blue) (from Wittkopp & Beldade 2009). **B.** Schematic representation of the genetic and environmental regulation of body size and proportions. Increased temperature reduces body size while increased protein content in the larval food increases adult size. The control of body size is determined by signals sent from the fat body to the central nervous system (CNS), which in turn regulates the production of insulin-like peptides (dILPs) and ultimately determines the duration of the growth period. The diagrams also shows differences in size between genotypes and between organs of a given individual (from Mirth & Shingleton 2012).

The association between body size and body pigmentation and temperature found across geographical populations of many insect species, including *Drosophila*, is assumed in most cases, to be adaptive plasticity. Flies are darker and larger in colder environments presumably relating to thermo-regulation needs; darker to be able to absorb more solar radiation and larger because growth efficiency decreases with increasing environmental temperature (see Klowden 2007). The selective advantage of

a larger body in colder environment remains unclear but the prevalence of the temperature-size rule (i.e. higher temperatures leads to smaller bodies and vice versa) in natural and experimentally evolved populations, has been taken as a evidence of its adaptive significance (e.g. Atkinson & Sibly 1997; Adrian *et al.* 2016). However, the literature also offers alternative hypotheses to explain the relationship between body pigmentation (and body size), temperature and fitness which do not invoke an adaptive response, including biophysical and/or developmental constraints (Gibert, Moreteau & David 2000; Angilletta Jr *et al.* 2002).

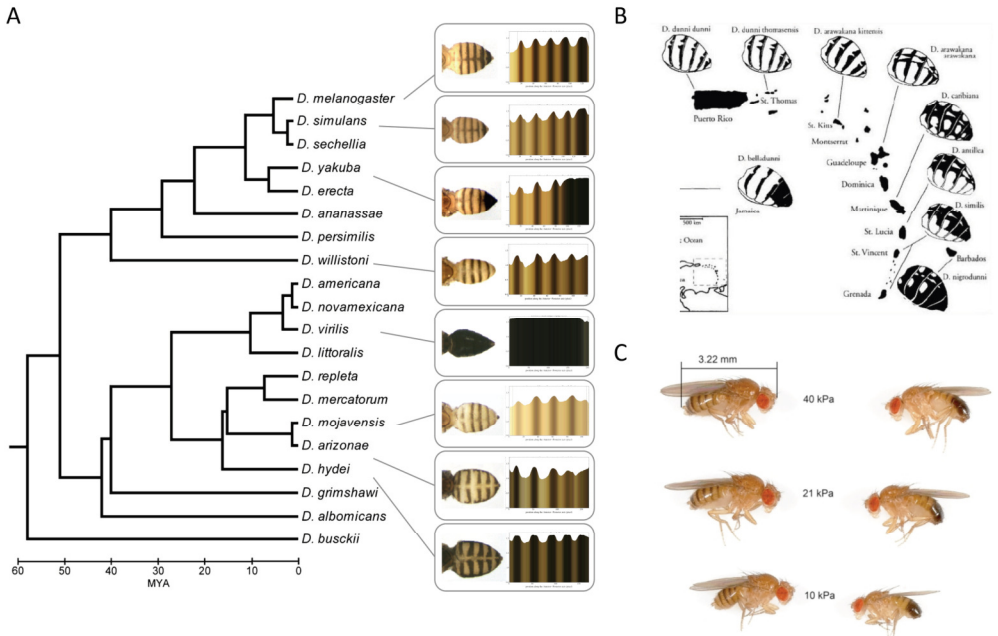


Figure 1.6. Drosophila body size and body pigmentation as a model to study plasticity. A. Phylogenetic tree and examples of diversity in abdominal pigmentation in females from different *Drosophila* species. **B.** Island of origin and drawing of illustrating pigmentation for females of each species in the *Drosophila dunni* subgroup showing a latitudinal cline in abdominal pigmentation (from Hollocher, Hatcher & Dyreson 2000). **C.** *Drosophila melanogaster* females (left) and males (right) from size-selected populations maintained under different conditions of hypoxia (from Klok & Harrison 2009).

Drosophila melanogaster, in particular, is a proven powerful model to map phenotypic variation to known locations in the genome given the availability of genetic tools and more recently, of panels of genotypes

representing naturally segregating alleles (King, Macdonald & Long 2012). This includes the Drosophila Genetic Reference Panel (DGRP), a wild-caught population from Raleigh (North Carolina, USA) composed of ca. 205 fully sequenced isogenic lines, which has been extensively used to map genetic variation for numerous complex traits (Mackay *et al.* 2012; Huang *et al.* 2014), including body size (Vonesch *et al.* 2016) and body pigmentation (Dembeck *et al.* 2015). Most of this work has been typically done by rearing genotypes under a single environment. Thus, despite great advances on our understanding of the genetic architecture and nature underlying variation in many quantitative traits, the genetic basis for plasticity remains largely unexplored. Given the availability of these mapping panels it is now possible to expose these lines/genotypes to different developmental environments, assess phenotypic plasticity for size and pigmentation, and identify which loci contributing to variation in plasticity.

THIS THESIS

This thesis investigates the molecular underpinnings and evolution of environmentally-induced phenotypic variation (Figure 1.7). It uncovers the genetic basis of thermal plasticity and explores the mechanisms by which environmental inputs affect development. By using *Drosophila* body size and body pigmentation as a model, we provide insight into some of the outstanding questions in the field of development plasticity. What are the loci that carry allelic variation determining that some genotypes are less or more plastic? Do these loci affect the sensing of environmental cues, the transmission of these external signals or the implementation of the information in developing tissues? Are the loci regulating plasticity the same for different plastic traits? Do these loci also contribute to inter-individual variation observed within a given environment? How is multifactorial environmental information integrated during development?

In *Chapter 2* we explore the effects of genetic, environmental and genotype-by-environment effects on body coloration. Because pigmentation

is composed of different elements related with color and color pattern, and those can vary in different ways, we developed a quantitative method that decomposes pigmentation into different traits (Figure 1.6). We studied how those traits differ across *Drosophila* species and between genetic backgrounds, sexes, and temperatures. Our data allowed us to characterize correlations across traits and body parts, and to analyze the effects of genotype and environment on those correlations. We also explored how different genotypes of *D. melanogaster* vary in their windows of thermal sensitivity. We expand some of our findings to other natural populations of *D. melanogaster* from a European latitudinal cline.

In *Chapter 3* we used the DGRP to unravel the genetic basis of thermal plasticity for different pigmentation components. To do so, we quantify five pigmentation traits, related with color and color pattern, of two body parts (thoracic and abdominal) from flies reared at two temperatures. We documented effects of genotype, environment and genotype-by-environment on the different pigmentation components, and we identified and validated loci contributing to variation in thermal plasticity in those. We explored the extent of overlap between validated and putative QTLs for plasticity in different pigmentation traits, as well as between QTLs underlying variation within and between environments.

Using a similar approach as used in Chapter 3, in *Chapter 4* we identified the genetic basis for thermal plasticity in body size in *D. melanogaster*. We characterized genetic variation for thermal plasticity in thorax and abdomen size, and identified loci contributing to variation in the slope of thermal reaction norms. We again explored at the extent of overlap between QTLs or size and for size plasticity, and between QTLs for size plasticity in different body parts.

In the *Discussion* chapter, I give an overview of our integrated results and discuss both their significance and possible future research directions. I also present preliminary data I have collected, including the contribution of environment-by-environment (ExE) interactions to phenotypic variation, and

the potential role of RNA editing to thermal plasticity in *D. melanogaster.* All together this thesis aims at shedding light on the extent and genetic basis of inter-genotype variation in plasticity that is required for the evolution of plasticity under heterogeneous environments.

The data chapters of this thesis have been written in view of later submission for publication. For that reason, there might be some repetition, particularly between this chapter and the introductory sections of other chapters.

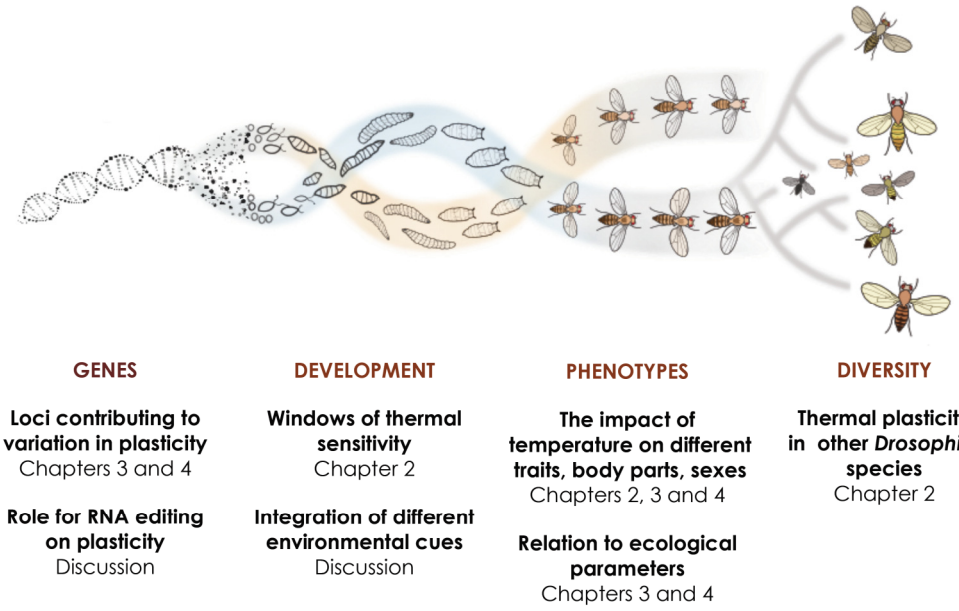


Figure 1.7. Thesis outline. Diagram showing variation in *Drosophila,* from genotypes to species diversity, and summarizing the main topics of this project.

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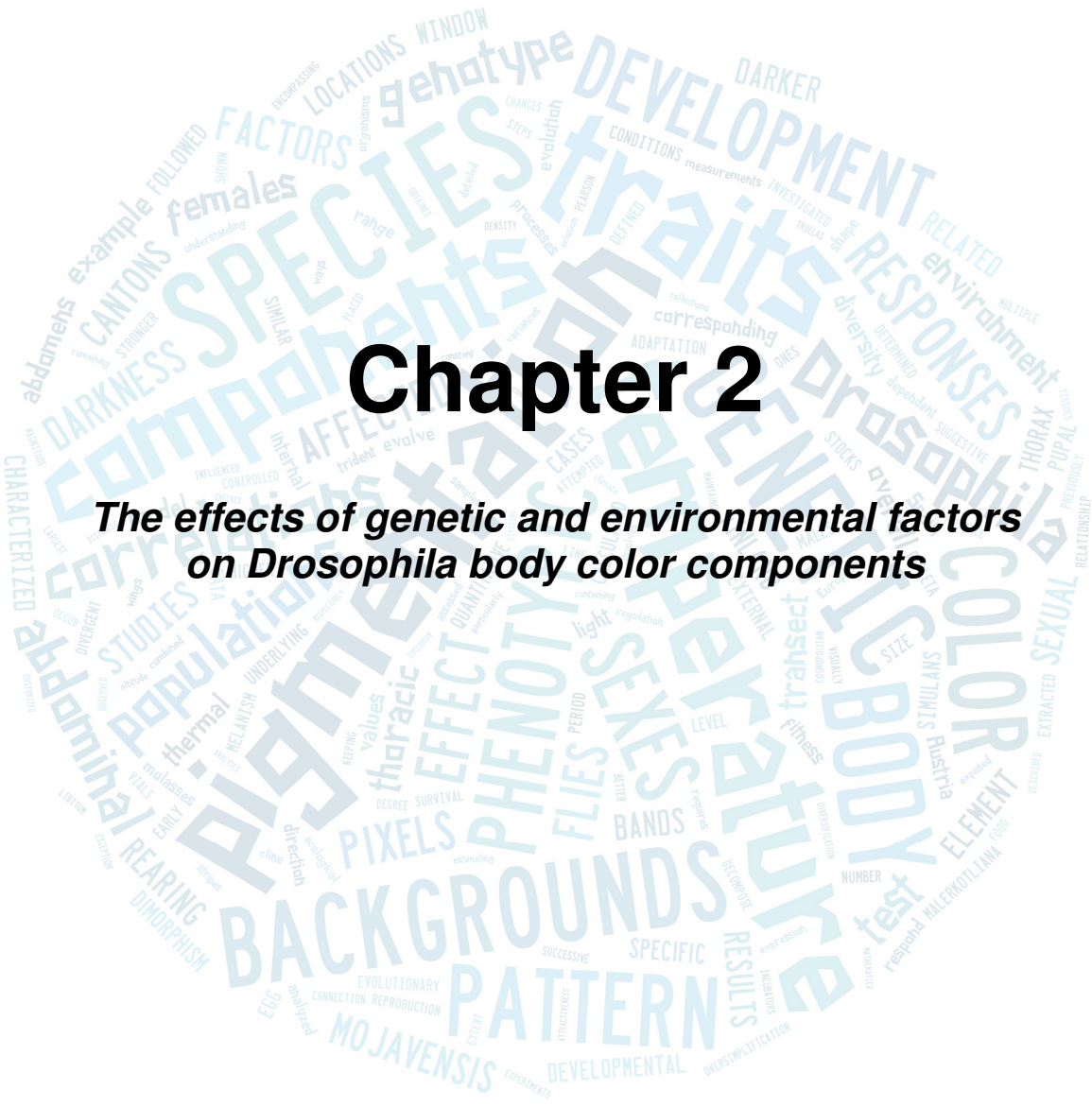

Chapter 2
The effects of genetic and environmental factors on Drosophila body color components

ABSTRACT

Body pigmentation is a diversified trait that plays important roles in the survival and reproduction of insect species. Pigmentation results from the combined effect of many traits that have the potential to vary in their response to internal and external factors and can develop and evolve more or less independently. However, due to the lack of quantitative methods to accurately measure pigmentation, body pigmentation is typically reduced to one trait in many species, including *Drosophila*. Here we develop a method to quantify color and color pattern, decompose the pigmentation phenotype into different components and explore the effect of genetic background, sex and temperature on each component. We found that genetic and environmental factors have different effects on trait associations, namely variance and correlations, in *D. melanogaster.* Pigmentation components show diverse responses to sexual and environmental differences. We also show that the stages of development that are sensitive to temperature differ between genetic backgrounds and across pigmentation traits. Finally, we characterize patterns of pigmentation variation in other natural populations and in five *Drosophila* species and investigate how pigmentation traits are affected by genetic and environmental effects to create the diversity in body color.

INTRODUCTION

Body coloration is an emblematic system of how adaptive evolution can shape phenotypes, given its essential role in the survival and reproduction of many species. Classic examples of adaptation via the evolution of color and color patterns include mate attraction (e.g. Mundy *et al.* 2004; Hill & McGraw 2006), mimicry (e.g. Mallet & Joron 1999; Nadeau 2016) and camouflage (e.g. Cook & Saccheri 2013) which all involve visual communication between individuals of the same or other species. Pigmentation also plays a role in thermoregulation (e.g. Clusella-Trullas *et al.* 2008), where fitness benefits arise from physiological processes that improve tolerance to adverse environmental conditions. The diversity of pigmentation found across species, populations, sexes and even individuals of same sex, has been the focus of many ecological and evolutionary studies, of which some have provided exciting insights on the sources of inter- and intraspecific variation in pigmentation (e.g. Pool & Aquadro 2007; Massey & Wittkopp 2016), including studies on co-option (e.g. Shirai *et al.* 2012) and cis-regulatory evolution (e.g. Miyagi *et al.* 2015). Attempts to unravel the relationship between genotype and (pigmentation) phenotypes typically focus on the genetic mechanisms underlying phenotypic variation, and this has led to the development of sophisticated methods for the analysis of genomic data in comparison to the rather coarse analytical tools available to quantify phenotypes.

Diversity in pigmentation is the product of different colors that are arranged in distinct spatial patterns. In insects, the development of pigmentation involves enzymes that produce pigments and transcription factors that regulate their temporal and spatial expression (see Wittkopp & Beldade 2009). Differences in pigmentation can be found between the body parts of an organism and even across pattern elements within the same body part. Moreover, body pigmentation is made up of many components, related with color and/or color pattern, which do not necessarily develop and evolve together (e.g. Beldade & Brakefield 2003; Linnen *et al.* 2013). These

pigmentation components can have divergent responses to genetic and environmental factors and be correlated and/or constrained by developmental and evolutionary processes. The inter-connection between traits, referred to as phenotypic integration in the case of a tight connection or phenotypic independence when traits are uncoupled, has been previously investigated in the context of body coloration. For instance, the serial pigmentation elements, called eyespots, present on the dorsal and ventral wing surfaces of the butterfly *Bicyclus anynana*, have been shown to respond independently to variation in temperature and to internal levels of ecdysone (Mateus *et al.* 2014).

Drosophila is perhaps one of the best-characterized insect species, with several studies exploring the genetic variants, and associations with environmental factors, that shape the patterns of variation found between species, populations and individuals (e.g. Hollocher, Hatcher & Dyreson 2000; Gibert, Peronnet & Schlötterer 2007; Pool & Aquadro 2007). In *Drosophila*, differences in pigmentation have been associated to desiccation resistance (Parkash, Rajpurohit & Ramniwas 2008) and UV protection (Matute & Harris 2013). Moreover, there is also an association between pigmentation and other phenotypes, such as behavior (Takahashi 2013) and immunity (Dombeck & Jaenike 2004), mediated by multiple roles of melanin and melanogenesis genes (see Wittkopp & Beldade 2009). Pigmentation in *Drosophila* shows developmental plasticity in relation to a variety of environmental cues, such as nutrition (Shakhmantsir, Massad & Kennell 2014) and temperature (David, Capy & Gauthier 1990). Thoracic pigmentation in flies is often characterized by the presence of a trident — a darker pigmented element — or longitudinal stripes. Abdominal pigmentation is composed of longitudinal stripes of dark pigment that vary in width and color. The wings of some *Drosophila* species can show melanic patches that vary in number, size and shape (e.g. True *et al.* 1999). Traditionally, studies on body coloration have used qualitative measurements of pigmentation or quantitative analysis of single body regions, leading to oversimplification of

the phenotypic data. A better understanding of the evolution and development of these pigmentation components requires more detailed and quantitative methods for phenotyping. In fact, the need for sophisticated methods to quantify phenotypic data has become a general quest in biology (Houle, Govindaraju & Omholt 2010) and researchers have begun to develop and implement techniques that quantify phenotypic variation, including pigmentation (Saleh Ziabari & Shingleton 2017), in more detail.

Here, we characterize different aspects of pigmentation in *Drosophila*, encompassing color and color pattern in different body parts and explore environmental and genetic effects on each of these. To do so, we developed a method to quantify body pigmentation components and analyzed these components in two different genetic backgrounds of *D. melanogaste*r, natural populations of the same species collected along a latitudinal cline and, finally, for five additional *Drosophila* species. We discuss our results in the context of the potential evolutionary and developmental independence of different pigmentation components.

RESULTS

We developed a method to quantify five pigmentation traits (Figure 2.1) and analyzed how these components, and the associations between them, vary between body parts, sexes and temperatures in two genetic backgrounds of *D. melanogaster* (Figure 2.2). We attempted to determine the stages of development that respond plastically to the environment (i.e. the windows of sensitivity for plasticity) (Figure 2.3). We then studied patterns of variation in pigmentation in natural populations of *D. melanogaster* which were collected along a latitudinal cline (Figure 2.4) and, finally, for five additional *Drosophila* species (Figure 2.5).

Associations between components of *Drosophila* body pigmentation.

We used a quantitative method to define five distinct pigmentation components (see Materials and Methods) and analyzed how these vary

between body parts, in females and males of two genetic backgrounds of *D. melanogaster* (CantonS and OregonR) that were reared at either 17°C or 28°C. These five components encompass different aspects of the pigmentation phenotype that are related to color and color pattern (see Figure 2.1 for examples and Materials and Method for detailed description of the traits).

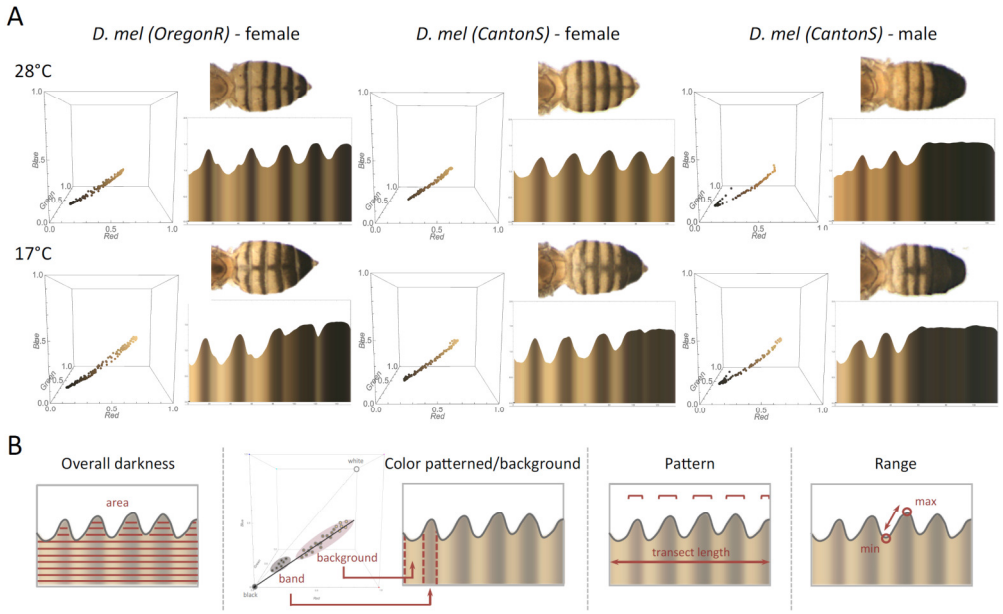


Figure 2.1. Quantitative analysis of body pigmentation. A. Abdomens from females and males from two *D. melanogaster* genetic backgrounds (OregonR and CantonS) reared at 17°C (upper panel) or 28°C (lower panel). For each pixel in the transect we extracted RGB values that are represented in the RGB plots (cube plots on the left side of each fly). By calculating the distance of each of those pixels to the black (see Materials and Methods) we converted the RGB vectors into two dimensional information and represented the distance of each pixel (Y axis) from the anterior to the posterior extremes of the transect (X axis) (plots below abdomens). **B.** Diagram showing the different pigmentation traits. From left to right, overall darkness (Odk), the color of the pattern element (Cpa), the color of the background (Cbk), the pattern (Pat) and the range (Ran).

We assessed the relationship between pigmentation components by estimating Pearson correlations (significance level 0.05). Globally, there were few significant correlations and those were dependent on the genetic background, the sex and the temperature analyzed (Figure 2.2A).

Pigmentation traits were more correlated with each other within a given body part than between body parts and trait correlations were stronger for abdominal female traits.

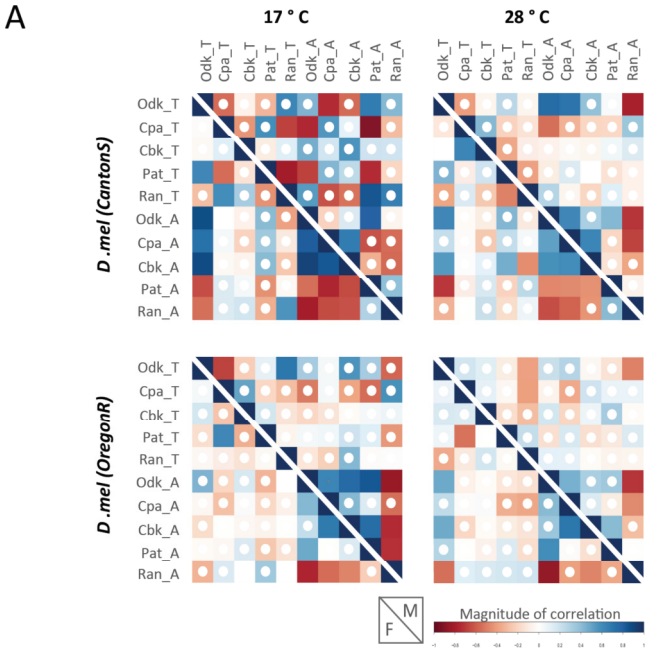
Correlations between traits were dependent on the genetic background; pigmentation traits were more integrated (i.e. more and stronger correlations) in CantonS when compared to OregonR. For a given genetic background, the relationship between pigmentation components also differed between sexes. For instance, female pigmentation traits showed stronger correlations than males in CantonS.

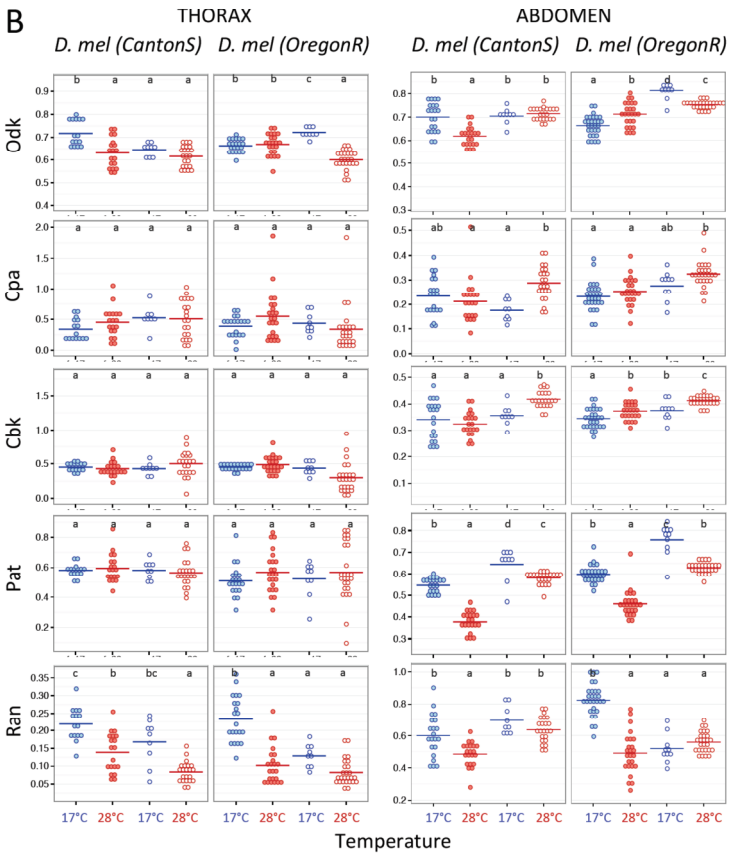
The temperature experienced during development also affected the correlations between traits, with higher temperature decreasing the strength and number of significant correlations. Interestingly, in some cases the effect of temperature was not found only in the magnitude of the correlation but also in the direction. For instance, patterning and band color in thoraxes of OregonR females was positively correlated at 17°C while negatively at 28°C.

Variation in components of body color: effects of genotype, environment and genotype-by-environment

Using flies from two genetic backgrounds (OregonR and CantonS), reared at either 17°C or 28°C, we assessed how the five pigmentation components (see Figure 2.1 and Materials and Methods) vary between genotypes, sexes and temperatures (Figure 2.2B, Table 2.S1 for the results of the model). We found significant differences between genetic backgrounds for most of the abdominal traits, but not for the thoracic ones. Differences between sexes were evident in all abdominal pigmentation traits. Here, sexual dimorphism was largest for pattern (Pat) and this was presumably determined by the presence of darker and wider bands in males. In the thorax, only overall darkness (Odk) and range (Ran) were sexually dimorphic.

We demonstrated that thermal plasticity for pigmentation in *Drosophila* compromises responses of all five components and that all of these, in their


A
17 ° C
28 ° C
Odk_T
Cpa_T
Cbk_T
Pat_T
Ran_T
Odk_A
Cpa_A
Cbk_A
Pat_A
Ran_A
D .mel (CantonS)
D .mel (OregonR)
M
F
Magnitude of correlation


B
THORAX
ABDOMEN
D. mel (CantonS)
D. mel (OregonR)
Odk
Cpa
Cbk
Pat
Ran
17°C
28°C
Temperature

own way, contributed to darker pigmentation phenotypes at lower temperatures. This finding held true for both sexes, although the extent of plasticity was higher in females than in males (estimated by the differences in mean values between temperatures). We also found differences in plasticity between body parts, with most abdominal traits showing a significant response to variation in developmental temperature while such responses where only observed for overall darkness and range in the thorax.

Overall, comparisons between pigmentation components across body parts revealed that abdominal traits have higher degree of sexual dimorphism and higher plasticity than thoracic ones. Indeed, most thoracic traits do not differ between genetic backgrounds, sexes and rearing temperatures. Comparison between pigmentation components across genetic backgrounds revealed that color traits were less variable than patterning ones between genetic backgrounds, sexes and environments.

In some cases, we found genotype-specific differences in pigmentation traits between sexes and/or temperatures. For instance, thoracic pigmentation is plastic in females of CantonS while it is not thermo-sensitive in OregonR. Similarly, darkness in abdominal pigmentation is plastic in both genetic backgrounds but the direction of the response goes in opposite directions.

Figure 2.2. Variation and co-variation in pigmentation traits with genetic background, sex and temperature in *D. melanogaster*. A. Heat map of Pearson's correlation coefficients for all pigmentation traits in abdomens and thoraxes of CantonS (upper panel) and OregonR (lower panel) of flies reared at 17°C or 28°C. For each matrix, females are in the left corner and males in the right. Positive correlations are denoted in blue and negative correlations in red. Non-significant correlations (p-value > 0.05) are indicated with a white dot. **B.** Pigmentation trait values in females (open circles) and males (closed circles) reared at 17°C (blue) or 28°C (red) degrees. The bar represents the mean value of all individuals per sex and temperature. We tested for the effect of sex and temperature on the trait using the model lm (*Trait ~ Sex *Temperature).* Results of the *post-hoc* multiple comparisons using the Tukey honest significance test are indicated in the figure with letters (p-value < 0.05).

The sensitive period for pigmentation plasticity differs between traits and genetic backgrounds

We investigated the thermally sensitive stages of development for pigmentation plasticity by characterizing the impact of changing the rearing temperature at different stages of development. For that, we exposed developing flies from OregonR and CantonS to 17℃ or 28℃ during one window of development while keeping them at 23℃ for the remaining stages. We tested four different treatments at 17℃ and at 28℃ (see

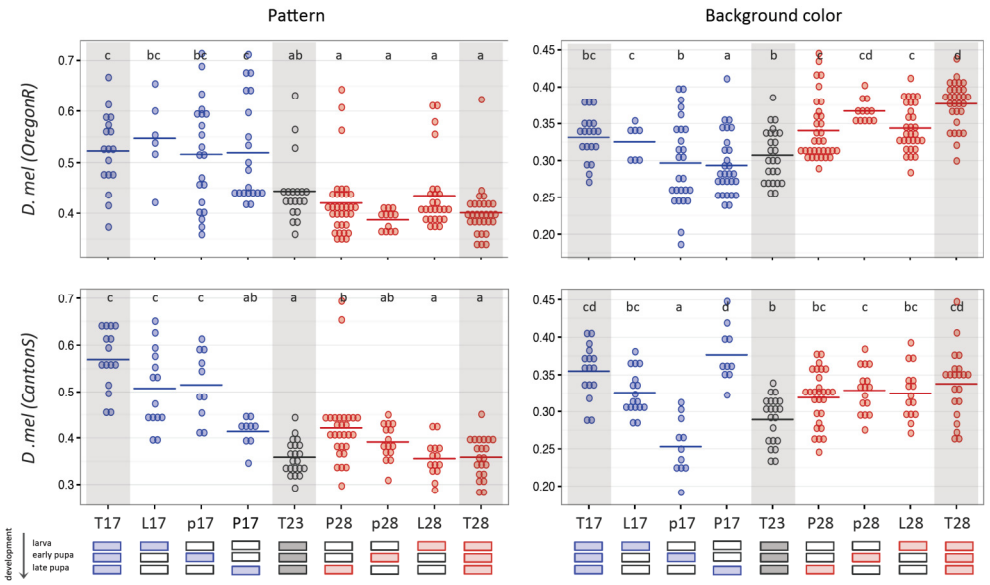


Figure 2.3. Windows of sensitivity for abdominal pigmentation plasticity in *D. melanogaster*. Pigmentation traits (Y axis) pattern (left plots) and background color (right plots) in females of two *D. melanogaster* genetic backgrounds (OregonR and CantonS) exposed to each of the treatments (X axis). The treatment codes and corresponding stages that were exposed to either 17℃ or 28℃ degrees 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 individual females, and the horizontal bar is the mean of those values. We tested for the effect of genotype and treatment and found a significant effect. We then tested for the effect of treatment on each genetic background independently using the model lm *(Trait ~Treatment).* Results of the *post-hoc* multiple comparisons using the Tukey honest significance test are indicated in the figure with letters (p-value > 0.01).

Material and Methods) and found that that all five pigmentation traits were affected by genotype and treatment (Table 2.S2) and that the window of thermal sensitivity differed between pigmentation traits and between genetic backgrounds (Figure 2.3). For instance, pattern in OregonR was affected by treatments at all developmental stages tested while in CantonS it seems to be determined by larval and early pupal stages, but not by the late pupal stage. On the other hand, the color of the background in OregonR was only affected by early pupal treatment and in CantonS did not show significant differences in flies reared at 17°C or 28°C. Accordingly, none of the thermal treatments seem to affect this trait, with the exception of early pupal treatment at 17°C, which showed a very extreme response, with flies being lighter than in any other treatment.

Body pigmentation differences between *Drosophila* populations

We explored the patterns of variation in pigmentation components in three natural populations, from Finland, Austria and Spain that together represent a latitudinal cline in Europe (Figure 2.4). For five different genetic backgrounds from each of those locations, we analyzed our pigmentation components in female flies reared at either 17°C or 28°C. We found differences between genetic backgrounds and rearing temperatures for all pigmentation components in each of the three locations. We also found that most pigmentation components and plasticity therein differed between locations, with the exception of color traits (i.e. color of pattern element and color of the background) (Figure 2.4A). At 17°C, flies were darker (thoracic darkness and pattern) in the population from Finland, followed by the Spain and Austria populations. However, at 28°C the situation was reversed, with the Finnish population demonstrating lighter components. Analysis of abdominal components revealed that darkness did not differ between populations, while pattern showed higher values in the populations from Finland in each of the two temperatures.

Body pigmentation differences between *Drosophila* species

We analyzed pigmentation components in five *Drosophila* species (*D. simulans, D. malerkotliana, D. repleta*, *D. mojavensis baja* and *D. mojavensis mojavensis)* that show interspecific variation in body coloration (see Figure 2.4A for an example). For instance, *D. simulans* and *D. malerkotliana* are phenotypically more similar to *D.* melanogaster than the other species, with abdomens showing a pattern of dark bands on a light background and the thoracic pigmentation is characterized by the presence (and darkness) of the trident. *D. repleta*, *D. mojavensis baja* and *D. mojavensis mojavensis,* which are closely related species, have heavily melanized dorsal thoraxes and patterned abdomens. However, both the shape and color of abdominal bands and background in the abdomens, of these three species, was is different from the ones found in *D. melanogaster, D. simulans* and *D. malerkotliana* (Figure 2.5A).

Quantification of the pigmentation components in the *Drosophila* species revealed clear differences between species (Figure 2.S1, Table 2.S3). Similarly to what was found for *D. melanogaster* pigmentation components differ between sexes and temperatures (Table 2.S3). Pigmentation components show divergent responses when we looked at differences between sexes and rearing temperature (Figure 2.4B). Comparison of Cohen's D coefficient shows that the direction and magnitude of the sexual response differs between traits and species (Figure 2.4C). The largest differences were found between sexes, were most abdominal traits show sexual dimorphism. Pattern and background color in the abdomens of *D. melanogaster* and *D. simulans* show a much stronger effect of sex than in all other species. Interestingly, different traits seem to contribute to the phenotypic differences between females and males in the various *Drosophila* species (Figures 2.4B, 2.4C, 2.S1 and 2.S2).

A

Trait	Effect of Location (Cline) 17°C Thorax	Effect of Location (Cline) 17°C Abdomen	Effect of Location (Cline) 28°C Thorax	Effect of Location (Cline) 28°C Abdomen	Effect of Location*Temperature Thorax	Effect of Location*Temperature Abdomen
Odk	(F=S)>A	F=A=S	F<(A=S)	F=A=S	(F<A)=S	F=A=S
Pat	(F>A)=S	(F=A)>S	F=(A>S)	F>(A=S)	F=A=S	(F<A)=S
Cbk	F=A=S	F=A=S	F=A=S	(F=A)>S	F=A=S	(F=A)<S
Cba	F=A=S	F=A=S	F=A=S	F>A>S	F=A=S	(F=A)<S
Ran	F<S<A	(F=S)<A	(F=S)<A	(F>A)=S	F<(A=S)	(F=S)<A

B

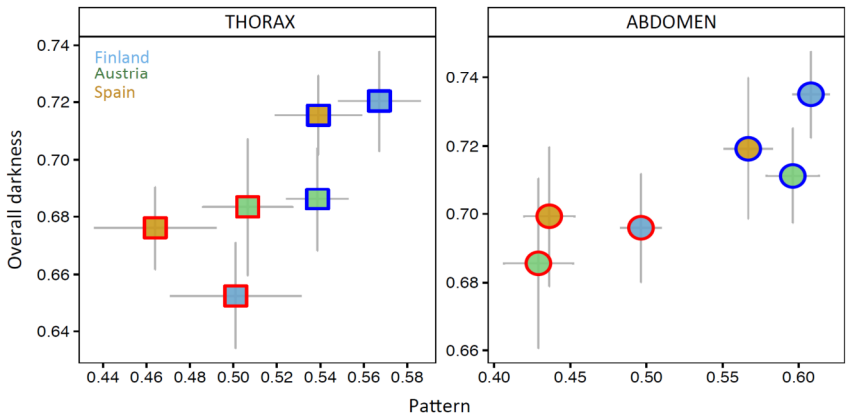


Figure 2.4. Variation in pigmentation between *D. melanogaster* populations. A. Differences in pigmentation components between populations from the three locations: Finland (F), Austria (A) and Spain (S). Significant (p-value < 0.01) differences in traits (columns under "Effect of Location") and in plasticity of the traits between locations (columns under "Effect of Location*Temperature) are shown in black. Non-significant effects are shown in grey. The models tested were lm *(Trait ~ Location)* for within-environment comparisons and lm *(Trait ~ Location * Temperature)* for plasticity comparisons. In all cases, significant differences among groups were estimated by post hoc comparisons (Tukey's honest significant differences) and are indicated by "<" and ">" symbols, depending on the direction of the difference. **B.** Means and confidence intervals of darkness (Odk; Y axis) and pattern (Pat; X axis) in thoraxes and abdomens of females from the three European populations (dot filling represent the location: Finland in blue, Austria in green and Spain in orange), reared at two temperatures (dot outline represents the temperature: 17℃ in and 28℃ in red).

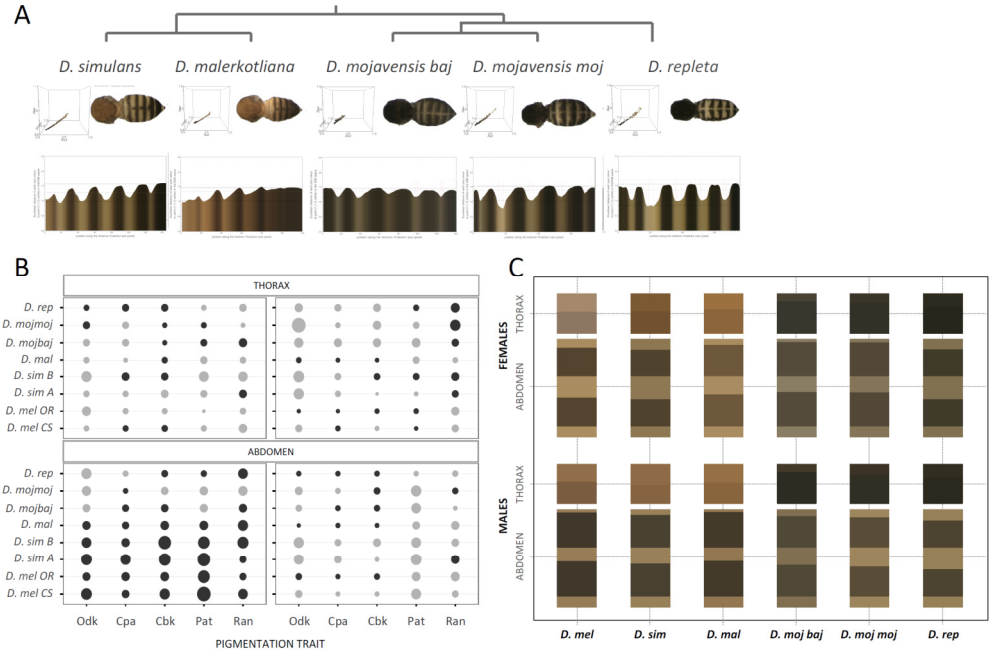


Figure 2.5. Inter specific variation in pigmentation. A. Example of abdominal pigmentation in females, RGB (left) and AP plots (below) in females of five *Drosophila* species: *D. simulans, D. malerkotliana*, two subspecies of *D. mojavensis* (*D. mojavensis baja* and *D. mojavensis mojavensis*) and *D. repleta*. **B.** The effect size (Cohen's D coefficient) of sex (left panel) and temperature (right panel) on the thoracic and abdominal pigmentation traits of the different *Drosophila* species. Cohen's D coefficient for the effect of sex or temperature is represented by the size of the dots for each species and trait. Black and grey dots represent positive and negative coefficients, respectively. **C.** Schematic representation of the thoracic and abdominal pigmentation in females and males of *Drosophila* species**.** The size of the boxes represents the size of the patterning relative to the/background and the color is the mean R, G, B value for each sex and species.

Temperature also affects pigmentation components in the different *Drosophila* species. In the thorax, variation in rearing temperature leads to larger differences in overall darkness and range while in the abdomen it is the pattern that shows the strongest response to temperature (Figures 2.4B and 2.4C).

DISCUSSION

Pigmentation is, as a visually compelling trait that is evolutionary diverse, ecologically relevant and developmentally tractable. Insects show a fascinating diversity of body colors and this variation is striking at both the inter- and intra-specific level. Variation in pigmentation between genotypes, populations and species, can be caused by differences in color composition and/or color pattern that occur across the entire body and/or in specific body regions. These components of body pigmentation can respond in different manners to internal and external factors, and consequently have the potential to develop and evolve more or less independently.

Decomposing phenotypic variation in *Drosophila* pigmentation

The lack of quantitative methods for phenotyping has led to an oversimplification of complex phenotypes that consist of different traits that can develop and evolve more or less independently. We show that the patterns of variation we observe between sexes, species or environments are ultimately the outcome of different traits, some related with pattern, such as the degree of banding in the abdomens, but also differences in the actual colors of these patterns (i.e. the color of the pigment that is deposited in the cuticle). By decomposing the pigmentation phenotype into different traits we were able to disentangle the extent and direction of the response of those components to internal and external cues. Analysis of the variation in pigmentation traits showed that i) pigmentation components respond in different ways to the same factor (for example, sex or genetic background) and ii) that different factors affect a given trait in different ways. For instance, abdominal traits in *Drosophila* were overall more responsive to sexual and environmental differences than thoracic traits.

Correlations between pigmentation components differ between genotypes, sexes and environmental conditions. The fact that trait associations, such as trade-offs, are dependent on genetic and environmental factors, could potentially influence adaptation (e.g. Marquez &

Knowles 2007; Manenti *et al.* 2016). This was previously shown for example, in the case of a thermally-driven switch in the association between longevity and body size in *D. melanogaster* (Khazaeli, Vanvoorhies & Curtsinger 2005). We found that correlations between pigmentation components were stronger within abdomens which can be suggestive of a tighter link between the developmental networks of traits in this body part. Overall, the divergent responses to genetic and environmental factors that we found in the pigmentation components and in their associations are suggestive of a complex scenario in which the final phenotype is determined by trait-specific fine-tuning, eventually allowing them to evolve independently.

Windows of sensitivity

For the environment to affect development, external environmental cues need to be sensed and these signals need to be transmitted to the developing tissues where changes in, for example, gene expression will alter developmental processes, leading to the production of alternative phenotypes from the same genotype. Differences in plasticity between populations and/or species can affect any of the steps in this cascade. Here we have explored whether one of the ways in which different genetic backgrounds show differences in plasticity is by having different periods of sensitivity to temperature. Indeed, we see that not only the sensitive period varies between genetic backgrounds but also that different pigmentation components react to temperature at different time points. For instance, for OregonR, all treatments at low temperatures that individuals were exposed to, resulted in phenotypes resembling those of individuals that spent all of development at 17ºC. On the other hand, in CantonS only treatments during early developmental stages had such an effect.

Because development is organized in internally fixed successive stages, that are contingent on previous steps, and dependent on the environment in which those occur (Lewontin 2000), these different windows of sensitivity to temperature have the potential to explain some of the

differences in plasticity found between traits and genotypes. It is yet unclear to what extent pigmentation plasticity in flies requires an active sensing of temperature or whether it is a more indirect effect. Environmental responses of both types have been described in flies and other organisms (see DeWitt & Scheiner 2004). A better understanding of the plastic responses should involve characterizing the genetic and molecular mechanisms underlying variation in plasticity in natural populations.

Clinal variation in *D. melanogaster* pigmentation

Our analysis of the pigmentation patterns from populations of a European cline (from Finland, Austria and Spain), showed darker pigmentation in the most northern population (i.e. Finland). This follows the expectation of the thermal melanism hypothesis whereby darker individuals tend to occur in areas with lower temperatures and lower solar radiation (Clusella Trullas *et al.* 2007; Clusella-Trullas & Terblanche 2010). However, the differences in pigmentation between Austria and Spain populations do not follow this. Because the sampling locations of our populations differ also in altitude (Spain>Austria>Finland), which is also associated with pigmentation in *D. melanogaster* (Pool & Aquadro 2007), it is possible that the combination of both latitude and altitude), with its corresponding ecological conditions, might lead to different pigmentation patterns.

Diversification of *Drosophila* pigmentation

Attempts to understand the general principles that govern adaptation and diversification of species have mainly focused on the genetics underlying phenotypic variation. Here we explored this problematic by characterizing variation at the phenotypic level between *Drosophila* species. From the species used in this study, *D. melanogaster, D. simulans* and *D. malerkotliana* belong to the melanogaster group, which originally inhabited tropical climates, though they have become cosmopolitan species. In contrast, *D. mojavensis and D. repleta* belong to the repleta group and which

inhabits desert climates. We explored whether the same type of responses found in *D. melanogaster* (e.g. sexual differences), are found in other *Drosophila* species and whether traits are affected in a similar way to genetic and environmental effects. We observed that the traits respond to sex and temperature in a species-specific manner. Altogether, these results suggest that different pigmentation traits contribute to the differences found between species and between sexes and those are also dependent on the body part. Overall, abdominal pigmentation traits show lower degree of sexual dimorphism in desert species while the level of plasticity is similar to the ones in cosmopolitan species (assessed by the effect size). However, thoracic traits in *D. mojavensis* subspecies seem to be more affected by temperature.

All together, the patterns of variation in pigmentation components and their responses to genetic and environmental factors are suggestive of, at least some degree, of phenotypic independence between traits which might have influenced the evolution and diversity of body coloration in *Drosophila*.

MATERIAL AND METHODS

Fly stocks

D. melanogaster genetic backgrounds *CantonS* and *OregonR* and *Drosophila* species *D. simulans, D. malerkotliana, D. repleta*, *D. mojavensis baja* and *D. mojavensis mojavensis* were obtained from C. Mirth's lab. *D. melanogaster* lines from populations of Finland, Austria and Spain are part of the collections from the DrosEU consortium (www.droseu.net) and were obtained from E. Sucena's lab.

All stocks were maintained in molasses food (45 gr. molasses, 75gr sugar, 70gr cornmeal, 20 gr. Yeast extract, 10 gr. Agar, 1100 ml water and 25 ml of Niapagin 10%) in incubators at 25ºC, 12:12 light cycles and 65% humidity until used in this study. For the experiments, we performed over-night egg-laying from ~20 females of each stock in vials with *ad libitum*

molasses food. Eggs were then placed at either 17°C or 28°C throughout development. We controlled population density by keeping between 20 and 40 eggs per vial.

For the experiment of the windows of sensitivity for pigmentation, we exposed developing flies to 17°C or 28°C during one window of development while kept at 23°C for the remaining stages. We tested four different treatments at 17°C and at 28°C: T (flies always kept at constant temperature), L (late larval development; staging done by using traqueal and the mouth hooks 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).

Phenotyping pigmentation components

Adult flies (8-10 days after eclosion) were placed in 2 ml Eppendorfs and frozen in liquid nitrogen. The tubes were shaken immediately after submersion in liquid nitrogen to remove wings, legs and bristles. Bodies of female flies were then mounted on 3% Agarose in Petri dishes, dorsal side up, and covered with water to avoid light reflection upon imaging. Images containing 10 to 20 flies were collected with a LeicaDMLB2 stereoscope and a Nikon E400 camera under controlled imaging conditions of light, contrast, and white-balance. Images were later processed with a customize Mathematica macro to extract pigmentation measurements. For this purpose, two transects were drawn for each fly, one in the thorax and one in the abdomen, using body landmarks (as shown in Figure 2.S1A) and extracted RGB (Red, Blue, Green) values from each pixel along the transects. For abdominal transects, when necessary, another step was performed and involved the removal of the pixels corresponding to the membranous tissue that occasionally is visible between abdominal segments. Using the customized Mathematica macro, RGB values were extracted from every pixel along the transect and these were used to define each of the five pigmentation components as follows. Overall darkness

(Odk) was calculated as the sum of the Euclidean distances of each pixel to black divided by the number of pixels. Color of the pattern element (Cpa) is the angle between the best-fitted line going through the pixels that correspond to the pattern element (trident in the thorax and darker bands in the abdomen) in the transect and the grey vector (a constant diagonal in the RGB space). Similarly, color of the background (Cbk) was calculated as the angle between the best-fitted line that goes through the background pixels in the transect and the grey vector. Pixels corresponding to pattern element and/or background were defined by dividing all RGB values in the transect into two clusters each containing 95% of the light or dark pixels respectively. Pattern (Pat) was extracted by calculating the proportion of pixels corresponding to the pattern element (thoracic trident and/or darker abdominal bands) relative to the transect length, being the number of pixels corresponding to the pattern element those above a threshold defined by an adjusted median line throughout all pixels. Range (Ran) was calculated as the Euclidean distance between the median value for the 20 darkest and the 20 lightest pixels along the transects.

Statistical analyses

All statistical analyses were performed with R Statistical Package v 3.1.1 (R Development Core Team 2014). To assess whether parametric test could be performed for the analysis of data, the underlying assumptions of normality and homogeneity of variances among samples were checked by using the Shapiro-Wilk test and Bartlett's test, respectively. Pearson's correlations (confidence $\alpha = 0.95$) were used to check correlations between traits and across temperatures. Linear regression models were used to test for the effect of genotype, sex, temperature and interaction terms (model lm *(Trait ~ Genotype*Sex*Temperature)* on each pigmentation trait of the two genetic backgrounds of *D. melanogaster.* A similar analysis was performed for the data on the sensitive stages of development, testing for the effects of genotype and treatment (model lm *(Trait ~ Genotype*Treatment))*, and for

the comparisons between Drosophila species, testing for the effects of species, sex and temperature (model lm *(Trait ~ Species *Sex*Temperature))*. Similarly, for the European populations, we used we used linear models (for each body part and pigmentation component), to test for the effect of genetic background, location and temperature. The models tested were lm *(Trait ~ Location)* for within-environment comparisons and lm *(Trait ~ Location*Temperature)* for plasticity comparisons. Post hoc multiple comparisons to identify differences between genotypes, sexes, temperatures and/or species were done using *post-hoc* Tukey's honest significant differences (Tukey HSD). Cohen's D coefficient was calculated using the R package *effsize* and Hedges' correction was applied to control for differences in sample sizes. The size of effect of temperature and/or sex on each trait was calculated independently for every genotype or species.

AUTHOR CONTRIBUTIONS

Elvira Lafuente and Patrícia Beldade conceived and designed the experiments; Elvira Lafuente, Carolina M Peralta and Jessica King performed the experiments, Elvira Lafuente and Carolina Peralta analyzed the data. Elvira Lafuente and Patrícia Beldade wrote the manuscript.

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SUPPLEMENTARY MATERIAL

Figure 2.S1. Variation in abdominal pigmentation traits with genetic background, sex and temperature in *Drosophila* species.

Figure 2.S2. Variation in thoracic pigmentation traits with genetic background, sex and temperature in *Drosophila* species.

Table 2.S1. Effect of genotype, sex and temperature on pigmentation components in *D. melanogaster.*

Table 2.S2. Effect of genotype and thermal treatment on abdominal pigmentation components in *D. melanogaster*.

Table 2.S3. Effect of species, sex and temperature on pigmentation components of five *Drosophila* species.

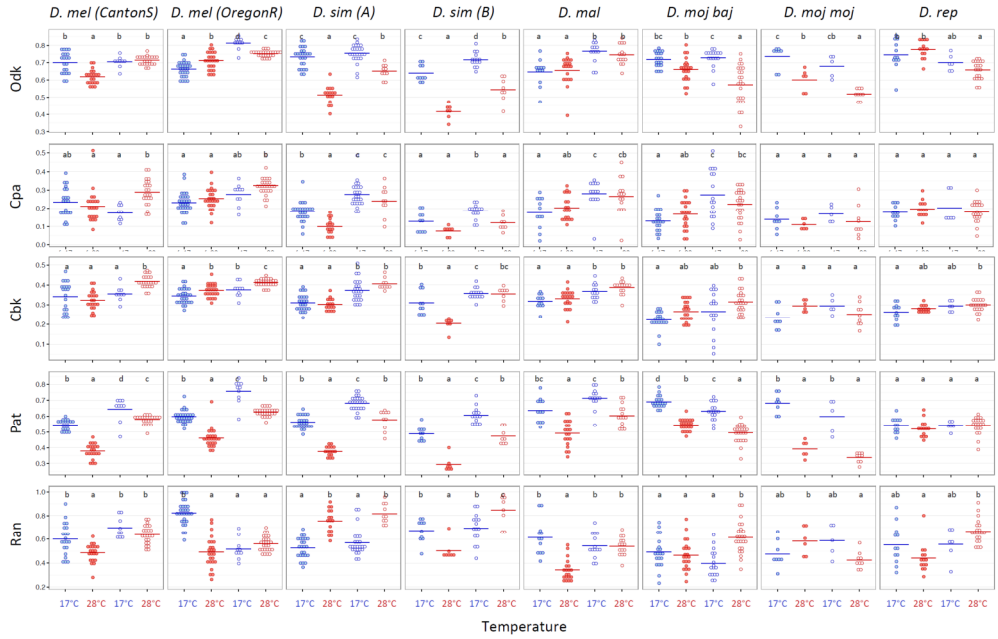


Figure 2.S1. Variation in abdominal pigmentation traits with genetic background, sex and temperature in *Drosophila* species. Pigmentation trait values in females (open circles) and males (closed circles) reared at 17℃ (blue) or 28℃ (red) degrees. The bar represents the mean value of all individuals per sex and temperature. We tested for the effect of sex and temperature on each trait Results of the *post-hoc* multiple comparisons using the Tukey honest significance test (significance 0.95) are indicated in the figure with letters.

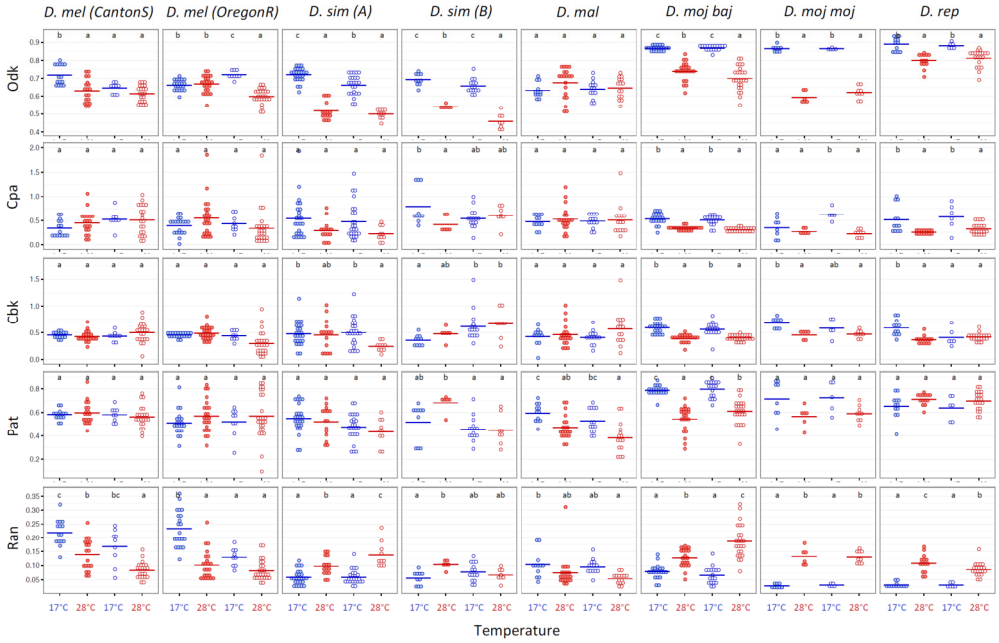


Figure 2.S2. Variation in thoracic pigmentation traits with genetic background, sex and temperature in *Drosophila* species. Pigmentation trait values in females (open circles) and males (closed circles) reared at 17℃ (blue) or 28℃ (red) degrees. The bar represents the mean value of all individuals per sex and temperature. We tested for the effect of sex and temperature on each trait Results of the *post-hoc* multiple comparisons using the Tukey honest significance test (significance 0.95) are indicated in the figure with letters.

Table 2.S1. Effect of genotype, sex and temperature on pigmentation components in *D. melanogaster*

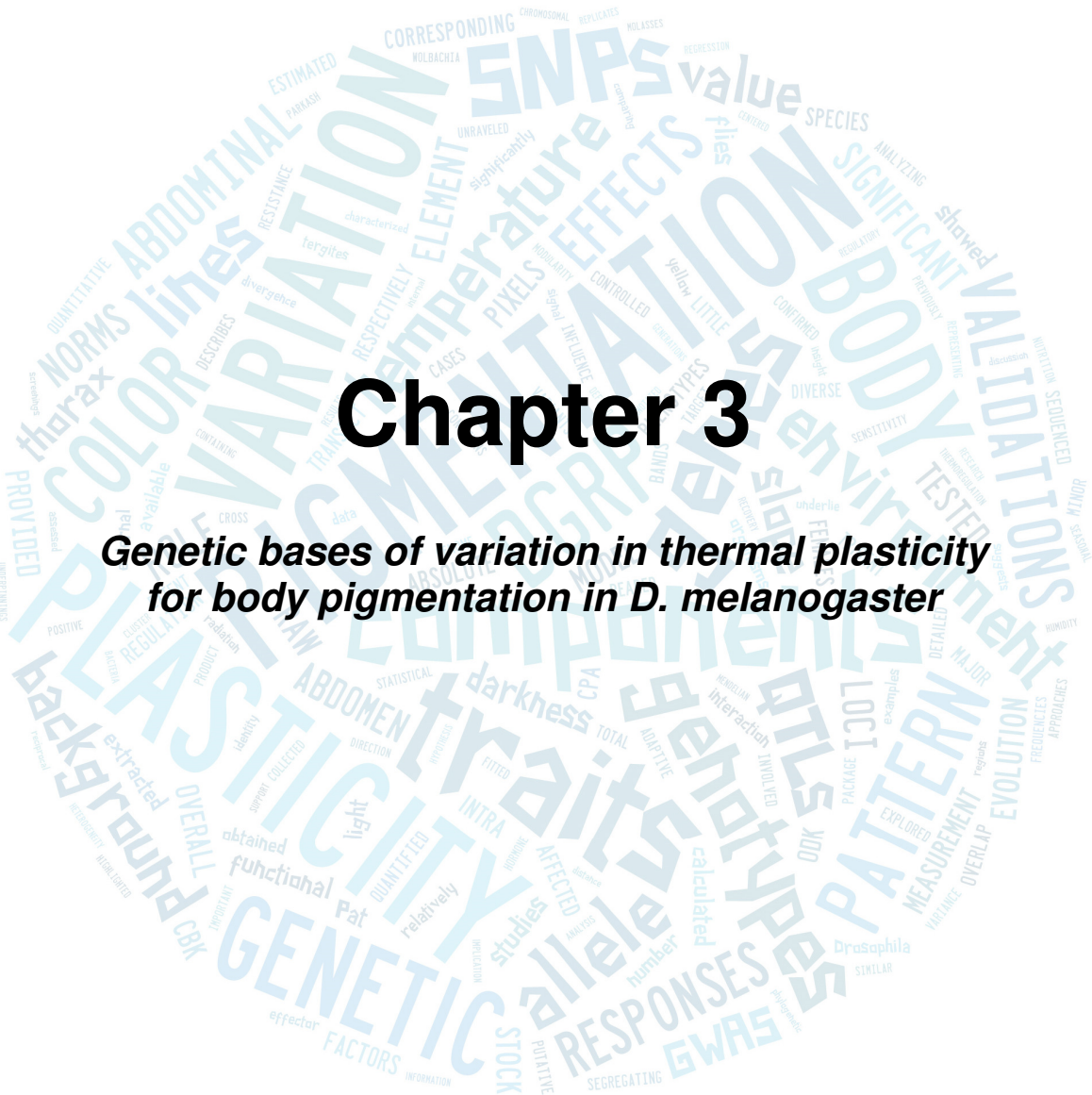
aov (trait ~ genotype*sex*temperature)		ABDOMEN						THORAX					
Trait	**Factor**	Df	Sum Sq	Mean Sq	F value	Pr(>F)		Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Odk	genotype	1	0,058	0,058	33,95	3,11E-08	***	1	0,000	0,000	0,14	0,71170	
	sex	1	0,190	0,190	112,01	<2,00E-16	***	1	0,054	0,054	25,54	0,00000	***
	temperature	1	0,009	0,009	5,37	0,0218	*	1	0,087	0,087	40,84	0,00000	***
	genotype:sex	1	0,009	0,009	5,50	0,0203	*	1	0,002	0,002	0,72	0,39810	
	genotype:temperature	1	0,030	0,030	17,46	0,0000486	***	1	0,003	0,003	1,64	0,20260	
	sex:temperature	1	0,002	0,002	1,42	0,235		1	0,013	0,013	6,31	0,01320	*
	genotype:sex:temperature	1	0,086	0,086	50,68	3,67E-11	***	1	0,067	0,067	31,42	0,00000	***
	Residuals	157	0,267	0,002				136	0,289	0,002			
Cpa	genotype	1	0,044	0,044	10,15	0,001744	**	1	0,055	0,055	0,31	0,58190	
	sex	1	0,112	0,112	25,79	0,00000107	***	1	0,081	0,081	0,45	0,50500	
	temperature	1	0,034	0,034	7,92	0,005509	**	1	0,004	0,004	0,02	0,88680	
	genotype:sex	1	0,012	0,012	2,73	0,10036		1	1,026	1,026	5,65	0,01890	*
	genotype:temperature	1	0,000	0,000	0,00	0,968356		1	0,004	0,004	0,02	0,88460	
	sex:temperature	1	0,051	0,051	11,68	0,000804	***	1	0,207	0,207	1,14	0,28790	
	genotype:sex:temperature	1	0,026	0,026	5,95	0,015837	*	1	0,049	0,049	0,27	0,60400	
	Residuals	157	0,682	0,004				136	24,707	0,182			
Cbk	genotype	1	0,008	0,008	4,71	0,03154	*	1	0,017	0,017	0,34	0,56000	
	sex	1	0,121	0,121	69,81	3,25E-14	***	1	0,016	0,016	0,32	0,57300	
	temperature	1	0,019	0,019	10,85	0,00122	**	1	0,001	0,001	0,02	0,89900	
	genotype:sex	1	0,006	0,006	3,61	0,05936		1	0,123	0,123	2,43	0,12100	
	genotype:temperature	1	0,003	0,003	1,81	0,18041		1	0,002	0,002	0,04	0,84000	
	sex:temperature	1	0,015	0,015	8,77	0,00354	**	1	0,000	0,000	0,00	0,96600	
	genotype:sex:temperature	1	0,011	0,011	6,44	0,01217	*	1	0,067	0,067	1,33	0,25100	
	Residuals	157	0,272	0,002				136	6,885	0,051			
Pat	genotype	1	0,179	0,179	84,79	<2,00E-16	***	1	0,038	0,037	2,46	0,11900	
	sex	1	0,696	0,696	329,13	<2,00E-16	***	1	0,000	0,000	0,00	0,96700	
	temperature	1	0,655	0,655	309,69	<2,00E-16	***	1	0,030	0,030	1,95	0,16500	
	genotype:sex	1	0,000	0,000	0,02	0,87699		1	0,011	0,011	0,70	0,40500	
	genotype:temperature	1	0,000	0,000	0,20	0,6539		1	0,024	0,024	1,58	0,21100	
	sex:temperature	1	0,023	0,023	10,66	0,00135	**	1	0,004	0,004	0,24	0,62800	
	genotype:sex:temperature	1	0,019	0,019	9,18	0,00286	**	1	0,001	0,001	0,04	0,83700	
	Residuals	157	0,332	0,002				136	2,069	0,015			
Ran	genotype	1	0,036	0,036	3,70	0,05624	.	1	0,003	0,003	1,31	0,25460	
	sex	1	0,010	0,010	0,98	0,32346		1	0,166	0,166	69,92	0,00000	***
	temperature	1	0,909	0,909	94,31	<2,00E-16	***	1	0,272	0,272	114,63	<2,00E-16	***
	genotype:sex	1	0,506	0,506	52,51	1,82E-11	***	1	0,000	0,000	0,02	0,90180	
	genotype:temperature	1	0,106	0,106	10,99	0,00114	**	1	0,002	0,002	1,01	0,31600	
	sex:temperature	1	0,459	0,459	47,62	1,2E-10	***	1	0,014	0,014	6,02	0,01540	*
	genotype:sex:temperature	1	0,216	0,216	22,41	0,00000488	***	1	0,015	0,015	6,40	0,01250	*
	Residuals	157	1,514	0,010				136	0,323	0,002			

Table 2.S2. Effect of genotype and thermal treatment on abdominal pigmentation components in *D. melanogaster*

Trait	Factor	Sum Sq	Mean Sq	F value	Pr(>F)	
Odk	genotype	0,00	0,00	1,02	0,3130	
	treatment	1,25	0,16	40,31	<2,00E-16	***
	genotype:treatment	0,22	0,03	6,96	0,0000	***
	Residuals	1,35	0,00			
Cba	genotype	0,09	0,09	26,16	0,0000	***
	treatment	0,63	0,08	22,36	<2,00E-16	***
	genotype:treatment	0,17	0,02	6,15	0,0000	***
	Residuals	1,23	0,00			
Cbk	genotype	0,01	0,01	3,93	0,0482	*
	treatment	0,20	0,02	17,24	<2,00E-16	***
	genotype:treatment	0,10	0,01	8,98	0,0000	***
	Residuals	0,49	0,00			
Pat	genotype	0,08	0,08	17,32	0,0000	***
	treatment	1,07	0,13	28,65	0,0000	***
	genotype:treatment	0,17	0,02	4,42	0,0000	***
	Residuals	1,46	0,00			
Ran	genotype	1,73	1,73	101,40	<2,00E-16	***
	treatment	4,94	0,62	36,20	<2,00E-16	***
	genotype:treatment	0,45	0,06	3,33	0,0011	**
	Residuals	5,92	0,02			

Table 2.S3. Effect of species, sex and temperature on pigmentation components of five *Drosophila* species

aov (trait ~ specie*sex*temperature)		ABDOMEN						THORAX					
Trait	**Factor**	Df	Sum Sq	Mean Sq	F value	Pr(>F)		Df	Sum Sq	Mean Sq	F value	Pr(>F)	
Odk	genotype	5	0,524	0,105	28,46	<2,00E-16	***	5	2,218	0,444	214,73	<2,00E-16	***
	sex	1	0,021	0,021	5,76	0,0170	*	1	0,075	0,075	36,14	0,0000	***
	temperature	1	0,334	0,334	90,78	<2,00E-16	***	1	0,739	0,739	357,70	<2,00E-16	***
	genotype:sex	5	0,609	0,122	33,09	<2,00E-16	***	5	0,023	0,005	2,21	0,0536	
	genotype:temperature	5	0,454	0,091	24,63	<2,00E-16	***	5	0,563	0,113	54,53	<2,00E-16	***
	sex:temperature	1	0,077	0,077	20,96	0,0000	***	1	0,037	0,037	18,13	0,0000	***
	genotype:sex:temperature	5	0,051	0,010	2,78	0,0178	*	5	0,050	0,010	4,85	0,0003	***
	Residuals	333	1,227	0,004				322	0,665	0,002			
Cpa	genotype	5	0,759	0,152	31,78	<2,00E-16	***	5	1,805	0,361	5,13	0,0002	***
	sex	1	0,319	0,319	66,72	0,0000	***	1	0,150	0,150	2,13	0,1451	
	temperature	1	0,002	0,002	0,33	0,5673		1	0,905	0,905	12,85	0,0004	***
	genotype:sex	5	0,096	0,019	4,01	0,0015	**	5	0,205	0,041	0,58	0,7127	
	genotype:temperature	5	0,061	0,012	2,55	0,0278	*	5	1,140	0,228	3,24	0,0072	**
	sex:temperature	1	0,021	0,021	4,42	0,0362	*	1	0,000	0,000	0,00	0,9985	
	genotype:sex:temperature	5	0,035	0,007	1,48	0,1963		5	0,639	0,128	1,82	0,1095	
	Residuals	333	1,590	0,005				322	22,666	0,070			
Cbk	genotype	5	0,701	0,140	67,52	<2,00E-16	***	5	0,486	0,097	2,64	0,0235	*
	sex	1	0,185	0,185	88,92	<2,00E-16	***	1	0,001	0,001	0,03	0,8537	
	temperature	1	0,023	0,023	10,92	0,0011	**	1	0,306	0,306	8,32	0,0042	**
	genotype:sex	5	0,043	0,009	4,13	0,0012	**	5	0,648	0,130	3,52	0,0041	**
	genotype:temperature	5	0,060	0,012	5,76	0,0000	***	5	1,042	0,208	5,66	0,0001	***
	sex:temperature	1	0,001	0,001	0,44	0,5058		1	0,046	0,046	1,24	0,2668	
	genotype:sex:temperature	5	0,044	0,009	4,25	0,0009	***	5	0,224	0,045	1,22	0,3009	
	Residuals	333	0,691	0,002				322	11,861	0,037			
Pat	genotype	5	0,507	0,101	33,87	<2,00E-16	***	5	2,255	0,451	35,78	<2,00E-16	***
	sex	1	0,168	0,168	56,11	0,0000	***	1	0,022	0,022	1,73	0,1891	
	temperature	1	1,495	1,495	499,39	<2,00E-16	***	1	0,292	0,292	23,16	0,0000	***
	genotype:sex	5	0,783	0,157	52,31	<2,00E-16	***	5	0,267	0,054	4,24	0,0010	***
	genotype:temperature	5	0,308	0,062	20,56	<2,00E-16	***	5	1,151	0,230	18,26	0,0000	***
	sex:temperature	1	0,011	0,011	3,64	0,0574		1	0,002	0,002	0,15	0,6989	
	genotype:sex:temperature	5	0,007	0,001	0,43	0,8268		5	0,081	0,016	1,29	0,2701	
	Residuals	333	0,997	0,003				322	4,059	0,013			
Ran	genotype	5	1,637	0,327	25,73	<2,00E-16	***	5	0,220	0,044	29,39	<2,00E-16	***
	sex	1	0,098	0,099	7,74	0,0057	**	1	0,005	0,005	3,48	0,0630	
	temperature	1	0,337	0,337	26,50	0,0000	***	1	0,010	0,010	6,94	0,0088	**
	genotype:sex	5	0,834	0,167	13,11	0,0000	***	5	0,123	0,025	16,41	0,0000	***
	genotype:temperature	5	1,069	0,214	16,79	0,0000	***	5	0,449	0,090	59,88	<2,00E-16	***
	sex:temperature	1	1,180	1,180	92,73	<2,00E-16	***	1	0,012	0,012	8,23	0,0044	**
	genotype:sex:temperature	5	0,541	0,108	8,50	0,0000	***	5	0,055	0,011	7,29	0,0000	***
	Residuals	333	4,238	0,013				322	0,483	0,002			



Chapter 3

Genetic bases of variation in thermal plasticity for body pigmentation in D. melanogaster

ABSTRACT

Pigmentation is a classical example of adaptive evolution that shows great inter and intra-specific variation. This variation is the product of the combination of pigmentation elements, related with color and/or color pattern, that can be independently regulated and inherited and have divergent responses to environmental factors. We used a natural *D. melanogaster* population (the DGRP) to document effects of genotype, environment and genotype-by-environment in several pigmentation components of two body parts. We then performed genome wide association studies (GWAS) and unravel the genetic basis of variation in plasticity for these pigmentation components. First, we found that different QTLs contribute to variation in plasticity of different components (and body parts). Second, for any given pigmentation component, there is little overlap between loci contributing to variation between and within environments. Third, analyses of the identity of the loci underlying plastic responses, revealed QTL with diverse roles in the environmental regulation of pigmentation development. We then used different approaches to validate selected QTLs. Our results shed light onto the nature of genetic basis of inter-genotype variation for plasticity of pigmentation components. These are the loci that can provide the raw material for the evolution of pigmentation plasticity.

INTRODUCTION

Body pigmentation is a compelling example of morphological diversity and adaptive evolution, including textbook cases such as mimicry (e.g. Nadeau 2016) and/or industrial melanism (Cook & Saccheri 2013). Body coloration also provided examples of genetic dissection of intra and inter-species differences (Gompel *et al.* 2005; Mundy 2005; Hoekstra 2006). Pigmentation can influence thermoregulation, UV protection, predator avoidance (e.g. mimicry, aposematism, camouflage), mate choice (e.g. Llopart, Elwyn & Coyne 2002). In insects is also closely associated to various other traits including behavior and immunity (see Wittkopp & Beldade 2009). Studies in different organisms have provided insights into what shapes the patterns of variation in pigmentation found between species, populations, including seasonal and clinal variation, as well as differences between sexes (e.g. Honěk & Honek 1993; Scharf, Juanes & Rountree 2000; Yom-Tov & Geffen 2006).

Pigmentation diversity includes differences in actual color as well as in how color is distributed in space. It involves genes responsible for the biochemical synthesis of pigments, as well as genes that regulate those to determine where and when pigments are synthesized (see Hoekstra 2006; Wittkopp & Beldade 2009). Different pigmentation elements might differ between body parts and even regions within a given body part and have the potential to develop and evolve independently (e.g. butterfly eyespots coloration and/or different portions of vertebrate coat color; Beldade & Brakefield 2003; Linnen *et al.* 2013). Studies of body pigmentation in different species have provided many important lessons about the reciprocal interactions between evolutionary and developmental processes that shape variation as well as about developmental constraints (Gibert, Moreteau & David 2000) and modularity (Beldade & Brakefield 2003).

Studies of pigmentation have also provided important lessons about the genetic basis of intra-specific variation and inter-specific divergence, including examples of co-option (e.g. Shirai *et al.* 2012), cis-regulatory

evolution (e.g. Rogers *et al.* 2014) and of evolution in coding sequence at hotspot loci (Papa, Martin & Reed 2008). External factors, such as temperature and nutrition, have been shown to interact with the genotype and influence body pigmentation in different species (e.g. McGraw *et al.* 2002; Hansson 2004; Rosenblum 2005), including many insects (e.g. Brakefield *et al.* 1996; Bernardo, Pedata & Viggiani 2007; Ethier *et al.* 2015). Such plasticity in body pigmentation can help organisms cope with environmental heterogeneity (see West-Eberhard 2003; Ghalambor *et al.* 2007). Plasticity in pigmentation and in other traits can be considered as a heritable trait that is variable in populations and thus, can evolve (Scheiner 1993). While we know about some of the genes and hormones that underlie plastic responses (Nijhout 1998; Beldade, Mateus & Keller 2011), we know relatively little about the naturally-segregating alleles responsible for variation in plasticity.

Drosophila is a particularly good system to study the genetic basis of pigmentation components and of plasticity therein. In addition to well-characterized patterns of variation between species, populations and sexes, there is knowledge about the ecological relevance (Rajpurohit, Parkash & Ramniwas 2008; Parkash, Rajpurohit & Ramniwas 2008) and the genetic and developmental underpinnings (Pool & Aquadro 2007; Shakhmantsir, Massad & Kennell 2014; Massey & Wittkopp 2016). These patterns of variation are the product of different pigmentation components that can show diverse responses to genetic and environmental factors (Chapter 2). We have knowledge about the enzymes that biosynthesize pigments (effector genes) and about the transcription factors that regulate the spatial and temporal expression of those enzymes (patterning genes). We also know about the genetic basis of intra- and inter-species differences in pigmentation being assigned to patterning and effectors genes (e.g. Wittkopp, Carroll & Kopp 2003; Massey & Wittkopp 2016). Furthermore, there is insight about the molecular mechanisms regulating thermal plasticity in pigmentation (Gibert, Peronnet & Schlötterer 2007; Gibert, Mouchel-Vielh

& Peronnet 2017) and body-part specific genetic-by-environment effects. For instance, pigmentation of different abdominal tergites in *D. melanogaster* show differences in thermal reaction norms (David, Capy & Gauthier 1990).

Genetic studies have identified major effect loci associated with variation in abdominal pigmentation (Dembeck *et al.* 2015). Much less attention has been paid to the genetic basis of variation for other aspects of body pigmentation components and for plasticity therein. In fact, we know relatively little about the identity of the loci contributing to naturally-segregating variation in pigmentation plasticity, including their role in the development of different pigmentation components, and whether they are the same loci that contribute to intra-individual variation in pigmentation in one fixed environment. *D. melanogaster* is well suited to tackle these questions given the availability of tools that enable the genetic dissection of variation in plasticity and of methods to quantify variation in different pigmentation components (Gibert *et al.* 2007; Saleh Ziabari & Shingleton 2017).

Here we used a panel of *D. melanogaster* genotypes representing naturally-segregating alleles (Mackay *et al.* 2012; Huang *et al.* 2014) to characterize genetic variation in thermal plasticity for five pigmentation components. The target traits relate to color and color pattern of two body parts (thoracic and abdominal). We used the ca. 196 isogenic sequenced genotypes that make up the DGRP mapping panel to document effects of genotype, developmental temperature, and genotype-by-temperature interactions on the different pigmentation components and to identify loci contributing to variation therein. We then used different approaches to validate the role of selected plasticity QTLs.

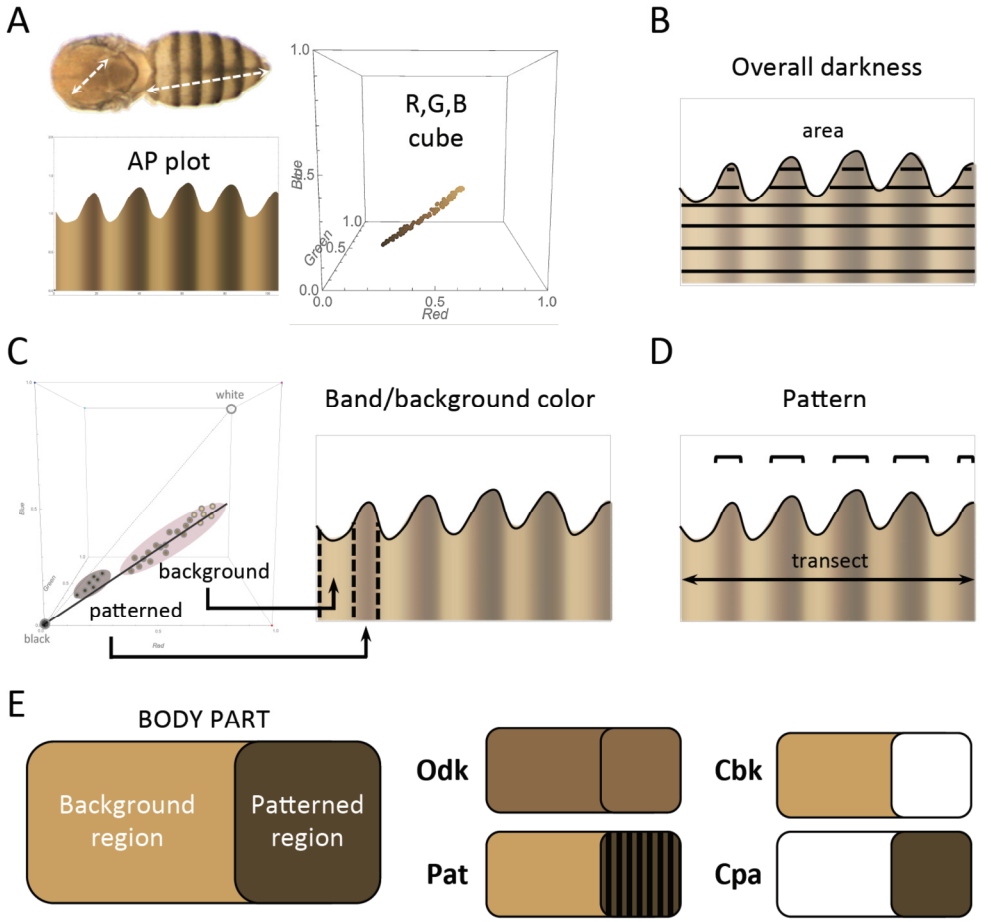


Figure 3.1. Pigmentation components. A. Thorax and abdomen of female adult fly showing the thoracic and abdominal transects and the corresponding pigmentation plots for the abdominal transect. Red, Blue, Green (RGB) plot shows all R,G,B values per pixel in the abdominal transect and the antero-posterior (AP) plot shows the distance from each pixel along the AP axis of the transect to the white vector (RGB 0,0,0). **B-D.** Pigmentation components extracted from DGRP adult females. From every pixel in the image, we extracted the RGB (red, blue, green) values to calculate the different pigmentation traits. **B.** Overall darkness (Odk) was calculated as the sum of the Euclidean distances of each pixel to black (RGB coordinates (0,0,0)) divided by the number of pixels. **C.** Color of the pattern element (Cpa) and color of the background (Cbk) were extracted by calculating the angle between the best-fitted line going through the pixels that correspond to the band (or the background) in our transect and the grey vector. **D.** Pattern (Pat) was extracted by calculating the proportion of pixels corresponding to the pattern element (thoracic trident or darker abdominal bands) relative to the transect's length. **E.** Schematic representation of each of the pigmentation components.

RESULTS

We assessed variation in pigmentation between genotypes and environments, by analyzing five pigmentation properties of abdomens and thoraxes of adult females from ~196 DGRP genetic backgrounds reared at either 17ºC or 28ºC. The pigmentation components analyzed were overall darkness (Odk), pattern (Pat), color of the background (Cbk) and color of the pattern element (Cpa; which corresponds to the trident in the thorax and the longitudinal darker bands in the abdomen) (Figure 3.1). A fifth component we call Range (Ran) was also quantified and all analyses for this trait are available in Supplementary Material (Figure 3.S1 and 3.S2). A detailed description on how the pigmentation traits were quantified can be found in the Materials and Methods section (see also Chapter 2).

Genetic and environmental effects on different pigmentation components

We documented effects of genotype (DGRP lines) and environment (Temperature) on all pigmentation traits (Figures 3.2A, 3.S3 and 3.S4; Table 3.1) and studied correlations between the different traits (Figure 3.2B). Correlations between traits were similar between temperatures, with all traits positively correlated in the abdomen but not in the thorax (Figure 3.2B). Overall, abdominal pigmentation was more affected by temperature (larger difference in mean value between 17ºC and at 28ºC) than thoracic pigmentation (Table 3.1). The most thermally responsive traits were abdominal pattern and thoracic and abdominal darkness, while the least affected ones were both color traits (Cbk and Cpa), particularly abdominal Cbk (Figure 3.3B, Table 3.1). Analyses of the broad sense heritability estimates for each pigmentation component and at each temperature (Table 3.1), revealed that estimates were overall, higher for measurements at 17ºC than at 28ºC and higher for measurements of abdominal components than thoracic, with the exception of Odk.

Table 3.1. Genetic variation for pigmentation components and plasticity. Mean, variance components and broad-sense heritability estimates for pigmentation components in thoraxes and abdomen at 17°C, at 28°C and for plasticity in pigmentation components per body part.

Trait	Body part	17°C				28°C				Plasticity			
		Mean DGRP	Genetic Variance	Residual Variance	H^2	Mean DGRP	Genetic Variance	Residual Variance	H^2	Genetic Variance	Residual Variance	G x E Variance	H^2
Darkness	Thorax	0,70	5,08E-03	1,51E-03	0,77	0,62	4,86E-03	2,07E-03	0,70	2,00E-03	1,80E-03	2,70E-03	0,42
Background color		0,46	1,60E-03	1,43E-02	0,10	0,44	2,64E-03	2,23E-02	0,11	6,00E-04	1,80E-02	1,50E-03	0,07
Band color		0,45	8,36E-03	4,32E-02	0,16	0,43	6,81E-03	7,90E-02	0,08	3,30E-03	6,08E-02	3,60E-03	0,05
Pattern		0,55	5,36E-03	7,66E-03	0,41	0,52	4,83E-03	1,26E-02	0,28	1,40E-03	1,02E-02	3,10E-03	0,21
Darkness	Abdomen	0,73	5,41E-03	2,34E-03	0,70	0,65	7,28E-03	4,05E-03	0,64	1,10E-03	3,20E-03	4,60E-03	0,51
Background color		0,35	2,32E-03	1,83E-03	0,56	0,33	2,03E-03	2,09E-03	0,49	3,00E-04	2,00E-03	1,70E-03	0,43
Band color		0,24	2,64E-03	4,03E-03	0,40	0,21	3,14E-03	5,93E-03	0,35	5,00E-04	5,10E-03	2,20E-03	0,28
Pattern		0,59	5,15E-03	3,74E-03	0,58	0,46	5,33E-03	3,50E-03	0,60	2,70E-03	3,60E-03	2,30E-03	0,26

To explore whether there were several strategies to be darker, we looked at the relationship between different pigmentation components and overall darkness (Odk). The analyses of the other components (Pat, Cbk in Cpa) in genotypes with different darkness revealed that genotypes become more (or less) dark by altering different traits. For instance, some genotypes are very dark because of an increase in Pat (e.g. genotype highlighted in yellow in Figure 3.2B), while other genotypes are darker because either the color of the background (Cbk) or the color of pattern element (Cpa) is formed by darker pigment (e.g. genotypes highlighted in green in Figure 3.2B).

Genotype-by-environment effects on different pigmentation components

We explored the extent and properties of thermal plasticity of pigmentation components by analyzing the reaction norms (RN) of the different traits in the two body parts (Figure 3.3A). For each genotype and trait, we calculated the slope of the regression of each pigmentation trait across temperatures (see Material and Methods). From each reaction norm, we then extracted two properties of the thermal plasticity: the absolute value of the slope as a measurement of thermal sensitivity, describing only the magnitude of the response to temperature, and the raw value of the slope as a measurement which describes also the direction of that response.

We documented genetic variation for the intercept and slope of the thermal reaction norms of our pigmentation traits in each body part (Figure 3.3A). For some components, most DGRP genotypes were plastic (i.e. 81% of the thoracic Odk RNs with slope significantly different from zero) while for others, few genotypes were plastic (i.e. 19% of the RNs for the color thoracic trident) (Figure 3.3A). In most cases, thoraxes and abdomens of flies reared at low temperature (17°C) showed darker pigmentation components. However, we also found genotypes in which plasticity was in the opposite direction (Figure 3.3A). Differences in thermal plasticity were also evident between body parts. For instance, 80% of the DGRP genotypes were plastic

in relation to abdominal pattern while only 34% were plastic in relation to thoracic pattern (Figure 3.3A).

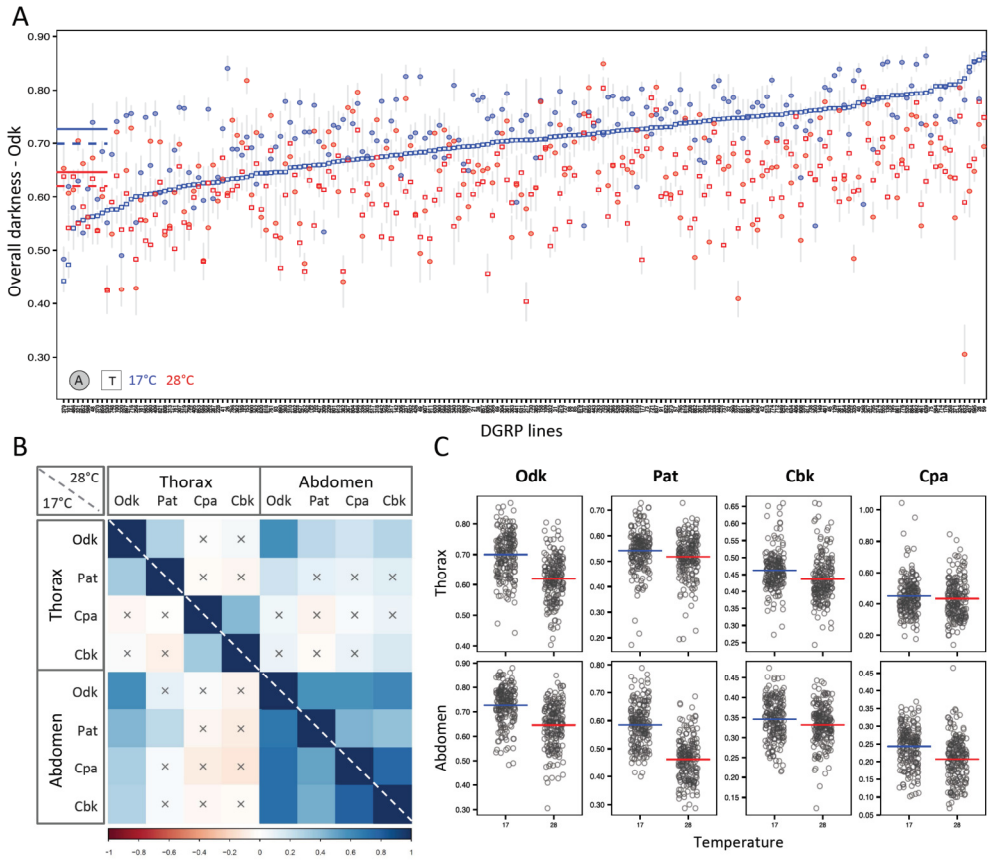


Figure 3.2. Variation in pigmentation components between genotypes and temperatures. A. Means and confidence intervals of overall darkness (Odk) in thoraxes (squares) and abdomens (circles) of the DGRP lines (X axis) reared at 17°C (blue) and 28°C (red). DGRP lines are ranked by the mean thoracic darkness at 17°C. Horizontal bars represent the mean Odk in thoraxes (dashed lines) and abdomens (solid line) of all DGRP lines at each temperature. **B.** Heat map of Pearson's correlation coefficients between pigmentation components at each temperature. Correlations at 17°C are shown in cells in the lower left off-diagonal and correlations at 28°C are given in the upper right off-diagonal. Positive correlations are denoted in blue and negative correlations in red. Non-significant correlations (p-value > 0.01) are indicated with an 'X'. **C.** Means values of the pigmentation components in thoraxes and abdomens of DGRP lines reared at 17°C and at 28°C. Horizontal bars represent the mean value of all DGRP lines at each temperature.

The correlations between properties of reaction norms for the different pigmentation components in the two body parts revealed few significant correlations within a given body part, and even fewer across body parts (Figure 3.3B).

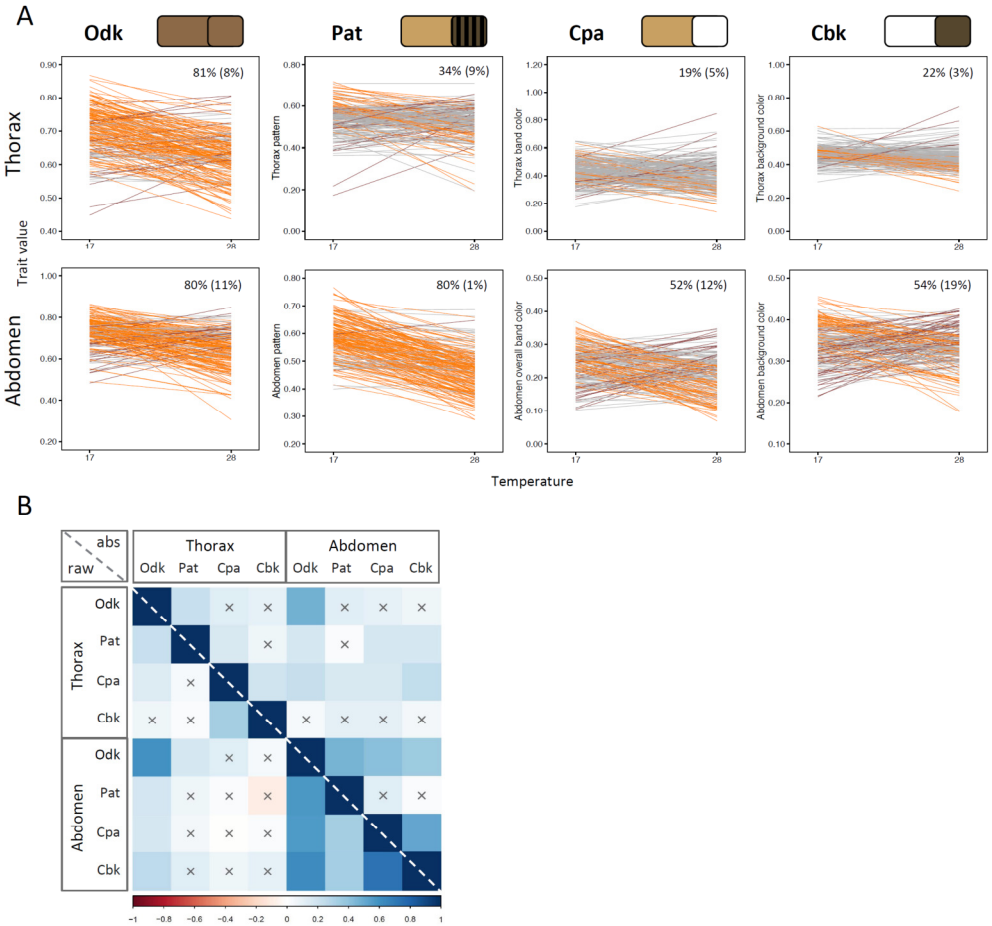


Figure 3.3. Variation in plasticity for pigmentation components. A. Reaction norms of each pigmentation component (Y axis) across temperatures (X axis) plotted as the regression fit for the model lm (*Trait ~ Temperature*) for each DGRP line. Colored lines are significantly different from zero (plastic) (positive slopes in orange, negative slopes in brown) while grey lines are non-significant (p-value>0.05). The percentage of plastic DGRP lines and among those, the percentage of lines with plasticity in the opposite direction, is shown in each plot. **B.** Heat map of Pearson's correlation coefficients between plasticity of pigmentation components. Correlations for the raw slopes of reaction norms are shown in cells in the lower left off-diagonal and correlations for the absolute slopes of reaction norms are given in the upper right off-diagonal. Positive correlations are denoted in blue and negative relationships in red. Non-significant correlations (p-value > 0.01) are indicated with an 'X'.

Genetic basis of variation in plasticity for pigmentation components

We explored the genetic basis of variation in thermal plasticity for our pigmentation components by running a GWAS using as quantitative trait the raw and absolute values of the slopes of the reaction norms (Figure 3.S6A). This was done for each pigmentation trait and body part independently. We did not find significant associations of *Wolbachia* infection nor inversion karyotype on any of our pigmentation components. We identified candidate QTLs significantly associated ($p<10e-5$) with variation in plasticity that were specific to each property of the reaction norm (raw and absolute slope), pigmentation component (Odk, Pat, Cbk, Cpa; Figures 3.4 and 3.5), and body part (thorax and abdomen) as well as QTLs associated with variation in plasticity that were common among traits and/or body parts. Table 3.S3 provides details about each of the significant SNPs/InDels ($p<10e-5$), including which genes they are putatively associated to as well as which gene regions they fall within (e.g. UTR, intronic, coding). Significant QTLs affecting variation in pigmentation plasticity corresponded to genes assigned to diverse functions (Table 3.S3, Figure 3.S6B), including genes well-documented effects on pigmentation biosynthesis, such as *ebony* (e) and *yellow* (y) and GO classes representing neuronal development and behavior (Figure 3.S6B).

Most significant QTLs where unique to trait (Odk, Pat, Cbk and Cpa), body part (thorax and abdomen) and plasticity property (raw and absolute slopes of reaction norms) (Figure 3.6A, Table 3.S3). The largest extent of overlap in identity of significant QTLs was found for plasticity between pigmentation traits more tightly correlated (Figures 3.3 and 3.6) and between the raw and absolute slopes of the reaction norms for several pigmentation components (Figures 3.4 and 3.5). Allelic variants influencing plasticity could do so by either buffering or increasing environmental responsiveness. To explore this, we looked at the effects and allele frequencies of our candidate plasticity QTLs in the DGRP and found that, in most cases, alleles that associated with increased plasticity tended to be at lower frequencies in the

population (assessed by the difference in mean value between the slope of all DGRP lines with the minor allele and the slope of all DGRP with the major allele at the candidate SNPs/InDels) (Figure 3.6C).

In order to assess to what extent the loci that carry allelic variation for pigmentation plasticity are the same as those contributing to within-environment pigmentation variation, we performed GWAS analyses on the pigmentation traits measured at each temperature (17°C and 28°C) (Figures 3.S7 and 3.S8). There was little overlap between QTLs contributing to variation in plasticity and QTLs contributing to within-environment variation for most traits, relatively higher when comparing thoracic vs. abdominal traits (Figure 3.S9). Analyses of the within-environment GWAS also revealed mostly "private" candidate QTLs; i.e. QTLs that were trait-specific, body-part-specific and environment-specific variation (Figures 3.S7 and 3.S8). There were few common QTLs, contributing to variation in multiple traits (Figure 3.6B) or body parts or temperatures (Figures 3.S7, 3.S8). Among the candidate QTLs affecting within-environment variation in pigmentation we found, once again, both genes previously implicated in pigmentation development (e.g. *bab1*, *y* and *e*) and genes not been previously associated with pigmentation (Table 3.S4). We did not find a phylogenetic signal on the genetic relatedness among DGRP lines (estimated by Blomberg's K and Pagel's λ coefficients) for any of our traits except for thoracic traits Odk and Pat. These traits also showed irregular Manhattan Plots with many highly significantly associated SNPs in chromosomal arm 3R (Figures 3.S7 and 3.S8), likely explained by a cluster of 16 DGRP lines harboring a particular haplotype with at least some, SNPs significantly associated to variation in these two traits (Figure 3.S12B).

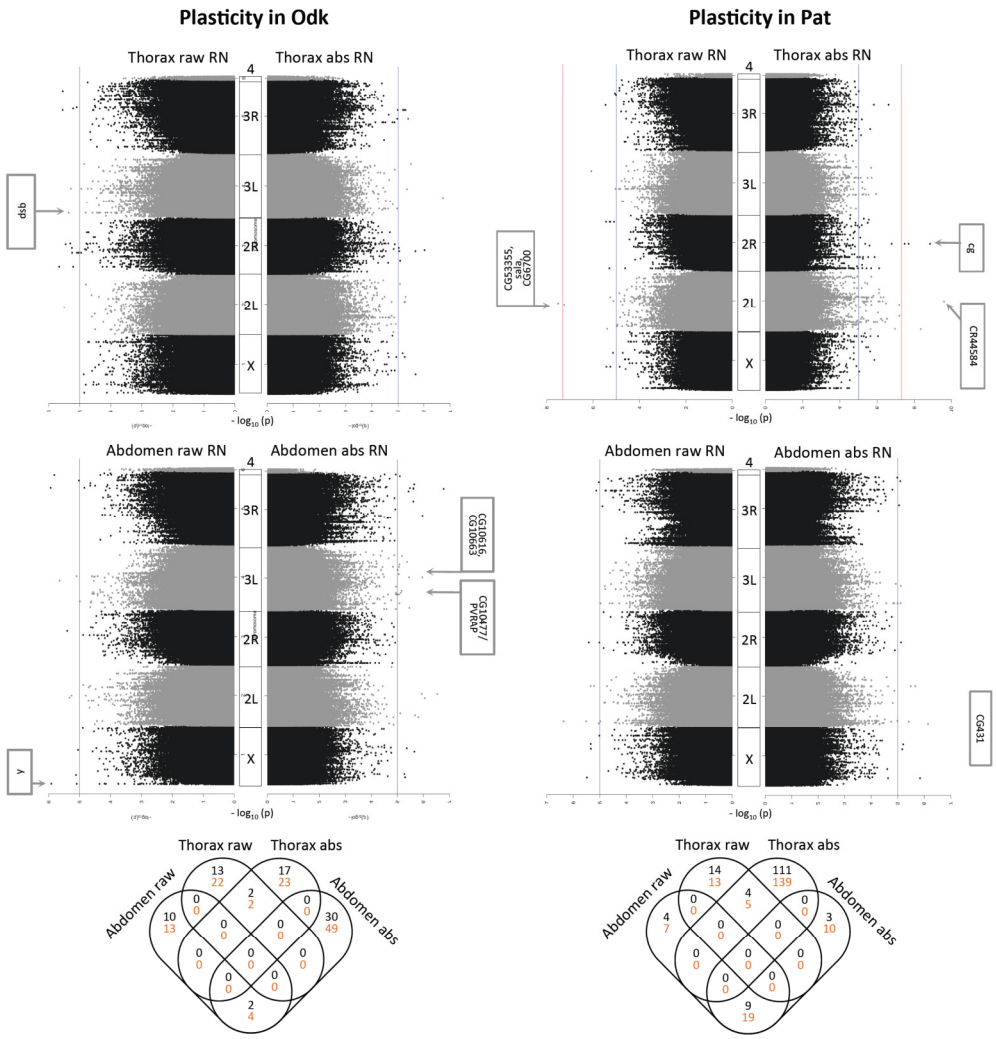


Figure 3.4. GWAS for variation in plasticity of overall darkness (Odk) and pattern (Pat). Manhattan plots and Venn diagrams corresponding to the GWAS performed for variation in plasticity of overall darkness (Odk) and Pattern (Pat). In the Manhattan plots, the significance level for each SNP along the chromosomal arms is shown as the $\log_{10}$ p-value. Horizontal lines are p-value < 10e-5 (blue) and p-value < 10e-8 (red). Some of the gene names associated to SNPs/InDels with a p-value < 10e-5 are shown in the plots. For each trait, upper and lower panels correspond to GWAS for thoracic and abdominal traits respectively, and left and right panels correspond to GWAS for raw and absolute slopes of reaction respectively. All GWAS were done testing the model lm (*Trait Slope ~ Allele + (1|Wolb|DGRP)*). Venn diagrams show overlaps in the identity of the SNPs/InDels with a p-value < 10e-5 (in black) and the putatively associated genes (in orange).

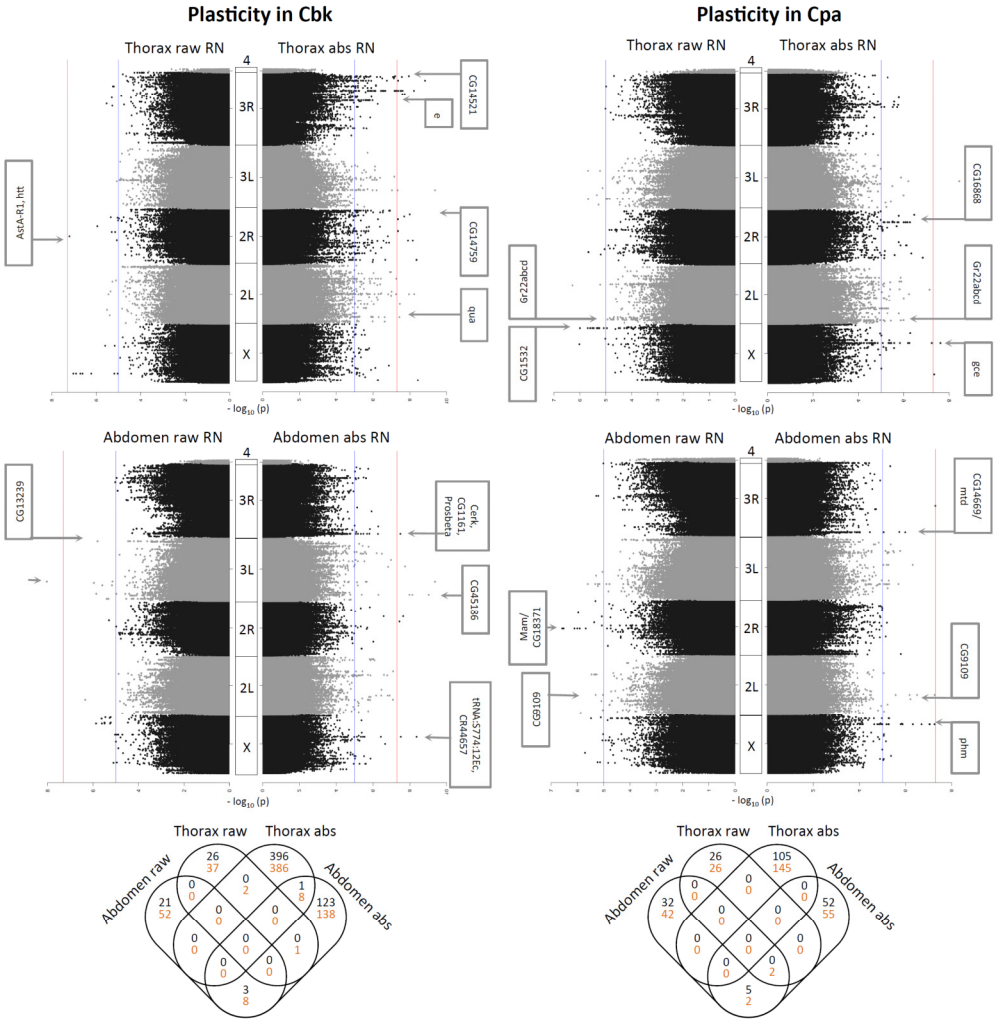


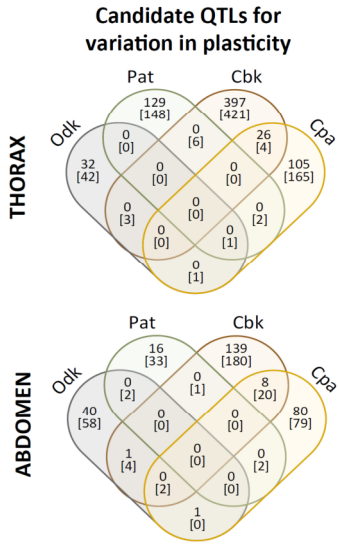
Figure 3.5. GWAS for variation in plasticity of color of the background (Cbk) and color of the pattern element (Cpa). Manhattan plots and Venn diagrams corresponding to the GWAS performed for variation in plasticity of color of the background (Cbk) and color of the pattern element (Cpa). In the Manhattan plots, the significance level for each SNP along the chromosomal arms is shown as the $\log_{10}$ p-value. Horizontal lines are p-value < 10e-5 (blue) and p-value < 10e-8 (red). Some of the gene names associated to SNPs/InDels with a p-value < 10e-5 are shown in the plots. For each trait, upper and lower panels correspond to GWAS for thoracic and abdominal traits respectively, and left and right panels correspond to GWAS for raw and absolute slopes of reaction respectively. All GWAS were done testing the model lm (*Trait Slope ~ Allele + (1|Wolb|DGRP)*). In the Venn diagrams show overlaps in the identity of the SNPs/InDels with a p-value < 10e-5 (in black) and the putatively associated genes (in orange).

Validation of selected GWAS hits

We selected a total of 18 SNPs/genes from our GWAS (based on their location, GWAS P-value, and putative SNP/gene function) for validation using two methods: a gene-centered method using available null mutants, or a SNP-centered method we are calling Mendelian Randomization (MR). While the former tests the hypothesis that abolishing protein production has an effect on phenotype, the later tests the hypothesis that individuals with one versus the other allele at the candidate SNP differ for the corresponding quantitative trait regardless of the genetic background. The MR approach involved, for each of the candidate SNPs, randomizing the genetic background between 10 same-allele genotypes and comparing the quantitative trait between flies carrying the minor versus the major allele (see Material and Methods).

Using these methods we confirmed the role of 12 out of 18 targeted SNPs/genes associated with either variation in pigmentation plasticity and/or with variation in pigmentation within-environment (Table 3.2). Using MR, we validated (i.e. significant difference in trait between major and minor allele when background is randomized) the role of 3 SNPs in thorax pigmentation plasticity (Figure 3.7A, Table 3.2) -- SNP-1 (in gene *sala*) for pattern, SNP-2 (in gene *gce*) for trident color and SNP-3 (in gene *CG14759*) for background color -- and 3 other SNPs in abdomen pigmentation plasticity (Figure 3.9B) -- SNP-4 (in gene *PVRAP*) in overall darkness, SNP-5 (in gene *CG9109*) in band color, SNP-6 (in gene *Cerk*) in background color (Figure 3.7B, Table 3.2). We did not validate the role in plasticity of 2 other SNPs: SNP-7 (in gene *qua*) and SNP-8 (in gene *CG12093*) (Figure 3.S4F, Table 3.2). Using available mutants, we confirmed (i.e. significant difference in trait between mutant and wild-type genotypes) the role of *ebony (e)* in plasticity of thoracic background color (Figure 3.S10A) but failed to validate the role of gene *gce* in plasticity of thoracic pattern (Figure 3.S10G). We attempted to perform validations for plasticity candidate genes *yellow* (*y*), *fruitless* (*fru*) and

A



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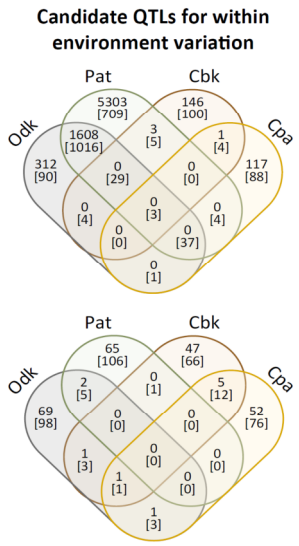


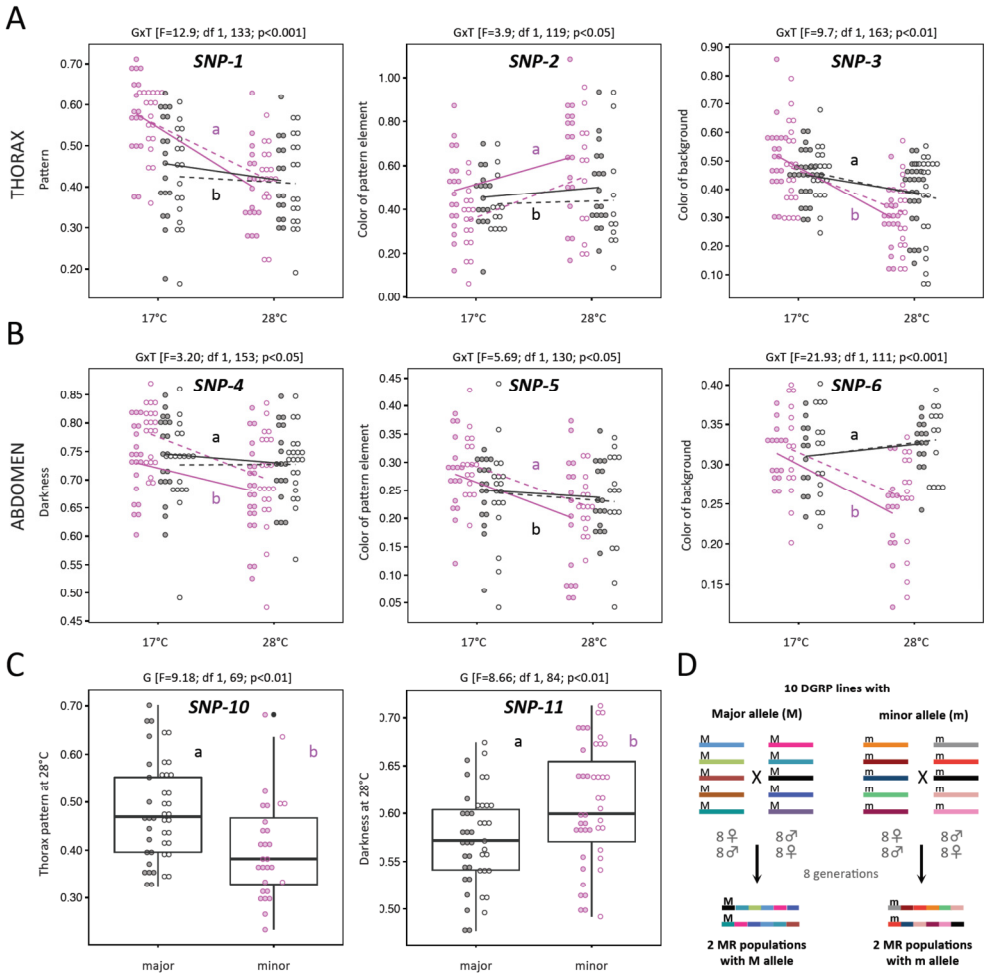
C

	Trait	Property	Body part	Number of SNPs/InDeLs	Number minor>major
	Odk	abs	thorax	18	8
			abdomen	31	15
		raw	thorax	12	4
			abdomen	12	4
	Pat	abs	thorax	4	3
			abdomen	2	1
		raw	thorax	5	5
			abdomen	2	1
	Cbk	abs	thorax	369	276
			abdomen	122	106
		raw	thorax	28	25
			abdomen	21	12
	Cpa	abs	thorax	105	101
			abdomen	56	31
		raw	thorax	23	23
			abdomen	37	10

Figure 3.6. Overlaps between QTLs for within and between-environment variation in pigmentation components. A. Venn diagrams showing overlaps in the identity of the SNPs/InDels with a p-value < 10e-5 (in black) and the putatively associated genes (in orange) for our different GWAS for variation in plasticity of pigmentation components. **B.** Venn diagrams showing overlaps in the identity of the SNPs/InDels with a p-value < 10e-5 (in black) and the putatively associated genes (in orange) for our different GWAS for within-environment variation in pigmentation components. **C.** For each pigmentation component, property of the reaction norms (raw and absolute (abs)) and body part (thorax and abdomen), the table shows the total number of significant candidate SNPs/InDels and out of those, the number of SNPs in which the mean slope of the reaction norms for DGRP lines with the minor allele was higher than the mean slope of the reaction norm for DGRP lines with the major allele (column named “Number minor>major”).

phantom (*phm*) but did not succeed due to high mortality of these genetic backgrounds at 28°C. Notably, for all our candidate SNPs, alleles that increase plasticity were always at lower frequency in the DGRP (Figure 3.S11). We also analyzed potential cross-trait effects of our candidate SNPs/genes and found that, in some cases, QTLs for plasticity in any given trait, also affected plasticity in other pigmentation components (Table 3.3).

We also validated 6 SNPs/genes implicated in within-environment pigmentation variation. Via MR, we confirmed the role of SNP-10 (in gene



Dop1R1) in thoracic pattern at 28°C and the role SNP-11 (in gene *e*) in overall darkness at 28°C (Figure 3.7C) and in thorax Ran at 17°C (Figure 3.S10C). We failed to validate the role of SNP-9 (in gene *Gr22d*) in thoracic pattern element at 17°C (Figure 3.S10H). Using mutants, we validated the role of genes *ebony (e)* in thoracic pattern at 17°C (Figure 3.S10B), *bric a brac 1* (*bab1)* in abdominal background color at 28°C (Figure 3.S10D), and *yellow (y)* in abdominal darkness at 17°C (Figure 3.S10E) and failed to validate the role of *gce* in color of thoracic pattern element at 17°C.

Figure 3.7. Functional validations of GWAS results. A-C. Validation via Mendelian Randomization (MR). In all the plots the two populations fixed for the major allele are shown in black and the two populations for the minor allele are shown in magenta. **A.** Validations for plasticity QTLs in thoracic pigmentation traits via MR. From left to right: individual phenotypic values and reaction norms for the traits: pattern, color of pattern element and color of background, in females from different MR populations corresponding to SNP-1 (in gene *sala*), SNP-2 (in gene *gce*) and SNP-3 (in gene *CG14759*). **B.** Validations for plasticity QTLs in abdominal pigmentation traits via MR. From left to right: individual phenotypic values and reaction norms for the traits: darkness, color of pattern element and color of background, in females from different MR populations corresponding to SNP-4 (in gene *PVRAP*), SNP-5 (in gene *CG9109*) and SNP-6 (in gene *Cerk*). **C.** Validations for within-environment QTLs. From left to right: individual phenotypic values for thoracic pattern at 28°C and abdominal pattern at 28°C in females from different MR populations corresponding to SNP-10 and SNP-11, respectively. For the validations of plasticity SNPs/genes (panels A and B), we tested the model lm (*Trait Slope ~ Genotype*Temperature)* and for the validations of within-environment SNPs/genes (panel C) we tested the model lm (*Trait ~ Genotype*). Results from the models are shown above each plot. In all cases, significant differences among groups were estimated by post hoc comparisons (Tukey's honest significant differences) and are indicated by different letters in each plot. **D.** Schematic representation of the Mendelian Randomization approach. For each candidate SNP, we first selected 10 DGRP lines with the minor allele and 10 with the major allele, not fixed for any other significant SNPs. These lines were used to generate four populations, two fixed for the major allele and two for the minor allele. Each population was established by crossing 8 virgin females from each of 5 of the same-allele lines to 8 males of the other 5 lines. The reciprocal crosses were used to set two independent populations per allele. These populations were allowed to cross for eight generations to randomize genetic backgrounds.

Beyond our pigmentation traits in the DGRPs

Widespread use of DGRPs, with multiple phenotypes measured independently for the same set of genotypes, allowed us to look at correlations between our pigmentation traits and a number of other DGRP traits, including pigmentation of last abdominal tergites (Dembeck *et al.* 2015) and a number of other traits fitness-related traits potentially associated to pigmentation (i.e. radiation resistance (Vaisnav *et al.* 2014), chill coma recovery (Mackay *et al.* 2012) and immune-defense traits (tolerance to infection with *Providencia rettgeri* bacteria (Howick & Lazzaro 2017) and resistance to infection with *Metarhizium anisopliae* fungi or with *Pseudomonas aeruginosa* bacteria (Wang, Lu & St. Leger 2017)).

We found significant (positive) correlations between abdominal pigmentation in tergites T5 and T6 and most of our abdominal pigmentation traits, but not thorax pigmentation traits (Figure 3.S12A), and we also found overlap between the candidate QTLs for variation in pigmentation in tergites 5 and 6 and at 25°C and some of our candidate QTLs for various pigmentation traits on the two body parts (14 SNPs and 34 genes). We also found that only chill coma recovery and resistance to *Pseudomonas aeruginosa* bacteria were significantly (negatively) correlated with our traits abdominal background color and overall thoracic darkness at 17°C, respectively (Figure 3.S12A).

DISCUSSION

Body pigmentation plays important roles in the survival and reproduction of many organisms (e.g. Llopart, Elwyn & Coyne 2002; Steiner, Weber & Hoekstra 2007; Ahlgren *et al.* 2013). In addition to genetic effects (e.g. Nachman, Hoekstra & D'Agostino 2003; Greenwood *et al.* 2011), pigmentation is also controlled by environmental factors, such as temperature (e.g. Solensky & Larkin 2009) or nutrition (e.g. Ethier *et al.* 2015), which have been shown to influence body color in different species, including *Drosophila* (David *et al.* 1990; Shakhmantsir *et al.* 2014). This

plasticity is heritable and adaptive, and, thus, can evolve. Intra-specific variation and inter-specific divergence in body pigmentation can be due to differences in color composition and/or color pattern that occur across the entire body and/or in specific body regions (Mundy 2005). Each of these components of body pigmentation can respond to internal and external factors more or less independently.

Partitioning variation in body pigmentation: genetic, environmental and genetic-by-environmental effects on pigmentation components

We have documented variation in different pigmentation components of two body parts and variation for thermal plasticity in those components in a population of *D. melanogaster* representing naturally segregating genetic variation. The DGRP showed substantial variation for the different pigmentation components with contributions of genetic (i.e. different DGRP lines) and environmental (i.e. temperature) effects having trait and body part-specific properties.

Analyses of trait-associations revealed few significant correlations between pigmentation components at any given environment, as well as between plasticity properties of the different pigmentation components. However, we did find that some traits were more tightly correlated; namely color traits (Cpa and Cbk) showed more similar responses to genetic and environmental inputs, and so did different pigmentation components within a given body part. The few significant correlations we found between pigmentation components and plasticity therein are suggestive of a potential for independent evolution and development of traits. A more detailed analysis of the differences between genotypes revealed that pigmentation components can contribute in different ways to the overall darkness. Flies can be overall darker because of darker color of background and/or of pattern element (trident in thorax or bands in abdomen) or because of larger area occupied by the darker pattern element.

Table 3.2. Identity of candidate SNPs/genes pursued for functional validation. Information for each of the SNPs/genes pursued for functional validations including details of the genomic position, the putatively affected gene and gene region, and the SNP identity and allele frequencies in the DGRP. **Table**

Validation Method	SNP/Gene code	Gene Affected	Positive validation	Target trait	GWAS class	Body part	Genomic position	Allele [frequency]	Affected Region
MR	*SNP_1*	*sala*	yes	Pat	plasticity	Thorax	2L:11486323	T [140] - A [54]	missense
MR	*SNP_2*	*gce*	yes	Cpa	plasticity	Thorax	X:15311088	T [187] - A [13]	missense
MR	*SNP_3*	*CG14759*	yes	Cbk	plasticity	Thorax	2R:8260391	C [151] - A [45]	missense
MR	*SNP_4*	*PVRAP*	yes	Odk	plasticity	Abdomen	3L:6029181	A [184] - G [14]	downstream
MR	*SNP_5*	*CG9109*	yes	Cpa	plasticity	Abdomen	2L:6024748	G [185] - T [14]	upstream
MR	*SNP_6*	*Cerk*	yes	Cbk	plasticity	Abdomen	3R:5361701	C [178] - G [20]	missense
MR	*SNP_7*	*qua*	n.s	Cbk	plasticity	Thorax	2L:17488875	T [186] - C [16]	missense
MR	*SNP_8*	*CG12093*	n.s	Cbk	plasticity	Abdomen	3L:2773539	A [114] - C [82]	missense
MR	*SNP_9*	*Gr22d*	n.s	Cpa	17C	Thorax	2L:1790336	C [190] - G [12]	missense
MR	*SNP_10*	*Dop1R1*	yes	Pat	28C	Thorax	3R:14186497	C [190] - G [12]	intronic
MR	*SNP_11*	*e*	yes	Odk	28C	Thorax	3R:21240071	C [168] - G [27]	upstream
mutant	*ebony*	*e*	yes	Cbk	plasticity	Thorax	several (see Tables S3 and S4)		
mutant	*ebony*	*e*	yes	Cbk	17C	Thorax			
mutant	*bric-a-brac*	*bab1*	yes	Cbk	28C	Abdomen			
mutant	*yellow*	*y*	yes	Odk	17C	Abdomen			
mutant	*yellow*	*y*	n.a (mortality at 28C)	Odk	plasticity	Abdomen			
mutant	*gce*	*gce*	n.s	Cpa	plasticity	Thorax			
mutant	*gce*	*gce*	n.s	Cpa	17C	Thorax			
mutant	*fru*	*fruitless*	n.a (mortality at 28C)	Ran	plasticity	Abdomen			
mutant	*phm*	*phantom*	n.a (mortality at 28C)	Cpa	plasticity	Abdomen			

We also documented variation in plasticity (genotype-by-environment) in different pigmentation components. All pigmentation components showed differences in properties of the reaction norms (raw and absolute values of the reaction norms) across DGRP genotypes (and heritabilities) different from zero.

Genetic basis of thermal plasticity in body pigmentation

Mutational screenings and/or mapping studies have provided considerable insight on the genetic underpinnings of inter- and intra-specific variation in pigmentation in *Drosophila* (Jeong, Rokas & Carroll 2006; Pool & Aquadro 2007; Rogers *et al.* 2014), including the effects disrupting canonical pigmentation genes on pigmentation plasticity (Gibert *et al.* 2007, 2017). Much less attention has been paid to unraveling the genetic basis of variation pigmentation plasticity in natural populations.

We were able to identify loci associated with variation in plasticity for several pigmentation components. Genes involved in plastic responses could potentially mediate environmental regulation of phenotypes by acting at the level of sensing (i.e. genes perceiving external cues), modulating (i.e. genes interpreting and/or transmitting external signals to developing organs) and/or executing (i.e. genes involved in pigmentation development, including patterning and effector genes). We report some polymorphisms that contribute to natural variation in plasticity of different pigmentation components, affecting canonical pigmentation genes (i.e. effector genes such as *e* and *y* and and/or patterning genes such as *bab1*), but many more genes not previously associated to pigmentation development. Gene-ontology enrichment analyses showed an overrepresentation of genes for neuronal development while some of our validated QTLs for plasticity are players of hormonal signaling, such as the juvenile hormone receptor *gce*. We also described a role in thermal pigmentation plasticity for genes for which there was very little prior information (i.e. genes *sala, CG14759* or *CG9109*).

3.3. Cross-trait effects of the candidate SNPs/genes pursued for functional validation. For each tested SNPs/genes, the results of the statistical analysis for each pigmentation component are shown. For the SNPs/genes associated with plasticity SNPs/genes, we tested the model lm (*Trait Slope ~ Genotype*Temperature*) and for the SNPs/genes associated within within-environment variation, we tested the model lm (*Trait ~ Genotype*). Non-significant effects are shown in red and significant are shown in green. The trait for which the SNP/genes was originally a candidate is highlighted in grey.

Validation Method	SNP/Gene code	Effect on pigmentation components				
		Odk	Cbk	Cba	Pat	Ran
MR	*SNP_1*	GxT [F=6.9; df 1, 133 p<0.01]	GxT [F=3.29; df 1, 133 p>0.05]	GxT [F=0.73; df 1, 133 p>0.05]	GxT [F=12.9; df 1, 133 p<0.001]	GxT [F=1.82; df 1, 133 p>0.05]
MR	*SNP_2*	GxT [F=15.04; df 1, 119 p<0.001]	GxT [F=0.02; df 1, 119 p>0.05]	GxT [F=3.92; df 1, 119 p<0.05]	GxT [F=11.3; df 1, 119 p<0.01]	GxT [F=0.13; df 1, 119 p>0.05]
MR	*SNP_3*	GxT [F=10.85; df 1, 163 p<0.01]	GxT [F=9.65; df 1, 163 p<0.01]	GxT [F=3.6; df 1, 163 p>0.05]	GxT [F=0.57; df 1, 163 p>0.05]	GxT [F=10.1; df 1, 163 p<0.01]
MR	*SNP_4*	GxT [F=6.09; df 1, 155 p<0.05]	GxT [F=1.09; df 1, 155 p>0.05]	GxT [F=1.43; df 1, 155 p>0.05]	GxT [F=0.4; df 1, 155 p>0.05]	GxT [F=2.04; df 1, 155 p>0.05]
MR	*SNP_5*	GxT [F=8.95; df 1, 130 p<0.01]	GxT [F=10.22; df 1, 130 p<0.01]	GxT [F=5.69; df 1, 130 p<0.05]	GxT [F=0.36; df 1, 130 p>0.05]	GxT [F=0.69; df 1, 130 p>0.05]
MR	*SNP_6*	GxT [F=2.36; df 1, 111 p>0.05]	GxT [F=21.9; df 1, 111 p<0.001]	GxT [F=2.72; df 1, 111 p>0.05]	GxT [F=0.76; df 1, 111 p>0.05]	GxT [F=0.49; df 1, 111 p>0.05]
MR	*SNP_7*	GxT [F=0.91; df 1, 128 p>0.05]	GxT [F=0.005; df 1, 128 p>0.05]	GxT [F=0.24; df 1, 128 p>0.05]	GxT [F=0.08; df 1, 128 p>0.05]	GxT [F=1.94; df 1, 128 p>0.05]
MR	*SNP_8*	GxT [F=0.7; df 1, 130 p>0.05]	GxT [F=0.7; df 1, 130 p>0.05]	GxT [F=0.2; df 1, 130 p>0.05]	GxT [F=0.03; df 1, 130 p>0.05]	GxT [F=0.05; df 1, 130 p>0.05]
MR	*SNP_9*	G [F=1.3; df 1, 69 p>0.05]	G [F=0.001; df 1, 69 p>0.05]	G [F=1.05; df 1, 69 p>0.05]	G [F=0.13; df 1, 69 p>0.05]	G [F=0.42; df 1, 69 p>0.05]
MR	*SNP_10*	G [F=0.26; df 1, 69 p>0.05]	G [F=2.9; df 1, 69 p>0.05]	G [F=2.24; df 1, 69 p>0.05]	G [F=9.18; df 1, 69 p<0.01]	G [F=7.74; df 1, 69 p<0.01]
MR	*SNP_11*	G [F=8.66; df 1, 84 p<0.01]	G [F=14.71; df 1, 84 p<0.001]	G [F=5.23; df 1, 84 p<0.05]	G [F=0.49; df 1, 84 p>0.05]	GxT [F=1.76; df 1, 84 p>0.05]
mutant	*ebony*	GxT [F=1.2; df 1, 78 p>0.05]	GxT [F=5.26; df 1, 78 p<0.01]	GxT [F=1.2; df 1, 78 p>0.05]	GxT [F=3.41; df 1, 78 p<0.05]	GxT [F=33.71; df 1, 78 p<0.001]
mutant	*ebony*	GxT [F=61.42; df 2, 43 p<0.001]	GxT [F=0.04; df 2, 43 p>0.05]	GxT [F=11.18; df 2, 43 p<0.001]	GxT [F=60.61; df 2, 43 p<0.001]	GxT [F=64.26; df 2, 43 p<0.001]
mutant	*bric-a-brac*	G [F=5.81; df 1, 22 p<0.05]	G [F=18.91; df 1, 22 p<0.001]	G [F=23.6; df 1, 22 p<0.001]	G [F=15.15; df 1, 22 p<0.001]	G [F=0.93; df 1, 22 p>0.05]
mutant	*yellow*	G [F=68.56; df 1, 30 p<0.001]	G [F=11.27; df 1, 30 p<0.001]	G [F=8.19; df 1, 30 p<0.01]	G [F=1.82; df 1, 30 p>0.05]	G [F=0.71; df 1, 30 p>0.05]
mutant	*yellow*	n.a	n.a	n.a	n.a	n.a
mutant	*gce*	G [F=1.85; df 2, 115 p>0.05]	G [F=1.17; df 2, 115 p>0.05]	G [F=0.57; df 2, 115 p>0.05]	G [F=6.71; df 2, 115 p>0.01]	G [F=3.79; df 2, 115 p>0.05]
mutant	*gce*	G [F=13.12; df 2, 65 p<0.001]	G [F=0.74; df 2, 65 p>0.05]	G [F=1.9; df 2, 65 p>0.05]	G [F=4.66; df 2, 65 p<0.05]	G [F=2.52; df 2, 65 p>0.05]
mutant	*fru*	n.a	n.a	n.a	n.a	n.a
mutant	*phm*	n.a	n.a	n.a	n.a	n.a

We have characterized a genetic architecture for pigmentation plasticity that is, to a large extent, different between pigmentation components, between body parts (thorax and abdomen) and even, between properties of the plastic response (raw and absolute slopes of the reaction norm). Moreover, these loci are not necessarily the same that underlie variation in pigmentation components at any given environment.

Evolution of thermal plasticity in body pigmentation

Pigmentation plasticity is quite common in nature, including seasonal differences in body color associated to seasonally-variable selective environments. Such plasticity can be triggered by different environmental factors (e.g. temperature, nutrition and photoperiod) that can differentially affect different pigmentation traits; color and color pattern of different body parts.

Pigmentation has been associated with increased fitness under different ecological pressures, such as predation, ultraviolet radiation, thermal stress, and pathogen resistance (Slagsvold, Dale & Andrzej 1995; Hill & McGraw 2006; Clusella-Trullas *et al.* 2008; Protas & Patel 2008). For instance, patterns of local adaption in *Drosophila*, where temperature and humidity worked as selective forces, suggest that a darker body can absorb solar radiation more efficiently, improve thermoregulation, and makes cuticle thicker, decreasing water loss under low humidity (Rajpurohit *et al.* 2008; Parkash *et al.* 2008). Despite this, we did not find evidence of a correlation between our pigmentation traits and radiation resistance, chill coma recovery, or immune-related responses. However, it is noteworthy that those phenotypes were measured in different environments than ours and are likely environmentally-sensitive.

There has been extensive debate of whether the genetic basis for plasticity was determined by the same or different loci than the ones controlling mean values at a given environment (see Via 1993). Our results shed light onto this discussion; most of the QTLs we identified for

pigmentation plasticity were not QTLs for pigmentation variation either 17°C or at 28°C. This suggests that the genetic basis of trait plasticity is, to a large extent, independent of genetic basis of the trait itself.

We found that allelic variants associated with increased plasticity (for any of the pigmentation components) were often at lower frequencies in the DGRPs, relative to the alleles associated with reduced plasticity. These allelic variants, likely to be different from the ones unraveled by mutational screenings (ref sleep in DGRP and Mackay SSE), have survived natural selection and are putative targets for the evolution of plasticity on this and possibly, other populations. Altogether the little degree of overlap between loci contributing to variation in plasticity for different pigmentation components, body parts and for between and within-environment QTLs, suggests a strong potential for independent development and evolution of traits under conditions of environmental heterogeneity. Both phenotypic and genetic data variation revealed a modular organization for pigmentation with body-part-specific and trait-specific responses to genetic and environmental factors and shed light onto the genetic basis by which external and internal information is integrated into functional, developmental and/or evolutionary.

MATERIALS AND METHODS

Fly stocks and rearing conditions

Data for the GWAS was collected from adult female flies of the Drosophila Genetic Reference Panel (DGRP) obtained from Bloomington Stock Center. The DGRP is a set of fully sequenced inbred lines collected from a single population in Raleigh, NC, USA (Mackay *et al.* 2012; Huang *et al.* 2014). The number and the details of the lines included in the GWAS for each trait can be found in Table 3.S2. Mutant stocks for the functional validations were: 1658 for *ebony*, 3039 for *yellow*, 37298 for *bric-a-brac*, 684 for *fruitless* and 2208 for *phantom,* all from Bloomington. Mutant line for *gce* was obtained from Marek Jindra's lab. Control genetic backgrounds were *w1118* (stock 5905, from Bloomington) and *Canton-S* (obtained from CK Mirth lab).

Data for the European cline was obtained from adult females flies of five isogenic lines from Finland, Austria and Spain. These lines are part of the collections from the DrosEU consortium (www.droseu.net) and were obtained from Elio Sucena's lab.

Experimental rearing of flies

Fly stocks were maintained in molasses food (45 gr. molasses, 75gr sugar, 70gr cornmeal, 20 gr. Yeast extract, 10 gr. Agar, 1100 ml water and 25 ml of Niapagin 10%) in incubators at 25ºC, 12:12 light cycles and 65% humidity until used in this study. For the experiments, we performed over-night egg lays from ~20 females of each stock in vials with *ad libitum* molasses food. Eggs were then placed at either 17ºC or 28ºC throughout development. We controlled population density by keeping between 20 and 40 eggs per vial. We quantified thorax and abdomen size of 5 to 20 females per line, per temperature and replicate. For 130 DGRP lines, we ran two replicates and for a subset of 33 lines we ran three replicates. The total number of flies used varied between lines due to mortality of some stocks at one of the temperatures. For some specimens, we could only quantify size of one body part but not of the other, for instance if part of the individual was not properly positioned in the image or if part of the body was damaged. Details on the stocks used and the number of flies used per stock and temperature can be found in Tables 3.S1 and 3.S2. Rearing conditions for the validations of candidate QTLs were similar to those used for the DGRP lines.

Phenotyping body pigmentation components

Adult female flies (8-10 days after eclosion) were placed in 2ml Eppendorf and killed in liquid nitrogen followed by shaking the tubes to remove wings, legs and bristles. Bodies were then mounted on 3% Agarose in Petri dishes, dorsal side up, and covered with water to avoid light reflection upon imaging. Images containing 10 to 20 flies were collected with a LeicaDMLB2 stereoscope and a Nikon E400 camera under controlled imaging conditions

of light, contrast, and white-balance. Images were later processed with a customize Mathematica macro to extract pigmentation measurements. For this purpose, we drew two transects per fly, one in the thorax and one in the abdomen, using body landmarks (as shown in Figure 3.S1A) and extracted RGB (Red, Blue, Green) values from each pixel along the transects. Those RGB values than were later used to define five pigmentation components (see also Chapter 2). Overall darkness (Odk) was calculated as the sum of the Euclidean distances of each pixel to black divided by the number of pixels. Color of the pattern element (Cpa) is the angle between the best-fitted line going through the pixels that correspond to the pattern element (trident in the thorax and darker bands in the abdomen) in the transect and the grey vector (a constant diagonal in the RGB space). Similarly, color of the background (Cbk) was calculated as the angle between the best-fitted line that goes through the background pixels in the transect and the grey vector. Pixels corresponding to pattern element and/or background were defined by diving all RGB values in the transect into two clusters each containing 95% of the light or dark pixels respectively. Pattern (Pat) was extracted by calculating the proportion of pixels corresponding to the pattern element (thoracic trident and/or darker abdominal bands) relative to the transect' length, being the number of pixels corresponding to the pattern element those above a threshold defined by an adjusted median line throughout all pixels. Range (Ran) was calculated as the Euclidean distance between the median value for the 20 darkest and the 20 lightest pixels in the transects.

Statistical analyses of G and E effects on body pigmentation

All statistical analyses were performed with R Statistical Package v 3.1.1 (R Development Core Team 2017). We checked assumptions of parametric test by using Shapiro test for normality and Bartlett test of homocedasticity. For each body part and pigmentation component, we used linear models to test for the effect of genotype (model lm (*Trait ~ DGRP genotype*)) or the

interaction between genotype and temperature (model lm (*Trait ~ DGRP genotype * Temperature*)). Reaction norms for each DGRP line were calculated by using the regression model lm (*Trait ~Temperature*). From that model we extracted two properties of the reaction norms per DGRP line and body part: the absolute value of the slope as a measurement of thermal sensitivity, describing only the magnitude of the response to temperature, and the raw value of the slope as a measurement which describes also the direction of that response. Linear mixed models were calculated using *lme4* R package.

Broad sense heritability for pigmentation components at each temperature was estimated as $H^2 = \sigma^2_A/(\sigma^2_A + \sigma^2_W)$ where σ^2_A and σ^2_W are the among-line and within-line variance components, respectively. Heritability of plasticity for each pigmentation components was calculated, as proposed in Scheider and Lyman (1989), as $H^2 = \sigma^2_{G*E}/\sigma^2_{TOTAL}$ where σ^2_{G*E} and σ^2_{TOTAL} are the variance associated with the genotype by environment interaction and total variance components, respectively. Variance components were extracted using *varcomp* R package.

For the functional validations of within-environment SNPs and genes we tested the model lm (*Trait ~ Allele)* and lm (*Trait ~ Genotype)*, respectively. For the validations of plasticity SNPs and genes we tested the model we tested the model lm (*Trait ~ Genotype*Temperature)* and lm (*Trait ~ Allele*Temperature)*, respectively. In all cases, significant differences among groups were estimated by post hoc comparisons (Tukey's honest significant differences).

Genome-Wide Association Study

For each pigmentation component (Odk, Pat, Cbk, Cba and Ran) and body part (thorax and abdomen), we performed four independent genome wide analyses (GWAS): two for thermal plasticity (raw and absolute values of the slopes of the reaction norms used as target quantitative trait), and two for within-environment variation (pigmentation at 17ºC and 28ºC as quantitative

traits). The GWAS for variation in thermal plasticity were testing the model lm (*Slope ~ allele + (1|Wolb|DGRP)*), *Wolb* being the *Wolbachia* status of the DGRP lines. The GWAS analyses for within-environment variation (at either 17°C or 28°C) were done by testing the model lm (*Trait ~ allele + (1|Wolb|DGRP)*). All the GWAS were performed by using SNPs where we had information for at least ten lines per allele. We did not find an effect of *Wolbachia* in any of our GWAS analysis.

We also tested for the effect of the chromosomal inversions that were present in at least 8 DGRP lines, namely inversions In_3R_K, In_3R_P, In_2L_t, In_2R_NS and In_3R_Mo, on each of our traits by using the models lm (*Mean Trait ~ inversion)* for within-environment (at 17°C and 28°C) variation in size or lm (*Slope ~ inversion)* for size plasticity, in each body part.

Genetic distance matrix for the DGRPs was obtained from http://dgrp2.gnets.ncsu.edu/data.html and was used to perform a cluster hierarchical dendogram using *ape* and *phylobase* R packages. We estimated the phylogenetic signal and statistical significance for each of our traits using Blomberg's K (Blomberg, Garland & Ives 2003) and Pagel's λ (Pagel 1999) metrics with the *phylosig* function in the *phytools* R package (Revell 2012). For each of the GWAS we annotated the SNPs with a p-value < 10e-5 using the FlyBase annotation (release 6; ref). Gene-ontology enrichment analysis was done with SNPs of p-value < 10e-5 using the publicly available *GOrilla* Software (ref 2x Eden).

Functional validations

SNPs with p-value < 10e-5 considered in relation to Manhattan plots (clear peaks prioritized), putative effect (missense and regulatory variants prioritized over inter-genic variants), associated genes (annotated and known function prioritized) were selected for functional validations. Two methods for validation were used: null mutants and Mendelian randomization. Validations by null mutants were done by comparing the

phenotype in the homozygous or heterozygous mutant stock with its respective genetic background. Validations by Mendelian randomization (MR) were done by selecting for each candidate SNP, 10 DGRP lines with the minor allele and 10 with the major allele, not fixed for any other significant SNPs. These lines were used to generate four populations, two fixed for the major allele and two for the minor allele. Each population was established by crossing 8 virgin females from each of 5 of the same-allele lines to 8 males of the other 5 lines. The reciprocal crosses were used to set two independent populations per allele. These populations were allowed to cross for eight generations to randomize genetic backgrounds. We confirmed by Sanger sequencing that those populations had our candidate allele fixed.

AUTHOR CONTRIBUTIONS

Elvira Lafuente and Patrícia Beldade conceived and designed the experiments; Elvira Lafuente performed the experiments, Elvira Lafuente and David Duneau analyzed the data. Elvira Lafuente and Patrícia Beldade wrote the manuscript.

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SUPPLEMENTARY MATERIAL

Figure 3.S1. Variation in Range between genotypes and temperatures.

Figure 3.S2. GWAS for variation in range.

Figure 3.S3. Phenotypic variation in darkness (Odk) and pattern (Pat).

Figure 3.S4. Phenotypic variation in color of the background (Cbk) and color of the pattern element (Cpa).

Figure 3.S5. Differences in pigmentation components between genotypes.

Figure 3.S6. Slope of the reaction norm as a quantitate trait.

Figure 3.S7. GWAS for in overall darkness (Odk) and pattern (Pat).

Figure 3.S8. GWAS for color of the background (Cbk) and color of the pattern element (Cpa).

Figure 3.S9. Overlaps between QTLs for within and between-environment variation in pigmentation components.

Figure 3.S10. Functional validations of GWAS results

Figure 3.S11. Effects of candidate SNPs in the reaction norms of the DGRP

Figure 3.S12. Variation in plasticity for pigmentation components.

The following documents are included in the digital supplement that accompanies this thesis.

Table 3.S1. Phenotypic variation in pigmentation components.

Raw data for measurements of Odk, Pat, Cbk, Cpa and Ran in thoraxes and abdomens of flies from DGRP lines reared at 17°C and at 28°C.

Table 3.S2. Summary of phenotypic variation in pigmentation components and plasticity of those.

Summary data from measurements of pigmentation components in thoraxes and abdomens of DGRP lines. Number of phenotyped flies (N), mean and standard deviation (SD) per temperature, DGRP line and body part.

Table 3.S3. GWAS for variation in plasticity of pigmentation components.

Nominally significant SNPs (p-value threshold of 10e-5) from GWAS for the raw slope of the reaction norms (Value=Raw) and absolute slope of the reaction norms (Value=Absolute) per body part and pigmentation component. The genomic position (from Genome Releases v.5 and v.6), type of SNP/InDel, potential impact, associated gene name (Flybase Gene ID) and putative consequence are also shown.

Table 3.S4. GWAS for variation in pigmentation components.

Nominally significant SNPs (p-value threshold of 10e-5) from GWAS for variation at 17°C and at 28°C per body part and pigmentation component. The genomic position (from Genome Releases v.5 and v.6), type of SNP/InDel, potential impact, associated gene name (Flybase Gene ID) and putative consequence are also shown.

Table 3.S5. Functional validations of GWAS candidates.

Raw data for measurements of Odk, Pat, Cbk, Cpa and Ran in thoraxes or abdomens of flies reared at 17°C and at 28°C from the different genetic backgrounds corresponding to each functional validation.

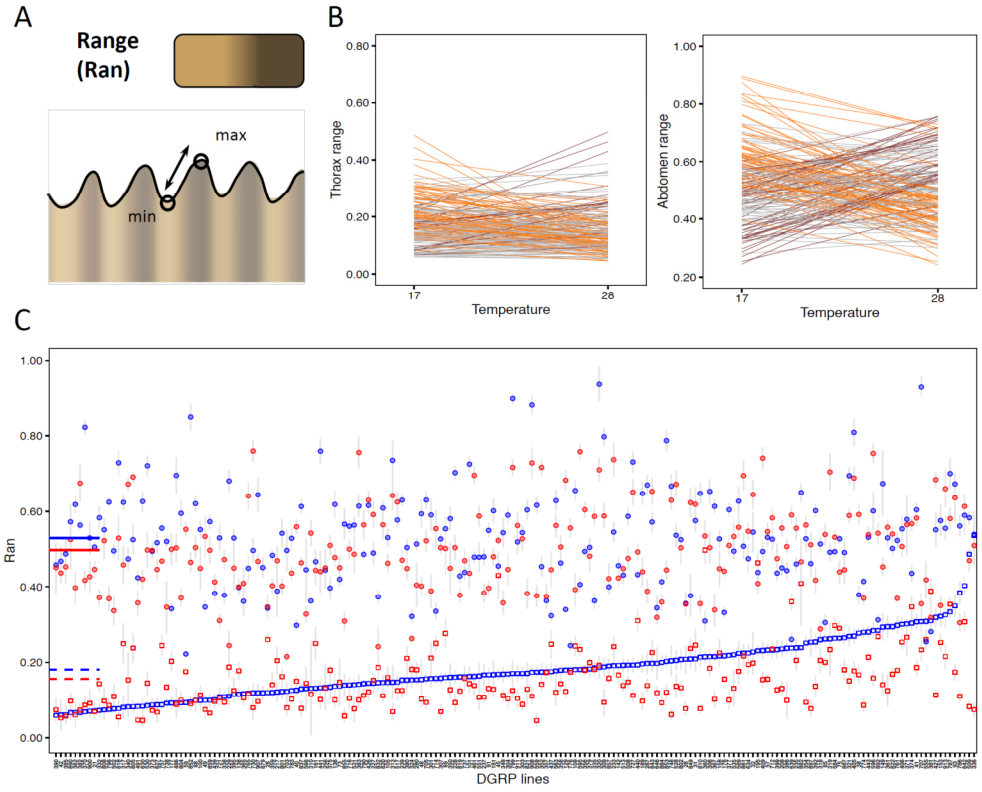


Figure 3.S1. Variation in Range between genotypes and temperatures. A. Range (Ran) was calculated as the Euclidean distance between the median value for the 20th darkest and the 20th lightest pixels in the transect. Antero-posterior (AP) plot shows the distance from each pixel along the AP axis of the transect to the white vector (RGB 0,0,0) highlighting the maximum and minimum values used to extract Ran. **B.** Reaction norms for Ran (Y axis) across temperatures (X axis) plotted as the regression fit for the model lm (Ran ~ *Temperature*) for each DGRP line. Colored lines are significantly different from zero (plastic) (positive slopes in orange, negative slopes in brown) while grey lines are non-significant (p-value>0.05). **C.** Means and confidence intervals of Ran in thoraxes (squares) and abdomens (circles) of the DGRP lines (X axis) reared at 17°C (blue) and 28°C (red). DGRP lines are ranked by the mean thoracic darkness at 17°C. Horizontal bars represent the mean darkness in thoraxes (dashed line) and abdomens (solid line) for all DGRP lines at each temperature.

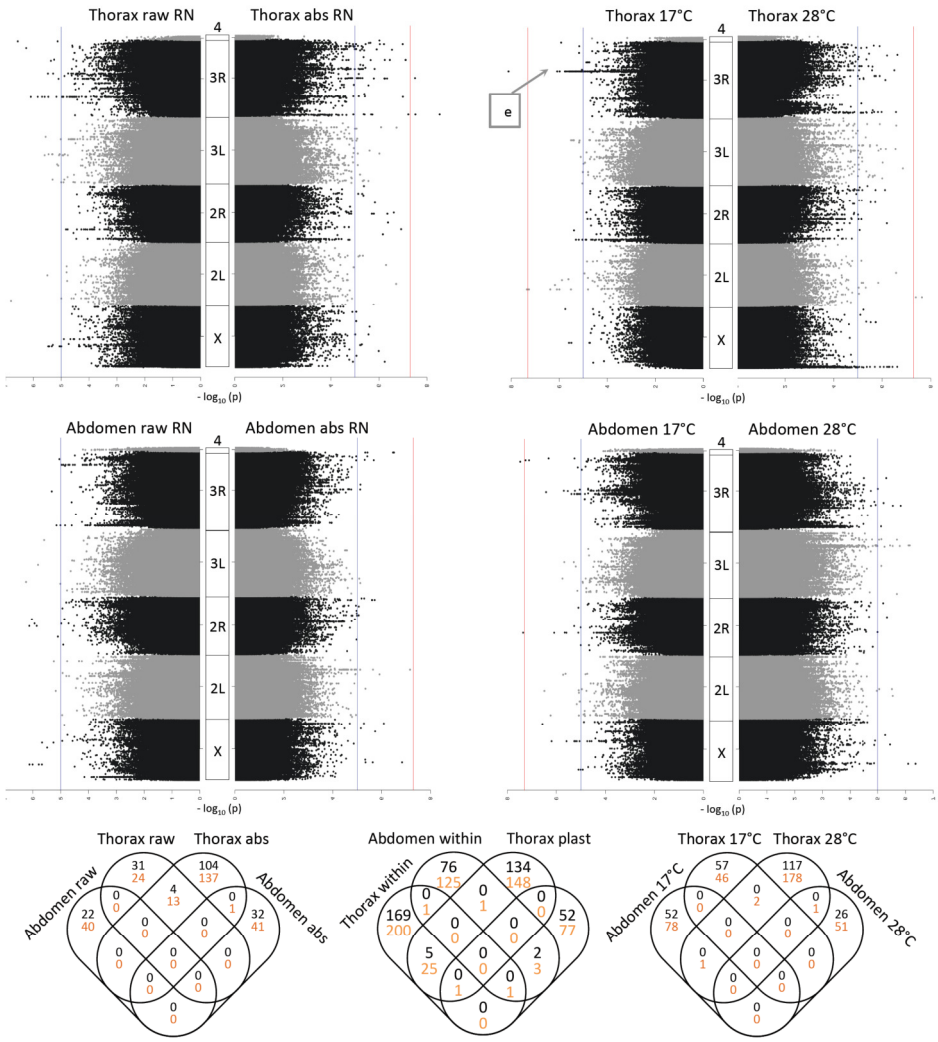


Figure 3.S2. GWAS for variation in range. Manhattan plots and Venn diagrams corresponding to the eight GWAS performed for variation in range (Ran). The significance level for each SNP along the chromosomal arms is shown as the $\log_{10}$ p-value. Horizontal lines are p-value < 10e-5 (blue) and p-value < 10e-8 (red). Some of the gene names associated to SNPs/InDels with a p-value < 10e-5 are shown in the plots. In the Venn diagrams show overlaps in the identity of the SNPs/InDels with a p-value < 10e-5 (in black) and the putatively associated genes (in orange). **A.** Manhattan plots and Venn diagrams corresponding for the four GWAS performed for variation in plasticity for Ran in thoraxes (upper panels) and abdomens (lower panels) and for either the raw (left panels) or the absolute (right panels) slopes of the reaction norms. For each body part and plasticity property, the GWAS was done testing the model lm (*Slope Ran ~ Allele + (1|Wolb|DGRP)*). **B.** Manhattan plots and Venn diagrams corresponding for the four GWAS performed for variation Ran in thoraxes (upper panels) and abdomens (lower panels) at 17°C (left panels) or 28°C (right panels). For each body part and temperature, the GWAS was done testing the model lm (*Ran ~ Allele + (1|Wolb|DGRP)*).

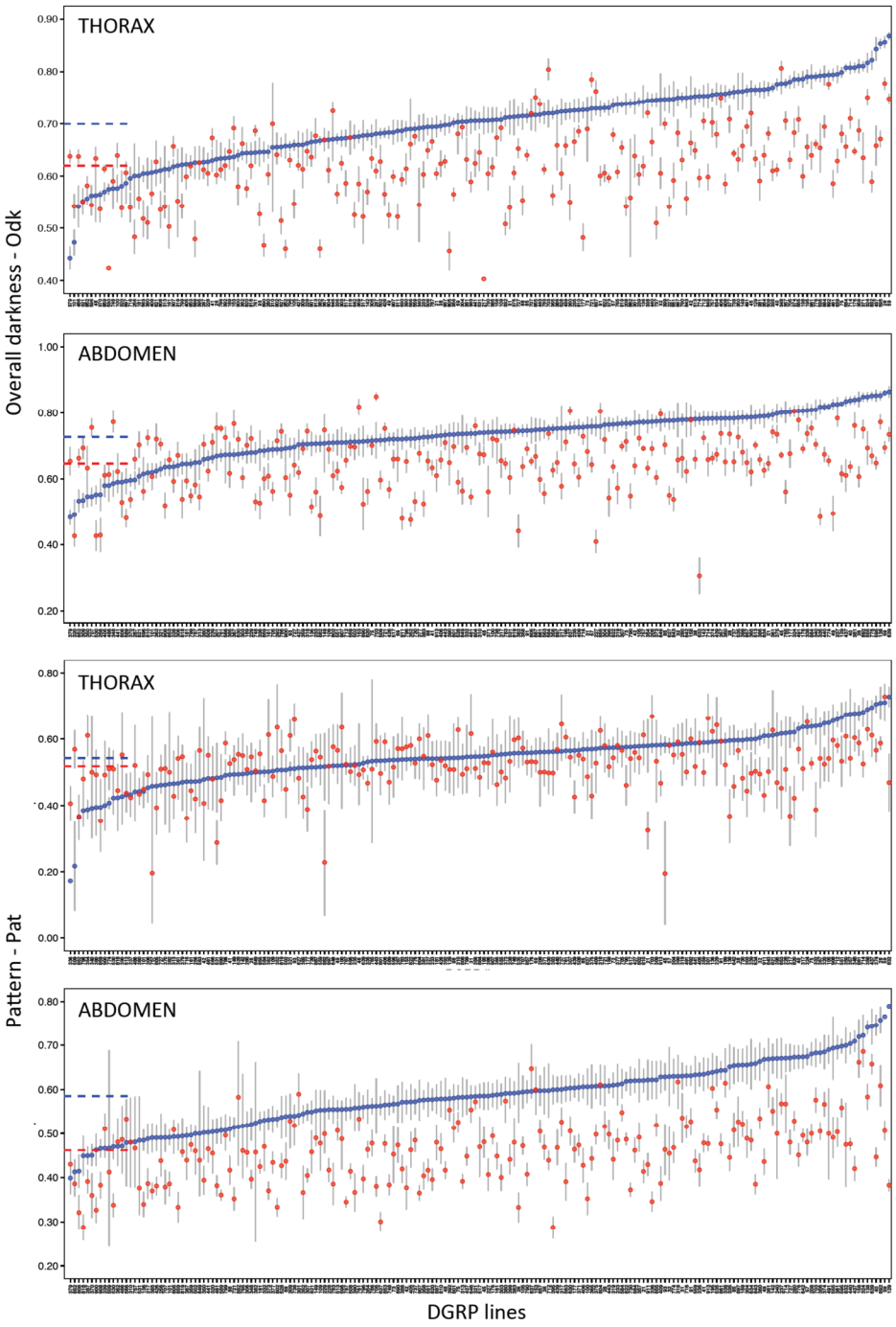


Figure 3.S3. Phenotypic variation in darkness (Odk) and pattern (Pat). Means and confidence intervals (Y axis) for Odk and Pat in thoraxes and abdomens of females from the DGRP lines (X axis) reared at 17℃ (blue) and 28℃ (red). DGRP lines are ranked by their mean size at 17℃. Dashed horizontal bar represents the mean value for all DGRP lines at a given temperature.

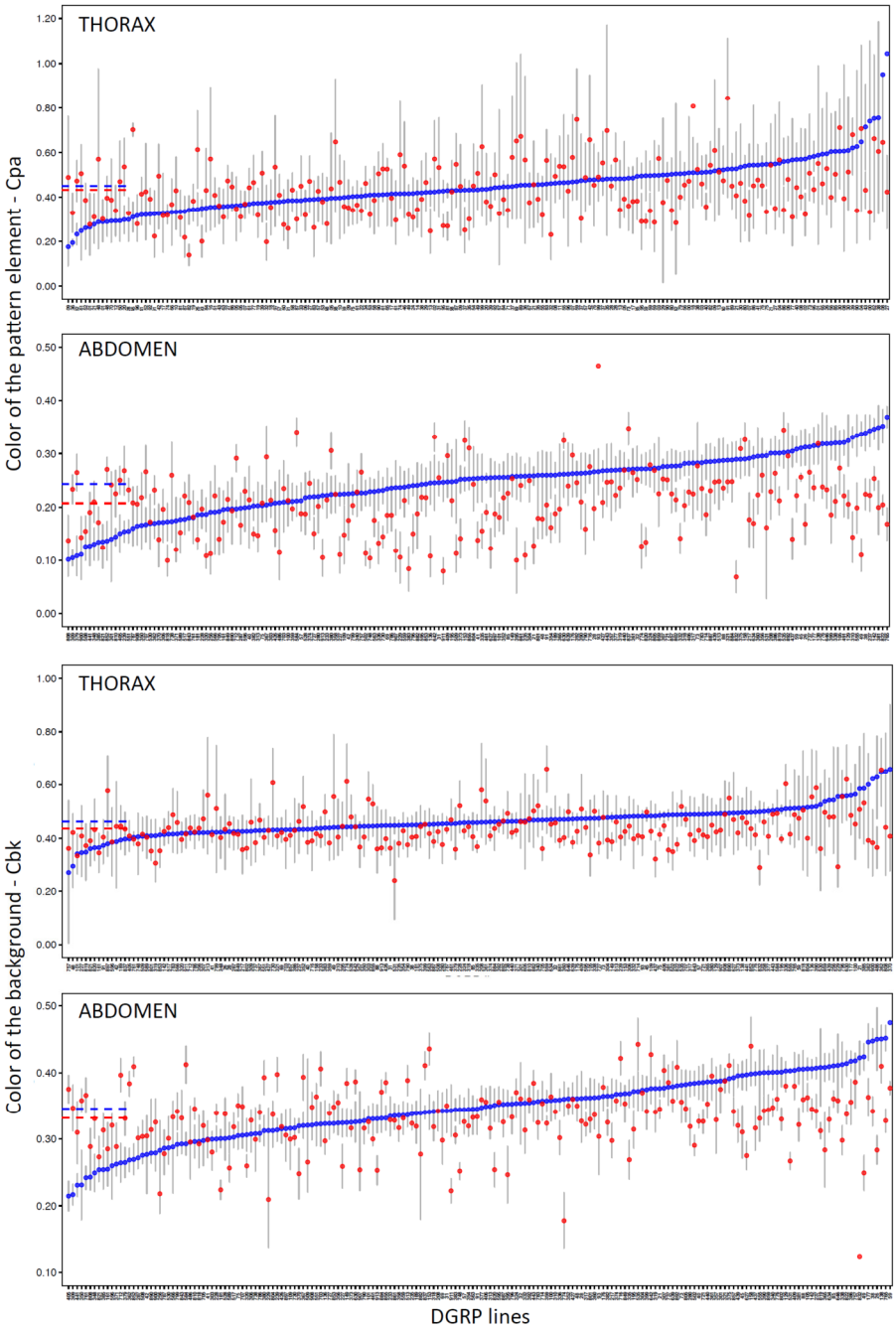


Figure 3.S4. Phenotypic variation in color of the background (Cbk) and color of the pattern element (Cpa). Means and confidence intervals (Y axis) for Cbk and Cpa in thoraxes and abdomens of females from the DGRP lines (X axis) reared at 17℃ (blue) and 28℃ (red). DGRP lines are ranked by their mean size at 17℃. Dashed horizontal bar represents the mean value for all DGRP lines at a given temperature.

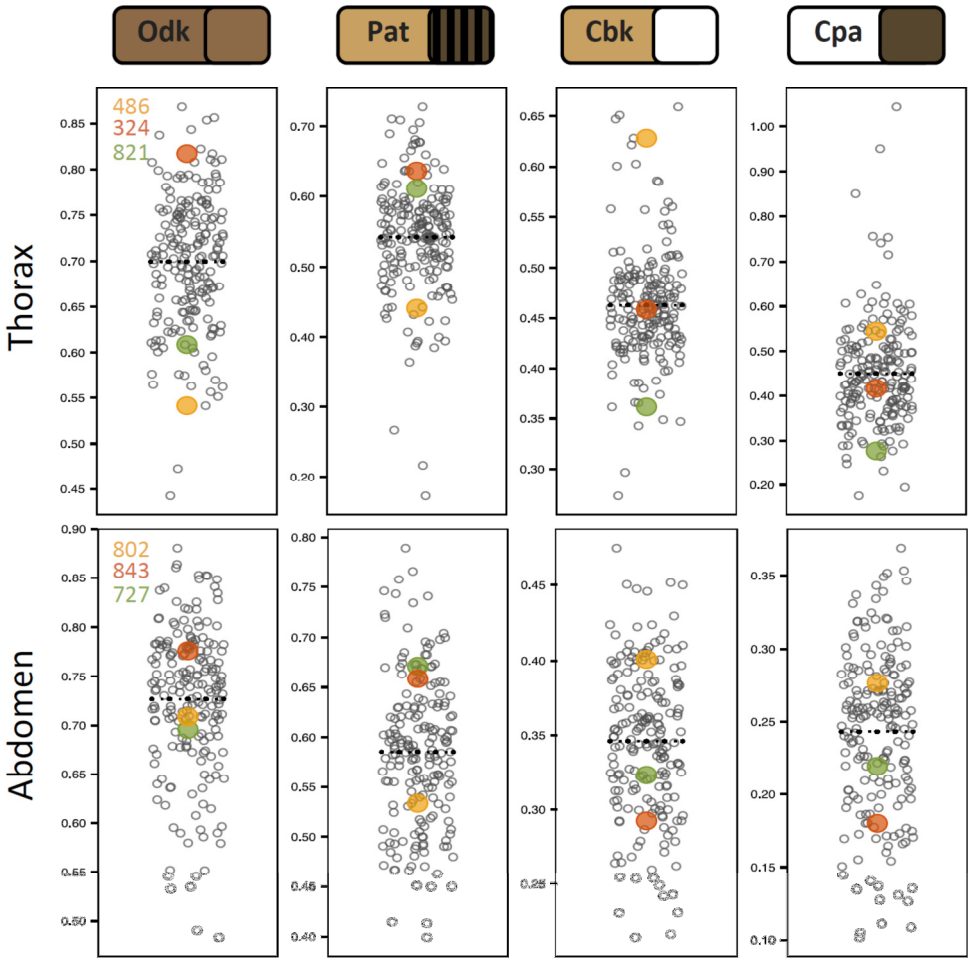
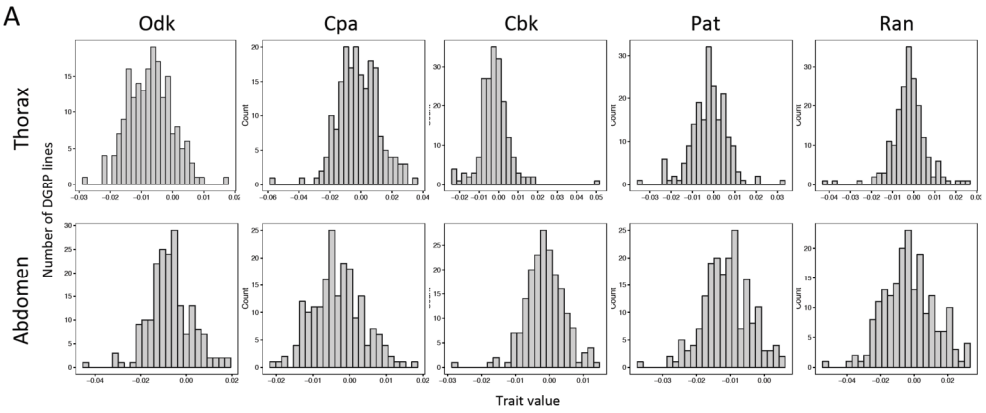


Figure 3.S5. Differences in pigmentation components between genotypes. Mean values of the pigmentation components in thoraxes and abdomens of DGRP lines reared at 17℃. Three genotypes with different trait contributions are highlighted.

A



B

GO term	Description	P-value	FDR q-value
GO:0007409	axonogenesis	6,14E-05	2,10E-01
GO:0045595	regulation of cell differentiation	4,67E-04	8,00E-01
GO:0007631	feeding behavior	4,70E-04	5,36E-01
GO:0051962	positive regulation of nervous system development	8,44E-04	7,23E-01
GO:0010453	regulation of cell fate commitment	9,76E-04	6,69E-01

Figure 3.S6. Slope of the reaction norm as a quantitate trait. A. Histograms for the raw value of the slope of the reaction norms for each of our pigmentation traits (Odk, Pat, Cbk, Cpa and Ran) in the DGRP lines, calculated as the slope of the regression lm (*Trait ~ Temperature*) per line. **B.** Results from the gene-ontology analysis performed with software GOrilla, using all the SNPs/InDels with a p-value < 10e-5 associated with variation in plasticity. Results from the GWAS for both raw and absolute slopes of the reaction norms of all pigmentation components were pooled for this analysis.

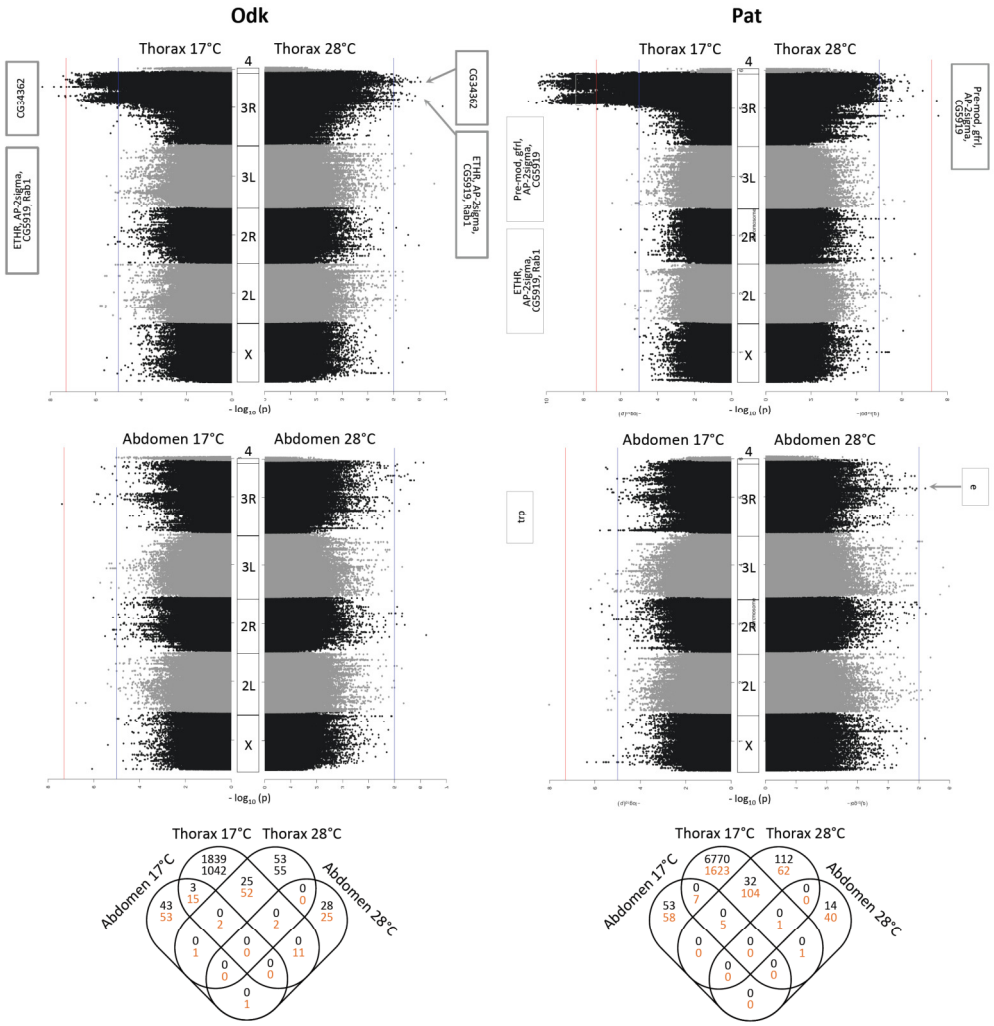


Figure 3.S7. GWAS for in overall darkness (Odk) and pattern (Pat). Manhattan plots and Venn diagrams corresponding to the GWAS performed for variation in overall darkness (Odk) and Pattern (Pat). In the Manhattan plots, the significance level for each SNP along the chromosomal arms is shown as the $\log_{10}$ p-value. Horizontal lines are p-value < 10e-5 (blue) and p-value < 10e-8 (red). Some of the gene names associated to SNPs/InDels with a p-value < 10e-5 are shown in the plots. For each trait, upper and lower panels correspond to GWAS for thoracic and abdominal traits respectively, and left and right panels correspond to GWAS for 17℃ and 28℃ respectively. All GWAS were done testing the model lm (*Trait ~ Allele + (1|Wolb|DGRP)*). Venn diagrams show overlaps in the identity of the SNPs/InDels with a p-value < 10e-5 (in black) and the putatively associated genes (in orange).

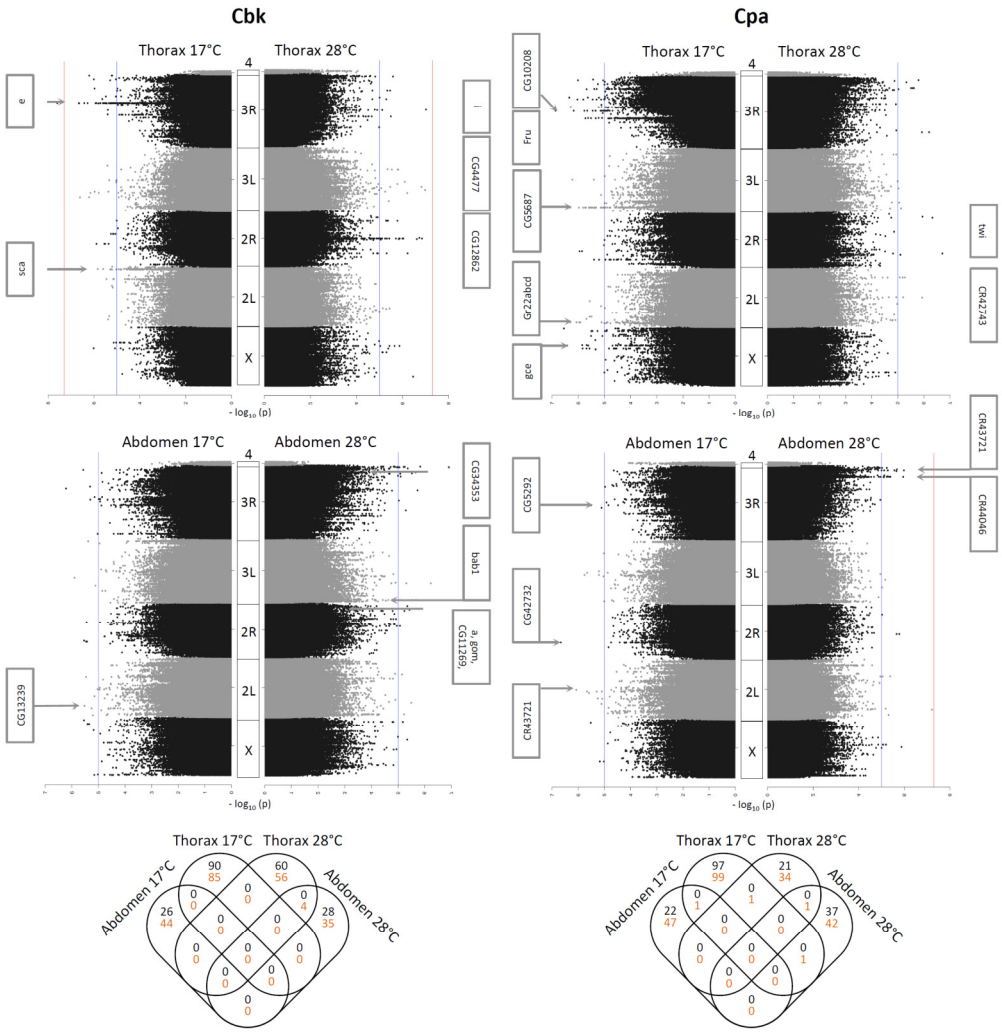


Figure 3.S8. GWAS for color of the background (Cbk) and color of the pattern element (Cpa). Manhattan plots and Venn diagrams corresponding to the GWAS performed for variation in color of the background (Cbk) and color of the pattern element (Cpa). In the Manhattan plots, the significance level for each SNP along the chromosomal arms is shown as the $\log_{10}$ p-value. Horizontal lines are p-value < 10e-5 (blue) and p-value < 10e-8 (red). Some of the gene names associated to SNPs/InDels with a p-value < 10e-5 are shown in the plots. For each trait, upper and lower panels correspond to GWAS for thoracic and abdominal traits respectively, and left and right panels correspond to GWAS for 17°C and 28°C respectively. All GWAS were done testing the model lm (*Trait ~ Allele + (1|Wolb|DGRP)*). Venn diagrams show overlaps in the identity of the SNPs/InDels with a p-value < 10e-5 (in black) and the putatively associated genes (in orange).

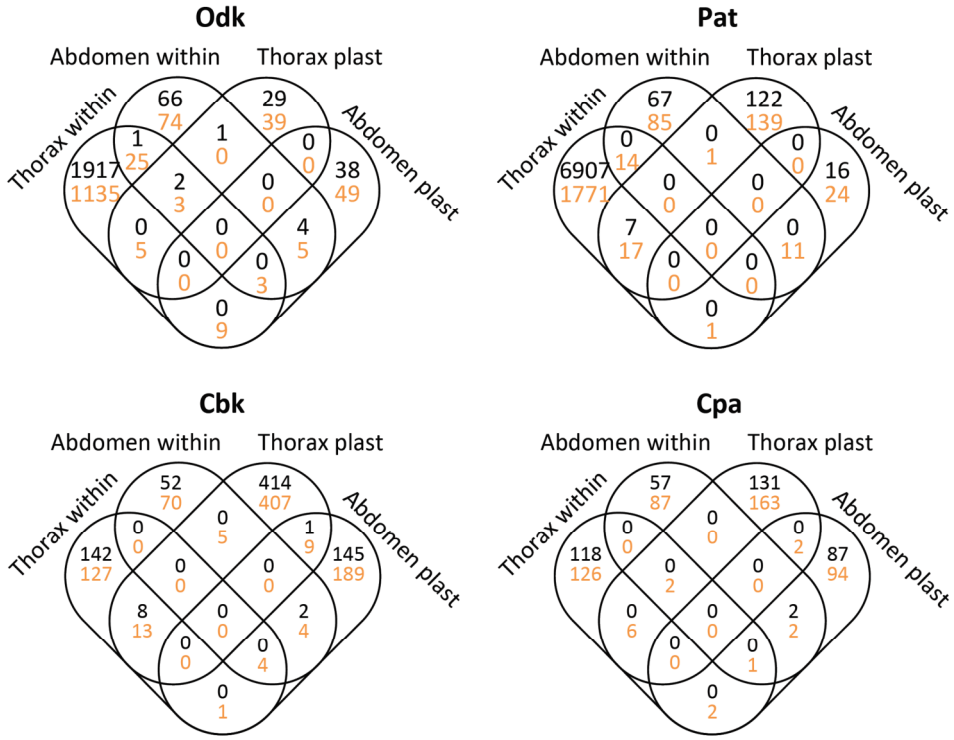


Figure 3.S9. Overlaps between QTLs for within and between-environment variation in pigmentation components. Venn diagrams showing overlaps in the identity of the SNPs/InDels with a p-value < 10e-5 (in black) and the putatively associated genes (in orange) for our different GWAS for variation in plasticity and for within-environment variation of all pigmentation components.

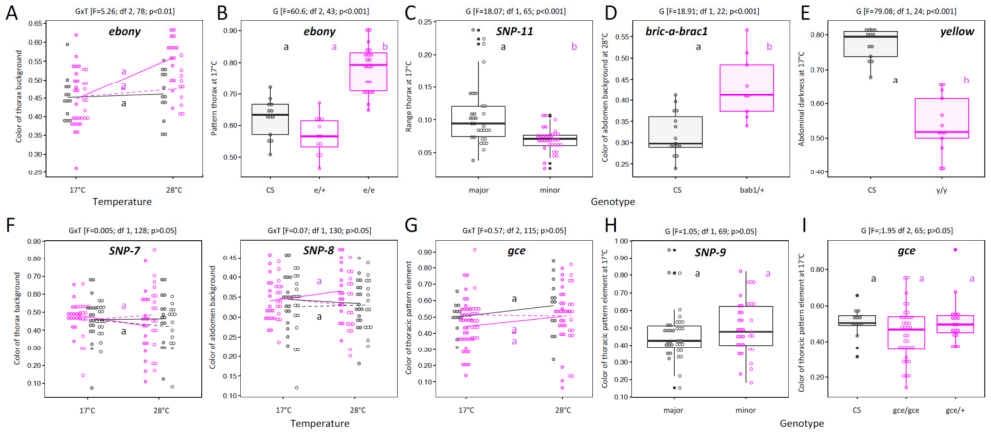


Figure 3.S10. Functional validations of GWAS results. A. Reaction norms for color of the thoracic pattern element in *ebony* homozygote mutants (*e/e*; magenta filled circles, solid line), *ebony* heterozygote (e/+; magenta empty circles, dashed line) and wild-type *Canton-S* (*CS*; black). **B.** Thorax pattern at 17℃ in *ebony* homozygote mutants (*e/e*; magenta filled circles), *ebony* heterozygote (*e/+*; magenta empty circles) and wild-type *Canton-S* (*CS*; black). **C.** Thorax range at 17℃ in females from MR populations corresponding to SNP-11 (genomic position 3R:21236915). **D.** Color of abdomen background in *bric-a-brac* heterozygote mutants (*bab1/+*; magenta) and wild-type *Canton-S* (*CS*; black). **E.** Abdominal darkness in flies reared at 17℃ from *yellow* homozygote mutants *(y/y*; magenta) and wild-type *Canton-S* (*CS*; black). **F.** Negative validations for QTLs associated with variation in plasticity. From left to right: individual phenotypic values and reaction norms for the traits: color of thorax background and color of abdomen background, in females from different MR populations corresponding to SNP-7 (genomic position 2L:17488875) and and SNP-8 (genomic position 3L:2773539), respectively. **G-I:** Negative validations for QTLs associated with within-environment variation. **G.** Phenotypic values and reaction norms for the color of thoracic pattern element in *gce* homozygote mutants (*gce/gce*; magenta filled circles, solid line), *gce* heterozygote mutants (*gce/+*; magenta empty circles, dashed line) and wild-type *Canton-S* (*CS*; black). **H.** Individual phenotypic values for the color of thoracic pattern element at 17℃ in females from different MR populations corresponding to SNP-9. **I.** Phenotypic values for the color of thoracic pattern element in *gce* homozygote mutants (*gce/gce*; magenta filled circles, solid line), *gce* heterozygote mutants (*gce/+*; magenta empty circles, dashed line) and wild-type *Canton-S* (*CS*; black).

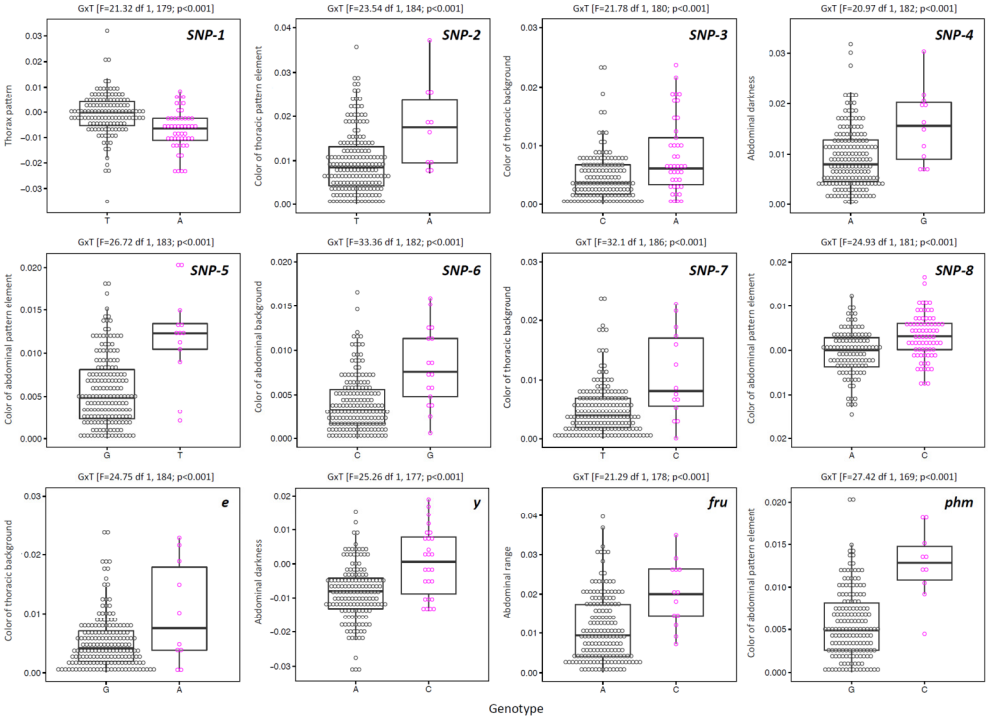


Figure 3.S11. Effects of candidate SNPs in the reaction norms of the DGRP. For each candidate SNP/gene, plots show the slope of the reaction norm for correspondent trait in the DGRP lines with the major (black) and the minor (magenta). Details about the SNP position and identity can be found in Table 3.2 and Tables 3.S3 and 3.S4. The result of the model lm (*Slope ~ Genotype*Temperature)* is shown above the plot. The significant differences among groups were estimated by post hoc comparisons (Tukey's honest significant differences) and are indicated by different letters in each plot.

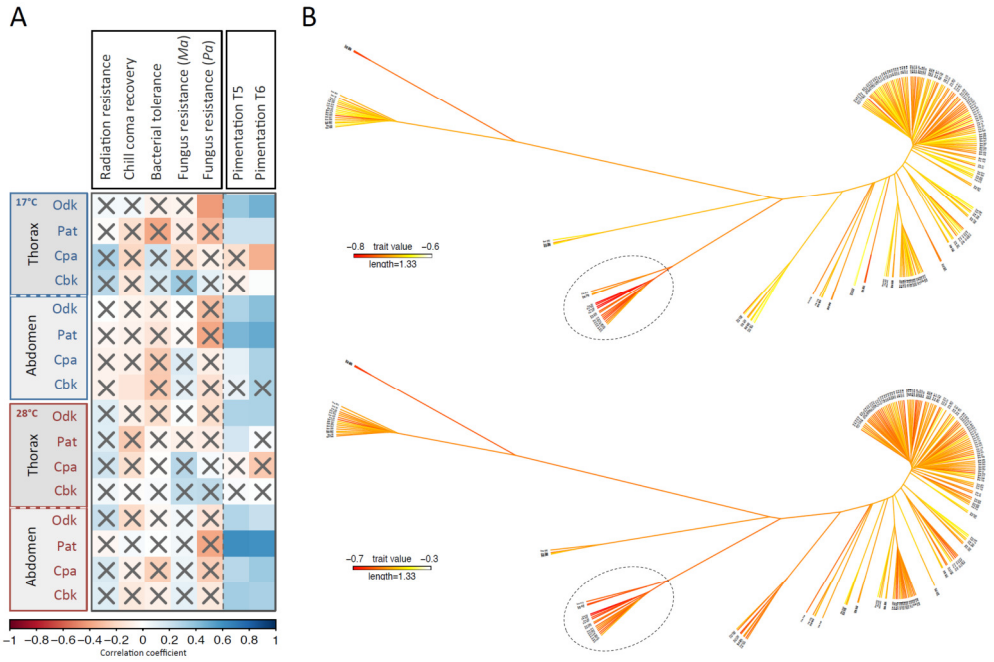
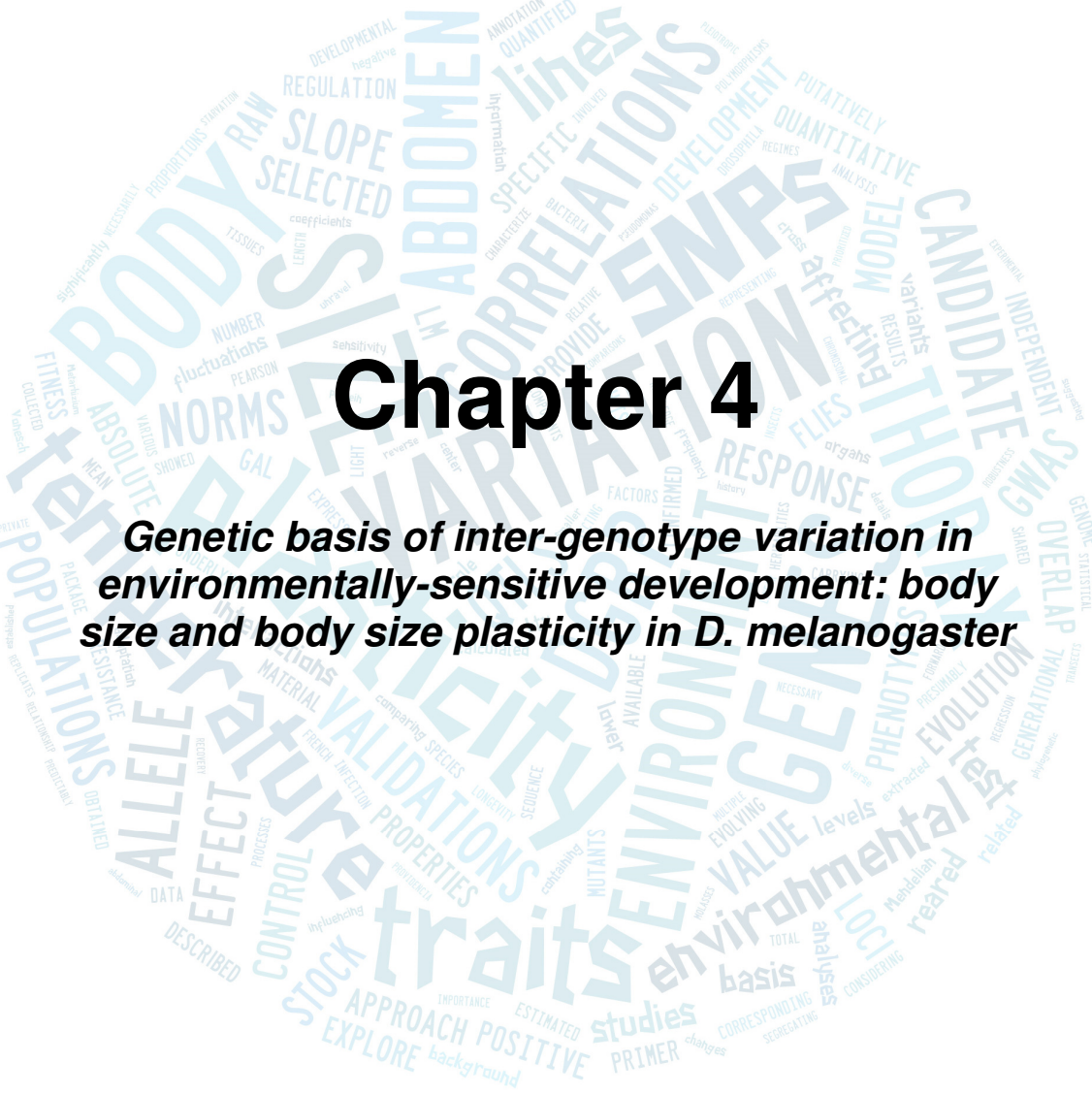


Figure S12. Variation in plasticity for pigmentation components. A. Heat map of Pearson's correlation coefficients between pigmentation components at each temperature and traits quantified in the DGRPs by others – this includes traits previously associated with pigmentation differences (radiation resistance, chill coma recovery and immune related traits) as well as quantifications from abdominal pigmentation (in tergites T5 and T6) from Dembeck et al (Dembeck *et al.* 2015). Positive correlations are denoted in blue and negative relationships in red. Non-significant correlations (p-value > 0.01) are indicated with an 'X'. **B.** Cluster analysis of the genetic relatedness among DGRP lines represented as an unrooted tree. Continuous variation in Odk at 28°C (upper panel) and Pat at 17°C (lower panel) is mapped on the branches based on the mean trait values of each DGRP line. The clade containing DGRP lines 819, 352, 712, 59, 324, 28, 437, 555, 810, 374, 239, 714, 861, 908, 707 and 820 is encircled; these lines have relatively high values of Odk and Pat at 17°C.



Chapter 4

Genetic basis of inter-genotype variation in environmentally-sensitive development: body size and body size plasticity in D. melanogaster

ABSTRACT

Body size is a quantitative trait closely related to fitness that is under the control of both genetic and environmental factors. In insects, it is well known to be affected by the temperature at which development occurs. Developmental plasticity for this and other traits is heritable and under selection, but little is known about the genetic basis for variation in plasticity, which provides the raw material for its evolution. We quantified genetic variation for body size and body size plasticity in *Drosophila melanogaster* by measuring thoraxes and abdomens of females from a panel representing naturally segregating allelic variants, the DGRP. We found variation between genotypes for the size of both body parts, and also for the levels and direction of thermal plasticity therein. We also found few significant correlations between our traits, as well as with other fitness-associated traits measured for the same genotypes in other studies. We then performed genome wide association studies (GWAS) to unravel the genetic basis of variation in body size plasticity. We found that different QTLs contribute to variation in: 1) size plasticity in the thorax versus abdomen, 2) size versus size plasticity, 3) level and the direction of the plastic response. We used different approaches to validate selected QTLs and explore pleiotropic effects of the plasticity candidates. Our data sheds light onto the nature of the inter-individual variation in size plasticity, necessary for the evolution of plasticity under heterogeneous environments.

INTRODUCTION

Body size can have a great impact on individual performance (Peters 1986; Kingsolver & Huey 2008), as well as on species' extinction rate (Ripple *et al.* 2017). Diversity in body size is shaped by the reciprocal interactions between the developmental processes that regulate individual growth, and the evolutionary forces that determine which phenotypes increase in frequency across generations (see Smith & Lyons 2013). Studies in different animal models have provided insight about the molecular mechanisms for proper regulation of body size and body proportions during development (e.g. Twombly & Tisch 2000; Glazier 2008; Gokhale & Shingleton 2015; Nagashima, Ishiura & Suo 2017), and about the selection agents that shape the evolution of body size. The latter includes predators (Lafferty & Kuris 2002; Barnes *et al.* 2010), mates (e.g. Head, Kozak & Boughman 2013) and thermal regimes (Gibert & DeLong 2014; Mitchell *et al.* 2017). Body size is controlled by both genetic and environmental factors (D'Amico, Davidowitz & Nijhout 2001; Gadau, Page & Werren 2002; Nijhout 2003; Mirth & Shingleton 2012) and varies greatly within and between populations (Peters 1986; Woodward *et al.* 2005).

Body size is plastic in relation to different external factors, such as nutrition and temperature, and is a prime example of the environmental regulation of development, or developmental plasticity. Plasticity can provide the means by which organisms cope with environmental heterogeneity and thus, it can have important implications for population persistence. Thermal plasticity in body size has been described for different species of insects (see Harrison, Woods & Roberts 2012). Presumably advantageous for thermal-regulation, the development under colder temperatures results in larger bodies (Angilletta Jr *et al.* 2002; Kingsolver & Huey 2008). Thermal plasticity can be described in the form of thermal reaction norms (Schlichting & Pigliucci 1998) whose properties might vary between genotypes (Newman 1994; Lardies 2008). The genes underlying variation in plasticity (e.g. those affecting properties of reaction norms) can provide the raw material for the

evolution of plasticity. Little is known about what these genetic variants are and what functions they perform (e.g. perception of external environment, conveying information about that to developing organs, or effector genes expressed in plastic organs). It is also unclear to what extent the same loci contribute to thermal plasticity in size of different body parts, and whether the loci contributing to variation for size plasticity are the same that contribute to size variation within environments.

Studies in *D. melanogaster* have provided much insight about the evolution and development of body size and body size plasticity (Partridge *et al.* 1994; French, Feast & Partridge 1998; Robinson & Partridge 2001; Bochdanovits & de Jong 2003). The regulation of body size and body proportions involves the coordinated action of different endocrine systems (Oldham & Hafen 2003; Colombani *et al.* 2005; McBrayer *et al.* 2007; Mirth *et al.* 2014). Differences between populations, including latitudinal clines and seasonal differences, and among individuals within a population, are due to effects of genes, environment, as well as to genotype-by-environment interactions (French et al. 1998; Gockel et al. 2002; De Jong & Bochdanovits 2003; Mendes & Mirth 2016). We have knowledge on the genetic basis of adaptation and of natural variation for many adaptive traits (e.g. Orr & Irving 1997; Takahashi & Ting 2004; Pool & Aquadro 2007). More recently, widely-accessible mapping panels (e.g. DGRP and DSPR) allow characterization of correlations between different traits and their genetic basis (King, Macdonald & Long 2012; Mackay *et al.* 2012; Huang *et al.* 2014). This has been done for many traits (e.g. Mackay *et al.* 2012; Huang *et al.* 2014; Ivanov *et al.* 2015; Wang, Lu & St. Leger 2017), including size (Vonesch *et al.* 2016), typically under a single environment. Therefore, we still have little knowledge about the genetic basis under different environments as well as genetic basis of plasticity. These series of genotypes can be reared under different conditions to characterize reaction norms and ask about the genes that harbor allelic variation for their properties.

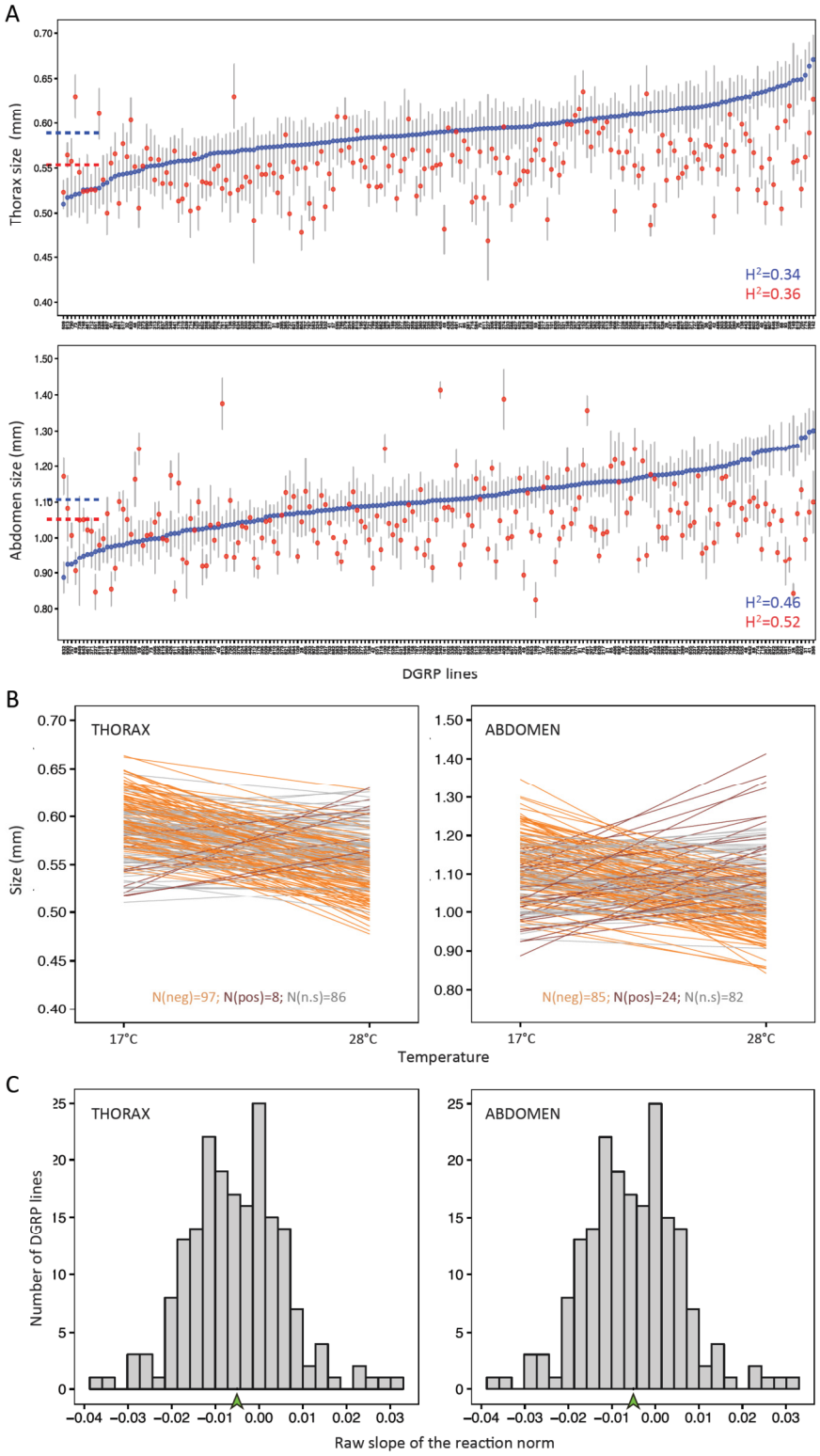
Here, we used a panel of isogenic lines representing naturally segregating alleles from one natural population, the DGRP, to characterize genetic variation for thermal plasticity in thorax and abdomen size, and to identify loci contributing to variation in the slope of thermal reaction norms. We document correlations between body size and body size plasticity, as well as correlations between these and other traits investigated in the same lines in other studies. We also ask about the extent of overlap between QTLs for size and for size plasticity, and between QTLs for size plasticity of the different body parts. We then use different approaches to validate the role of selected QTLs in body size at different temperatures.

RESULTS

We measured thorax and abdomen size in adult females from different genotypes reared at two different temperatures. We documented effects of genotype, environment and genotype-by-environment interactions on body size (Figure 4.1), and explored correlations between body parts and between temperatures (Figure 4.2). A GWAS then identified DNA sequence polymorphisms associated with variation in body size plasticity (Figure 4.3). Ensuing functional analyses of candidate QTLs validated and clarified their role in body size variation at different temperatures (Figure 4.4).

Between and within genotype variation for body size and body size plasticity

To assess body size variation and the contribution of genetic and environmental factors, we quantified length of abdomens and thoraxes (Figure 4.1A, Figure 4.S1A) of adult females from ~196 DGRP genetic backgrounds reared at either 17°C or 28°C (Table 4.S1 and Table 4.S2). We found significant differences between genotypes (DGRP) and developmental environments (rearing temperature), as well as significant genotype-by-environment interaction effects (thermal plasticity) for both thorax and abdomen sizes (Figure 4.1A, 4.S1C). We also found appreciable differences


A
Thorax size (mm)
H²=0.34
H²=0.36
Abdomen size (mm)
H²=0.46
H²=0.52
DGRP lines
B
THORAX
ABDOMEN
Size (mm)
N(neg)=97; N(pos)=8; N(n.s)=86
N(neg)=85; N(pos)=24; N(n.s)=82
17°C
28°C
Temperature
C
THORAX
ABDOMEN
Number of DGRP lines
Raw slope of the reaction norm

between individuals of (presumably) the same genotype and same rearing temperature; the coefficients of variation (CV), a measure of relative variation that accounts for the fact that variance increases with the mean, were, in average, lower for thorax (i.e. CV=6.25 at 17ºC and CV=6.8 at 28ºC) than for abdomen size (i.e. CV=7.1 at 17ºC and CV=7.7 at 28ºC). Broad-sense heritabilities calculated based on variance components (Table 4.S3) were of the same order but, to some extent, lower for thorax relative to abdomen size, lower for 17ºC relative to 28ºC, and lower for plasticity (between-environment variation) relative to within-environment variation.

To explore how the sizes of the body parts are related to each other and whether temperature influences their association, we investigated the correlations between different traits (Figure 4.2). First, we estimated correlations between body parts measured at the same temperature and found significant positive correlations between thorax and abdomen size (Figure 4.2A), both considering the mean for each DGRP line (Pearson correlation) and considering all individuals measured, controlling for genotype (partial Pearson correlation). Second, we investigated correlations between the extent of inter-individual variation, measured as the coefficient of variation (CV), across body parts and temperatures. We found thorax and abdomen size CV to be positively correlated for measurements at 28ºC, but

Figure 4.1. Phenotypic variation in size and size plasticity. Phenotypic variation for thorax size is shown in the upper panels for abdomen size in the lower panels. A. Means and confidence intervals (Y axis) for size in the DGRP lines (X axis) reared at 17ºC (blue) and 28ºC (red). DGRP lines are ranked by their mean size at 17ºC. Dashed horizontal bar represents the mean value for all DGRP lines at a given temperature. Mean values (μ) and broad sense heritability estimates (H^2) were: μ = 0.59 mm; H^2 = 0.34 (thorax at 17ºC), μ = 0.55 mm; H^2 = 0.36 (thorax at 28ºC), μ = 1.11 mm; H^2 = 0.46 (abdomen at 17ºC) and μ = 1.05 mm; H^2 = 0.52 (abdomen at 28ºC). B. Reaction norms for size (Y axis) across temperatures (X axis) plotted as the regression fit for the model lm (*Size* ~ *Temperature*) for each DGRP line. Colored lines are significantly different from zero (positive slopes in orange, negative slopes in brown) while grey lines are non-significant (p-value>0.05). Broad sense heritability estimates, H^2, were: 0.33 (thorax plasticity) and 0.49 (abdomen plasticity). C. Histograms for the raw value of the slope of the regression lm (*Size* ~ *Temperature*) in the DGRP lines. Mean value for the raw slope of all DGRP lines is indicated with a green arrowhead.

not 17°C (Figure 4.2B). Within temperatures, we found that the CV was negatively correlated with mean size for the thorax (both at 17°C and 28°C), but not abdomen (Figure 4.2B). Finally, we estimated correlations across temperatures and found a significant positive correlation for thorax but not for abdomen size (Figure 4.2B).

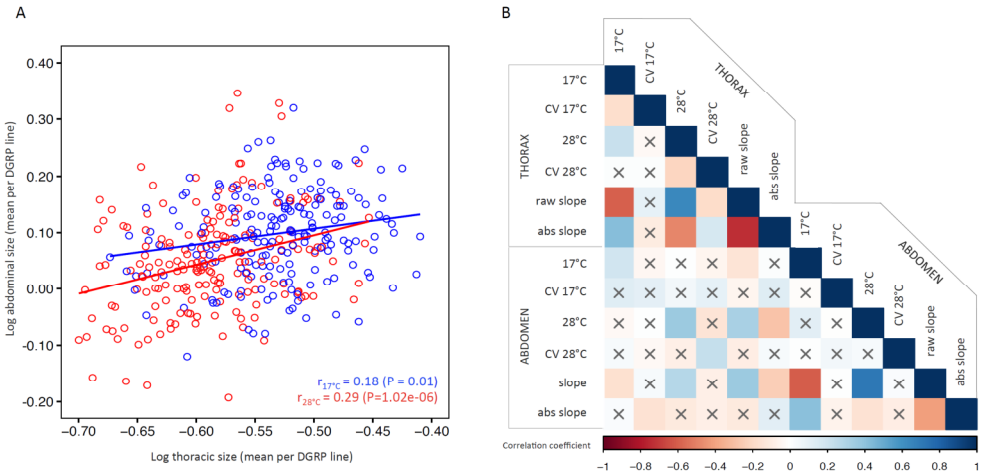


Figure 4.2. Phenotypic co-variation in size and size plasticity. A. Thoracic (X axis) and abdominal (Y axis) mean size per DGRP line and estimated for Pearson's correlation coefficient at 17°C (blue) and at 28°C (red). Partial Pearson correlation coefficient was r= 0.34 (p-value = 1.02e-06) for 17°C and r=0.33 (p-value < 2e-16) for 28°C. **B.** Heat map of Pearson's correlation coefficients between our traits: mean size and coefficient of variation (CV) per temperature and body part and plasticity (raw and absolute slopes of the reactions norms) per body part. Positive correlations are denoted in blue and negative correlations in red. Non-significant correlations (p-value > 0.01) are indicated with an 'X'.

Thermal reaction norms for body size

We studied the extent and properties of thermal plasticity for body size in the DGRP lines by analyzing thermal reaction norms (Figure 4.1B). We calculated the slope of the regression for size across temperatures for each body part and DGRP line and found genetic variation for both the intercept and slope of the reaction norms (Figure 4.1B, Table 4.S2). Taking into account the reaction norms obtained for 191 DGRP lines (those for which we could obtain sufficient individuals at both temperatures), we identified plastic and non-plastic genotypes; slope significantly different from zero (p-

value<0.05) for 55% for the thorax and 57% for the abdomen, with most of the plastic genotypes having smaller sizes for flies reared at higher temperature. However, we also found genotypes with plasticity in the opposite direction (i.e. smaller flies at lower temperature), corresponding to a positive significant slope for the thermal reaction norms: 8% for the thorax and 22 % for the abdomen. Note that there was not necessarily a match in thermal plasticity for the two body parts: genotypes could be plastic for only one body part and also plastic in different directions for the two body parts. (Figure 4.1B).

From each reaction norm, we extracted two properties of the thermal plasticity in body size: the absolute value of the slope, as a measurement of thermal sensitivity, describing only the magnitude of the response to temperature, and the raw value of the slope as a measurement which describes also the direction of that response (Figure 4.1C, Figure 4.S1D). We then estimated correlations between plasticity traits and a number of other traits (Figure 4.2B). For thorax but not abdomen size, we found that genotypes with higher levels of inter-individual variation (for same temperature and same genotype) had reaction norm slopes that were more negative. This was reflected in a correlation between CV that was negative for the raw values of the slope and positive for the absolute values. Finally, analysis of the genotypes individually showed that lines more plastic for one body part were not necessarily more plastic for the other body part (Figure 4.S1B). When considering all the DGRP lines together, we saw a significant positive correlation between the raw value of the slope of the thorax and abdomen reaction norms (Pearson correlation r=0.36, p-value<0.0001).

Genetic basis of variation in body size plasticity

We used a Genome-Wide Association Study (GWAS) approach to unravel the genetic basis of thermal plasticity for thorax and abdomen size (Materials and Methods). First, because the loci carrying allelic variation for the

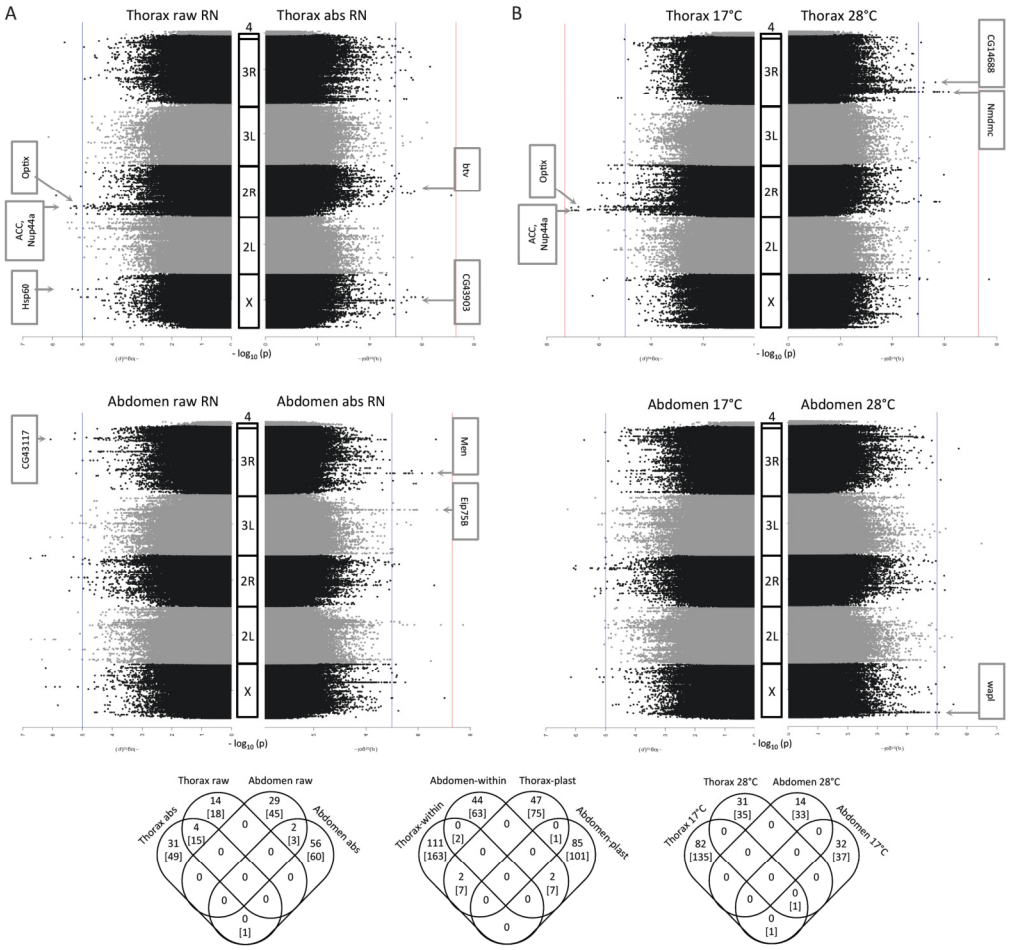


Figure 4.3. GWAS for variation in size and size plasticity. Manhattan plots and Venn diagrams corresponding to the eight GWAS performed for variation in size. The significance level for each SNP along the chromosomal arms is shown as the $\log_{10}$ p-value. Horizontal lines are p-value < 10e-5 (blue) and p-value < 10e-8 (red). Some of the gene names associated to SNPs/InDels with a p-value < 10e-5 are shown in the plots. **A.** Manhattan plots and Venn diagrams corresponding to the four GWAS performed for variation in size plasticity in thoraxes (upper panels) and abdomens (lower panels) and for either the raw (left panels) or the absolute (right panels) slopes of the reaction norms. For each body part and plasticity property, the GWAS was done testing the model lm (*Slope ~ Allele + (1|Wolb|DGRP)*). **B.** Manhattan plots and Venn diagrams corresponding for the four GWAS performed for variation in size in thoraxes (upper panels) and abdomens (lower panels) at 17°C (left panels) or 28°C (right panels). For each body part and temperature, the GWAS was done testing the model lm (*Size ~ Allele + (1|Wolb|DGRP)*).

direction and extent of environmental responsiveness are not necessarily the same, we used both the raw and absolute values of the slopes of the DGRP reaction norms as our quantitative traits (Figure 4.3A, Figure 4.S3). Second, to explore to what extent loci carrying allelic variation for plasticity in body size also contribute to within-environment variation in body size, we ran GWAS analysis using as quantitative traits body size for flies reared at 17°C and body size for flies reared at 28°C (Figure 4.3B, Figure 4.S4).

Even though natural variation in size had been previously shown to be affected by chromosomal inversions (Fernández Iriarte, Norry & Hasson 2003; De Jong & Bochdanovits 2003; Kapun *et al.* 2016), we did not find an effect on our traits (model lm (*Trait ~ Inversion);* p-value > 0.01). We confirmed that genetic relatedness among DGRP lines was not significantly associated with any of our traits (assessed by low and non-significant coefficients of phylogenetic signal Blomberg's K and Pagel's λ (Figure 4.S2C).

We identified candidate QTLs significantly associated (p<10e-5) with variation in size and size plasticity (raw and absolute slope) for both body parts (thorax and abdomen). Analysis of the overlap between significant SNPs/InDels and corresponding genes for raw versus absolute values of slopes of reaction norms, as well as for plasticity versus within-environment variation (Figure 4.3) revealed private QTLs were putatively associated with trait-specific, body-part-specific and environment-specific variation as well as common QTLs. For the two measurements of plasticity, the largest extent of overlap was seen for hits for raw versus absolute slopes of thorax reaction norms (4 SNPs, 15 genes). We had much less overlap for abdominal reaction norms (2 SNPs, 3 genes), and essentially no overlap between body parts (0 SNPs, 1 gene: *Sdc* for absolute slopes of thorax and abdomen). We also saw very little overlap for hits affecting body size variation in between the two temperatures, as well as for hits for plasticity and those for within-environment variation. Comparing hits for body size variation at 17°C and at 28°C, we found different SNPs (affecting gene *RunxB*) putatively affecting

thorax size at both temperatures and abdomen size at 17°C, and different SNPs (affecting gene *Dif*) putatively affecting abdomen and thorax size (at 17°C). Finally, we also compared candidate QTLs for plasticity variation with candidate QTLs for within-environment variation. We found 2 shared SNPs and 7 shared genes for the thorax, 2 shared SNPs and 50 shared genes for the abdomen, and no shared SNP or gene across body parts.

Figure 3 shows the number and overlap between significant SNPs/InDels (p-value threshold of 10e-5) for the eight different GWAS analyses. Tables S4 and S5 provide details about each of the significant SNPs/InDels, including which genes they are putatively associated to as well as which gene regions they fall within (e.g. UTR, intronic, coding). Note that not only can different SNPs/InDels affect the same gene (multiple sequence polymorphisms in the same locus), but the same SNP can also be associated to different genes (when there is no certainty of its putative effect based on the genome annotation). For the polymorphisms significantly associated with variation in our size and size plasticity traits, a protein-protein interaction network and gene ontology enrichment analyses revealed an over representation of genes from the Wnt and Notch pathways for the plasticity traits (Figure 4.S5A), and of genes involved in proteolytic processes and Wnt signaling for the within-environment size variation (Figure 4.S4B).

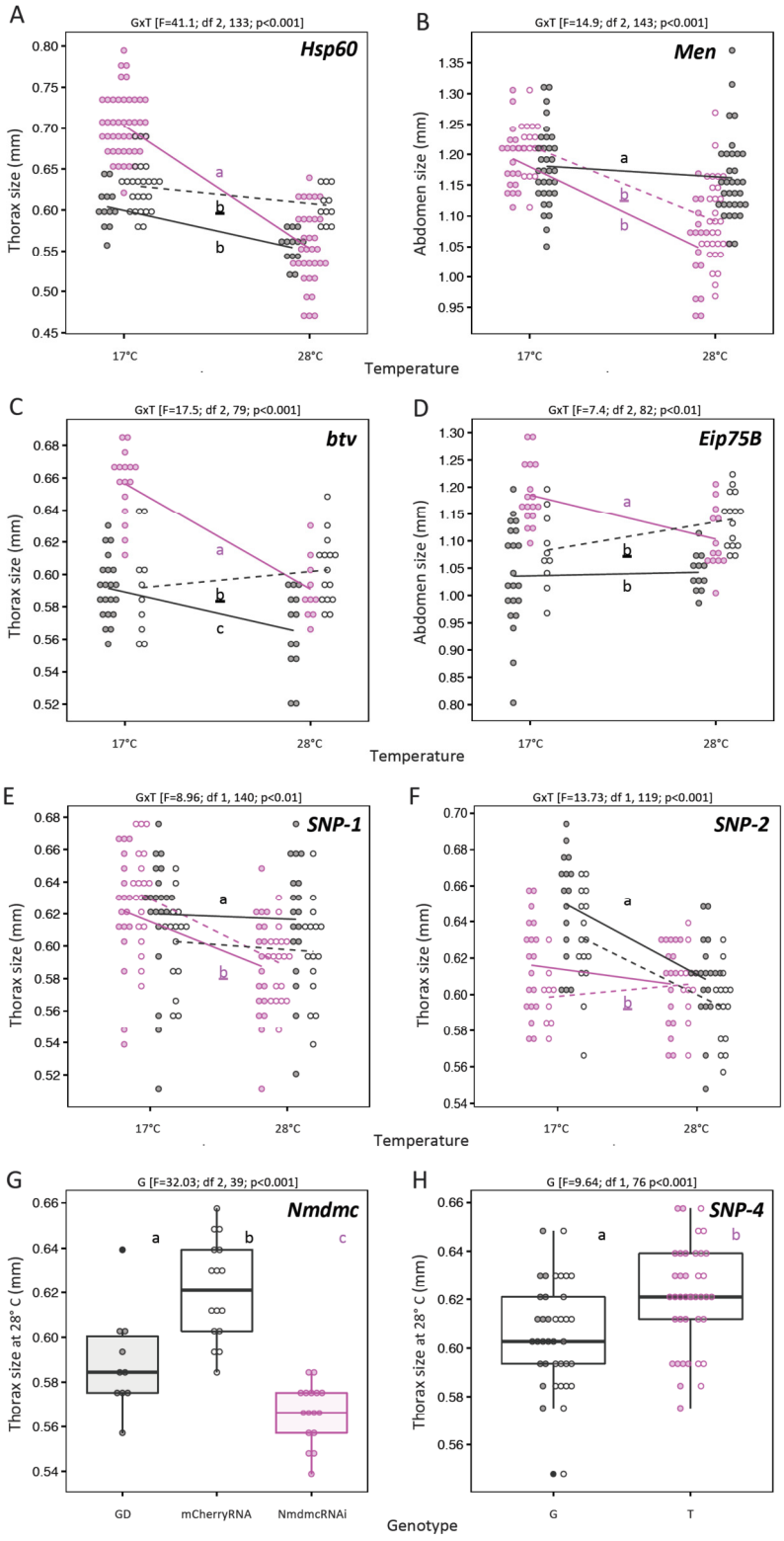
Finally, to explore whether allelic variants influencing plasticity tend to do so by either buffering or increasing environmental responsiveness, we looked at the effects and frequencies of the alleles at our candidate plasticity QTLs and found that, in most cases, alleles associated to increased environmental responsiveness were at lower frequency (Figure 4.S6).

Validation of selected GWAS hits

Potential false-positives require validation of GWAS hits. We selected a number of significant SNPs and significant genes for validation via different approaches. Available null mutants and inducible gene knock-down (with

RNAi-Gal4 system) were used to test the role of selected candidate genes in quantitative trait variation. To test specific significant SNPs (putatively associated to multiple genes) we used an approach we are calling Mendelian Randomization. For each of those SNPs, this involved randomizing the genetic background between different same-allele genotypes and comparing the quantitative trait between flies carrying the minor versus the major allele (see Material and Methods). This approach tests the hypothesis that individuals with one versus the other allele at the candidate SNP differ for the quantitative trait for which that SNP was identified as a GWAS hit and this effect is independent of the genetic background.

Upon validation, we confirmed a role in thermal plasticity for four of five candidate genes and for two of three candidate SNPs tested. Using mutants, we confirmed a role for plasticity in abdomen size for gene *Hsp60* (Figure 4.4A, Figure 4.S7A) and for plasticity in thorax size for gene *Men* (Figure 4.4B, Figure 4.S7A, 4.S7B). Using RNAi, we confirmed the role of *btv* in thermal plasticity for thorax size (Figure 4.4C, Figure 4.S7C) and of *Eip75B* in thermal plasticity for abdomen size (Figure 4.4D, Figure 4.S7C, 4.S7D). Using the Mendelian Randomization approach we validated the effect of two candidate SNPs (SNP-1 and SNP-2) on thorax plasticity (Figure 4.4E and 4.4F, Figure 4.S7E and 4.S7F) and did not validate the effect of one candidate SNP (SNP-3) on abdomen plasticity (Figure 4.S8, Table 4.S5). For all the confirmed candidates for plasticity, the mutants were more plastic than the respective controls, and the DGRP genotypes carrying the minor allele were more plastic than those carrying the major allele in the DGRPs (Figure 4.S7). For all these genes we found several SNPs (i.e. 8 different SNPs and 1 MNP in *Men,* 2 different SNPs in *btv* and 14 SNPs in *Eip75),* with the exception of *Hsp60*, for which we only found an insertion in the 5'UTR (Table 4.S4).


A
GxT [F=41.1; df 2, 133; p<0.001]
Hsp60
Thorax size (mm)
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
a
b
b
17°C
28°C
B
GxT [F=14.9; df 2, 143; p<0.001]
Men
Abdomen size (mm)
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
a
b
b
17°C
28°C
Temperature
C
GxT [F=17.5; df 2, 79; p<0.001]
btv
Thorax size (mm)
0.68
0.66
0.64
0.62
0.60
0.58
0.56
0.54
0.52
a
b
c
17°C
28°C
D
GxT [F=7.4; df 2, 82; p<0.01]
Eip75B
Abdomen size (mm)
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
a
b
b
17°C
28°C
Temperature
E
GxT [F=8.96; df 1, 140; p<0.01]
SNP-1
Thorax size (mm)
0.68
0.66
0.64
0.62
0.60
0.58
0.56
0.54
0.52
a
b
17°C
28°C
F
GxT [F=13.73; df 1, 119; p<0.001]
SNP-2
Thorax size (mm)
0.70
0.68
0.66
0.64
0.62
0.60
0.58
0.56
0.54
a
b
17°C
28°C
Temperature
G
G [F=32.03; df 2, 39; p<0.001]
Nmdmc
Thorax size at 28° C (mm)
0.66
0.64
0.62
0.60
0.58
0.56
0.54
a
b
c
GD
mCherryRNA
NmdmcRNAi
H
G [F=9.64; df 1, 76 p<0.001]
SNP-4
Thorax size at 28° C (mm)
0.66
0.64
0.62
0.60
0.58
0.56
a
b
G
T
Genotype

We also validated hits for within-temperature variation in body size: one of two candidate genes and one of one candidate SNP. The RNAi approach confirmed the role of gene *Nmdmc* in thorax size at 28°C (Figure 4.4G, Figure 4.S7G), knock-down had smaller thorax than controls, but not of gene *wings apart-like* (*wapl*) in abdomen size at 28°C (Table 4.S5), as we found no difference between knock-down and controls. The Mendelian Randomization approach validated the contribution of one SNP (SNP-4, in gene *CG14688*) to variation in thorax size at 28°C (Figure 4.4H and Figure 4.S7H).

In order to explore the pleiotropic effect of our candidate plasticity QTLs, we investigated whether the plastic response was also seen in the other body part, for which the SNP/gene had not been significantly associated to in the GWAS analysis (Figure 4.S7). For instance, for a

Figure 4.4. Functional validation of GWAS results. A. Thoracic reaction norms for size in mutant *Hsp60A/+* (magenta) and control lines *Canton-S* (filled circles, solid line) and *Fm7a/Canton*-S (empty circles, dashed line). **B.** Abdominal reaction norms for size in *Men* mutants *MenEx3/+* (magenta; filled circles, solid line) and *MenEx55/+* (magenta; empty circles, dashed line) and control line *w1118* (black). **C.** Thoracic reaction norms for size in *btv-RNAi/bab-Gal4* (magenta) and control lines *KK* (black; filled circles, solid line) and *mCherry-RNAi/bab-Gal4* (black; empty circles, dashed line). **D.** Abdominal reaction norms for size in *Eip75B-RNAi/bab-Gal4* (magenta) and control lines *KK* (black; filled circles, solid line) and *mCherry-RNAi/bab-Gal4* (black; empty circles, dashed line). **E.** Thoracic reaction norms for size in the four Mendelian Randomization populations corresponding to SNP-1 (in gene *CG43902*). The two populations fixed for the major allele are shown in black and the two populations for the minor allele are shown in magenta. **F.** Thoracic reaction norms for size in the four Mendelian Randomization populations corresponding to SNP-2 (in gene *ACC*). The two populations fixed for the major allele are shown in black and the two populations for the minor allele are shown in magenta. **G.** Thorax size at 28°C in *Nmdmc-RNAi/tub-Gal4* (magenta) and control lines GD (black; filled cicles) and *mCherry-RNAi/tub-Gal4* (black; filled circles). **H.** Thorax size at 28°C in the four Mendelian Randomization populations corresponding to SNP-4 (in gene *CG14688*). The two populations fixed for the major allele are shown in black and the two populations for the minor allele are shown in magenta. For the validations of plasticity SNPs/genes (panels A-F), we tested the model lm (*Size ~ Genotype*Temperature).* For the validations of within-environment SNPs/genes (panels G and H), the result from the model lm (*Trait ~ Genotype*). Results from the models are shown above each plot. In all cases, significant differences among groups were estimated by post hoc comparisons (Tukey's honest significant differences) and are indicated by different letters in each plot.

SNP/gene that became a candidate in the GWAS for variation in thorax plasticity, we quantified the effect of that SNP/gene in abdomen plasticity. We only found cross-body part effects for gene *btv* (Figure 4.S7); *btv*-knockdown flies showed differences in abdomen plasticity in comparison to control flies (Figure 4.S7C).

Relationship between size, size plasticity and ecological pressures

We benefited from the widespread use of *D. melanogaster* as a model organism to explore the correlation between our traits and other relevant phenotypes collected for the DGRPs. We also investigated the overlap between candidate QTLs for our traits and those identified in DGRP GWAS for other traits, as well as those identified underlying adaptation to different thermal regimes in experimental populations of *D. melanogaster* (Figure 4.S2).

First, we explored how our body size measurements related with previous quantifications of size in *D. melanogaster* DGRPs, by investigating correlations between our thorax and abdomen lengths (at 17°C and at 28°C) and measurements of head, wing, and thorax size (at 25°C) (Vonesch *et al.* 2016). We found significant positive correlations between our measurements of thorax but not abdomen size (both temperatures) and the size of those different body parts (Figure 4.S2A), and no overlap in the genes containing nominal SNPs underlying variation in head size, the trait for which there was available GWAS data.

Second, we explored the correlations between our traits and various fitness-related traits measured in the DGRPs (Figure 4.S2B), including life-history traits (longevity (Ivanov *et al.* 2015); starvation resistance, chill coma recovery (Mackay *et al.* 2012)) and immune-defense traits (tolerance to infection with *Providencia rettgeri* bacteria (Howick & Lazzaro 2017) and resistance to infection with *Metarhizium anisopliae* fungi or with *Pseudomonas aeruginosa* bacteria (Wang *et al.* 2017)). We found no significant correlation between our measurements of thermal plasticity in

body size and any of these traits. For our measurements of body size within-temperature, we found significant negative correlations only with chill coma recovery (thorax size at both temperatures and abdomen size at 28℃), and positive correlation with resistance to *M. anisopliae* fungi (abdomen size at 17℃).

Third, we asked about overlap in our candidate QTLs for body size and body size plasticity and the loci putatively selected in experimental populations of *D. melanogaster* evolving under different fluctuating thermal regimes (Tobler, Hermisson & Schlötterer 2015). Among our 210 candidate QTLs for thermal plasticity (both body parts and both raw and absolute values of thermal reaction norms), 8 genes (including *btv*) had changes in the populations evolving under hot and under cold temperatures fluctuations, 9 genes (including *Men*) had changes in the populations evolving under hot fluctuations, and 25 genes had changes in the populations evolving under cold temperatures fluctuations.

DISCUSSION

Body size and body size proportions are key life-history traits, closely associated to fitness (Peters 1986; Woodward *et al.* 2005). They show much diversity between species and populations, as well as between sexes and individuals of the same sex (e.g. Honěk & Honek 1993; Valenzuela-Sánchez, Cunningham & Soto-Azat 2015). Environmental conditions, such as temperature or food availability, can work both as inter-generational selective agents that filter body size variation and affect its evolution, and as intra-generational instructive agents that affect body size during development (Nunney & Cheung 1997; French *et al.* 1998; Nijhout 2003; Mirth & Shingleton 2012). Many studies have explored what shapes inter and intra specific differences in size for various species, including studies on the physiological basis of body size regulation (e.g. D'Amico *et al.* 2001) as well as the genetic basis of variation in body size (e.g. Robertson 1959; Gadau *et al.* 2002).

Partitioning (phenotypic) variation in body size

Studying a population representing naturally-segregating alleles, we quantified effects of genotype, environment, and genotype-by-environment interactions on the size of two body parts (thorax and abdomen), and we identified loci contributing to variation in size and in size plasticity. The correlations between size of different body parts and plasticity therein, are likely to be reflecting the tight regulation of body proportions, which is key for organismal performance (see Shingleton *et al.* 2007; Mirth & Shingleton 2012). In insects, low temperature and high protein content typically associates to larger bodies. We found this same pattern with most genotypes showing larger bodies at our lower temperatures and we also documented cases of no plasticity (i.e. robustness) and of plasticity with the opposite direction in the DGRPs. The relationship between plasticity levels and trait variance, by which genotypes with increased levels of plasticity also showed higher inter-individual variation in thorax size, could be reflecting an association between the responsiveness of some genotypes to micro environmental differences and their response to macro environmental differences (i.e. plasticity). Recent work using the DGRP, showed that inter-individual intra-environmental variation (also refer to as micro-environmental plasticity) can differ across traits and be under genetic control (Morgante *et al.* 2015). This inter-individual intra-environmental variation could also be explained by micro-environmental differences (i.e. due to small fluctuations in the developmental or social environment among flies), by micro-genetic variation (i.e. due to a certain degree of somatic mutations undergoing within lines) and/or stochasticity in phenotype expression, but could also be attributable to measurement error.

The widespread use of *D. melanogaster* as a model organism has built up extensive data for many phenotypes as well as for the genetic basis of phenotypic variation and of adaptation (e.g. Pool & Aquadro 2007; Tobler, Hermisson & Schlötterer 2015). Despite well-established associations between body size and fitness (Kingsolver & Huey 2008), we found no

correlations with longevity, starvation resistance or immune defense. Noteworthy, those phenotypes had been measured in different environments and are all environmentally-sensitive. The importance of the environment in which a trait is measured is illustrated in our results by weak correlations between body size measurements at different temperatures (e.g. no significant correlation between abdomen size measured at 17°C and measured at 28°C). In fact, trait associations, such as trade-offs, are dependent on genetic and environmental factors, as was shown for instance, for a thermally-driven switch in the association between longevity and body size in *D. melanogaster* (Norry & Loeschcke 2002). The extent to which internal and external factors influence these trait associations can have important implications for adaptation (Chevin 2013; Manenti *et al.* 2016).

Mostly private QTLs for size and size plasticity of different body parts

The physiological mechanisms affecting body size can change the duration of growth period or alter growth rates and can be affected by different genes (e.g. Robertson 1959, 1960; Partridge *et al.* 1994). Our GWAS identified candidate loci for inter-genotype variation in body size with little overlap between candidates QTLs for size at different temperatures (17°C versus 28°C), and between candidate QTLs for different body parts (thorax versus abdomen) at any specific temperature. Sex, body part and environment-specific QTL effects had been documented for various traits (e.g. Beldade, Brakefield & Long 2002; Linnen *et al.* 2013), including quantitative traits in *Drosophila* (e.g. Vonesch *et al.* 2016; Wang, Lu & St. Leger 2017). Different loci have been associated to bristle number of various body parts (Dilda & Mackay 2002), to thorax size in several environments (Norry & Gomez 2017) and even to fitness traits at different ages (Durham *et al.* 2014) in *D. melanogaster*. Such private QTLs can presumably facilitate the potential for independent evolution of the traits.

Our functional validations of candidate SNPs/genes were positive in most cases and the different approaches test different hypotheses: while

mutant and RNAi test that no or low levels of peptide affect variation in the quantitative trait for which the gene was identified as a candidate QTL, the Mendelian Randomization tests for sufficiency and independence from genetic background of the specific allele. The former is timely when there are several SNPs on the same genes (such as in gene *Men*) while the latter is a better approach to test specifically the effect of a given allele, as it is allelic replacement.

Previous work exploring the loci underlying genotype-by-environment interactions have mostly focused on investigating QTLs whose effect vary across environments (QTL-by-environment interactions) and this has been studied for a variety of traits in several species (e.g. Fry *et al.* 1998; Gurganus *et al.* 1998; Vieira *et al.* 2000; Leips & Mackay 2000; Bergland *et al.* 2008). Much less attention has been paid to unraveling the allelic variants contributing to differences in plasticity itself (but Ungerer *et al.* 2003; Gutteling *et al.* 2007). Expanding our knowledge about the natural allelic variation conferring differences in size plasticity is of key importance as those loci can provide the raw material for selection to act on during the evolution of environmentally-sensitive development.

We used the raw and absolute slopes of the reaction norms as quantitative traits in a GWAS, and identified loci associated with variation in size plasticity of two body parts. We documented very little overlap between QTLs for different properties of the reaction norms (raw versus absolute value of slopes) and for plasticity in different body parts (thorax versus abdomen), suggesting no general “plasticity QTLs”. The genes influencing size plasticity had diverse functions, potentially mediating environmental effects on size at different levels such as the perception of the environmental cue (e.g. *btv*), the transmission of that information to developing tissues (e.g. *Eip75B*) and/or the execution of the information on those tissues (e.g. *Men* and *Hsp60A*). Genetic variants affecting different aspects of the environmental response, such as different properties of reaction norms, have been previously shown in other systems. In *Manduca sexta*, for

instance, the acquisition of a single mutation in the juvenile hormone pathway conferred environmental sensitivity and enabled ensuing experimental evolution of a larval color polyphenism (Suzuki & Nijhout 2006).

We documented little overlap between candidate QTLs for body size and body size plasticity and for different properties of the plastic response (extent versus direction). There has been extensive discussion on the nature of the genetic basis for plasticity. Some models propose that the genetic control of phenotypic plasticity happens via specific loci determining plastic responses (Bradshaw, 1965, Scheiner and lyman 1989, 1991) while others suggest that plasticity could be regulated by the same loci that control trait values at a given environment (Via and Lande, 1985). Our results are suggestive of a genetic basis for plasticity very much independent of the one underlying trait variation.

Potential for independent evolution of size and size plasticity

Plasticity can help populations coping with environmental heterogeneity and can promote phenotypic and taxonomic diversification (West-Eberhard 2005). Theoretical models highlight the ecological conditions that should favor the evolution of plasticity, such as the predictability of the environmental fluctuations (Chevin & Lande) and the costs for plasticity (see Murren *et al.* 2015) and empirical work has provided evidence on the multigenic basis evolved differences in reaction norms (Wijngaarden & Brakefield 2000). Plasticity is generally presumed to be costly and only selected for in predictably heterogeneous environments, such as seasons. The absence of a correlation between our thermal plasticity traits and various fitness-related traits measured for the same genotypes could not identify any such cost for plasticity. We have identified loci contributing to variation in size plasticity that can provide the raw material for the evolution of plasticity under heterogeneous environments.

Loci can contribute to variation in plasticity by either increasing or decreasing environmental responsiveness; promoting robustness or plasticity, respectively. In the DGRPs, we found that the alleles contributing to increased levels of plasticity are most often at lower frequencies. Even though some degree of environmental responsiveness is maintained in this population, the alleles that provide robustness to body size in relation to variation in temperature are more frequent. The ability to respond or resist environmental perturbation, and the balance between both processes, can be for crucial for fitness in variable environments. Moreover, these loci are different of the ones that underlie variation in size and thus, size and size plasticity can have the potential to evolve independently. It is conceivable that different QTLs contribute to variation in plasticity in other populations. Notably, some of our candidate genes for plasticity where selected in populations evolving under fluctuation environments. Altogether, our results shed light onto the nature of the inter-individual variation in plasticity, necessary for the evolution of plasticity under heterogeneous environments.

Our data highlights the potential for independent evolution of trait and trait plasticity, whereby plasticity of different body parts and even properties of the environmental response, can be under distinct genetic control, and thus have the potential to respond to selection independently.

MATERIALS AND METHODS

Fly stocks and rearing conditions

Data for the GWAS was collected from adult female flies of the Drosophila Genetic Reference Panel (DGRP) obtained from Bloomington Stock Center. The DGRP is a set of fully sequenced inbred lines collected from a single population in Raleigh, NC, USA (Mackay et al. 2012; Huang et al. 2014). The number and the details of the lines included in the GWAS for each trait can be found in Table S2. Mutant stocks for the functional validations were: *Hsp60A* (stock 4689 from Bloomington), *MenEx3* and *MenEx55* (obtained from the T. Merritt lab). Control genetic backgrounds were *w1118* (stock

5905, from Bloomington) and Canton-S (obtained from C. Mirth lab). UAS-Gal4 and UAS-RNAi lines used for validations were: stocks 6803 for *bab-Gal4,* 5138 for *tub*-Gal4, 28737 for *btv*-RNAi, and 35785 for mCherry-RNAi, all from Bloomington stock center and stock v108399 for *Eip75B,* from VDRC stock center.

Fly stocks were maintained in molasses food (45 gr. molasses, 75gr sugar, 70gr cornmeal, 20 gr. Yeast extract, 10 gr. Agar, 1100 ml water and 25 ml of Niapagin 10%) in incubators at 25ºC, 12:12 light cycles and 65% humidity until used in this study. For the experiments, we performed over-night egg laying from ~20 females of each stock in vials with *ad libitum* molasses food. Eggs were then placed at either 17℃ or 28℃ throughout development. We controlled population density by keeping between 20 and 40 eggs per vial. We reared 200 DGRP lines and quantified thorax and abdomen size of 5 to 20 females per line, per temperature and replicate. For 130 DGRP lines, we ran two replicates and for 33 lines we ran three replicates. The total number of flies used varied between DGRP lines due to differences in mortality at one of the temperatures. For some specimens, we could only quantify size of one body part if, for example, the individual was not properly positioned in the image or was damaged. Full details on the stocks used and the number of flies used per stock and temperature can be found in Tables S1 and S2. Rearing conditions for the validations of candidate QTLs were similar to those used for the DGRP lines.

Phenotyping: body size and plasticity

Adult female flies (8-10 days after eclosion) were placed in 2ml Eppendorf and killed in liquid nitrogen followed by shaking the tubes to remove wings, legs and bristles. Bodies were mounted on Petri dishes with 3% Agarose, dorsal side up, and covered with water to avoid light reflections. Images containing 10 to 20 flies were collected with a LeicaDMLB2 stereoscope and a Nikon E400 color camera under controlled imaging conditions of light, contrast and white-balance. Images were later processed with a customized

Mathematica macro to extract size measurements. For this purpose, we drew two transects per fly, one in the thorax and one in the abdomen, using body landmarks (as shown in Figure 4.S1A). Size on each body part was quantified as the number of pixels in the transect, and converted to mm. For abdominal transects, when necessary, we performed another step to remove the pixels corresponding to the membranous tissue that is sometimes visible between abdominal segments.

Genome-Wide Association Study

For each body part (thorax and abdomen), we performed four independent genome wide analyses (GWAS): two for thermal plasticity (raw and absolute values of the slopes of the reaction norms), and two for within-environment variation (length at 17°C and length at 28°C). Slopes of the reaction norm were calculated as the slope of the regression model lm (*Size ~ Temperature)* for each body part and DGRP line. The GWAS for variation in thermal responsiveness were done by using the raw and absolute value of the reaction norms, testing the model lm (*Slope ~ Allele + (1|Wolb|DGRP)*), *Wolb* being the *Wolbachia* status of the DGRP lines (Mackay *et al.* 2012; Huang *et al.* 2014). The GWAS analyses for within-environment variation (at either 17°C or 28°C) were done by testing the model lm (*Size ~ Allele + (1|Wolb|DGRP)*). All the GWAS were performed by using SNPs where we had information for at least ten lines per allele. We did not find an effect of *Wolbachia* in any of our GWAS analyses.

We tested for the effect of the chromosomal inversions (In_3R_K, In_3R_P, In_2L_t, In_2R_NS and In_3R_Mo) on our thorax and abdomen traits by using the models lm (*Mean Size ~ Inversion)* for within-environment size variation and lm (*Slope ~ Inversion)* for size plasticity.

For each of the GWAS we annotated the SNPs with a p-value<10e-5 using the FlyBase annotation (release 6; ref). For the same SNPs, gene-enrichment and pathway-enrichment analyses were done using the publicly available NetworkAnalyst Software (Xia, Benner & Hancock 2014; Xia, Gill &

Hancock 2015); using all nodes from first order network generated with IrefIndex Interactome settings.

Genetic distance matrix for the DGRPs was obtained from http://dgrp2.gnets.ncsu.edu/data.html and was used to perform a cluster hierarchical dendogram using *ape* and *phylobase* R packages. We estimated the phylogenetic signal and statistical significance for each of our traits using Blomberg's K (Blomberg, Garland & Ives 2003) and Pagel's λ (Pagel 1999) metrics with the *phylosig* function in the *phytools* R package (Revell 2012).

Functional validations

Selection of significant SNPs (p-value<10e-5) to validate was based on: corresponding peaks in the Manhattan plots (clear peaks prioritized), putative effect (missense and regulatory variants prioritized over intergenic variants), associated genes (annotated and known function prioritized). We used three methods for validation, depending on SNP properties: null mutants and RNAi (Gal4-UAS system) for genes containing several significant SNPs and/or containing SNPs corresponding to missense variants, and Mendelian randomization for SNPs in genes with little or no information available. Following these criteria we tested a total of 10 candidate SNPs/genes.

Validations by null mutants were done by comparing the phenotype in the heterozygous mutant stock with its respective genetic background. Validations by RNAi were done by comparing, for each Gal4 driver line, the phenotype of the gene of interest knockdown with the corresponding control cross using UAS-mCherryRNAi. We always used three different driver lines for our validations by RNAi: tub-Gal4 which is ubiquitously expressed, bab2-Gal4 which has been described to resemble partially bab2 expression in different developing tissues but not in the nervous system (ref), and y-Gal4 which has been described to fully resemble the expression of yellow gene in different developing tissues, including the nervous system (ref). For all

candidate genes selected for RNAi validation, except *Nmdmc*, the crosses between RNAi line and tub-Gal4 were lethal.

We used Mendelian randomization to validate the effect of three SNPs. For each candidate SNP, we first selected 10 DGRP lines with the minor allele and 10 with the major allele, not fixed for any other significant SNPs. These lines were used to generate four populations, two fixed for the major allele and two for the minor allele. Each population was established by crossing 8 virgin females from each of 5 of the same-allele lines to 8 males of the other 5 lines. Reciprocal crosses were used to set two independent populations per allele. These populations were allowed to cross for eight generations to randomize genetic backgrounds. The identity of the SNPs tested by MR is given by their annotation with Genome Release v6. We confirmed by Sanger sequencing that those populations had our candidate allele fixed. Primer sequences used to confirm the allele in each population were:

Gene *CG43902*	forward primer: ACCACCAACATCAGCGTTTC;
	reverse primer: TGGTTTCGGCGTAGTTGTTG.
Gene ACC	forward primer: CGCTGGAGTTGTCTGTAAGC;
	reverse primer: TGGCCACCAGATAGCAGATT.
Gene CG43117	forward primer: TAAGCAAAATGTGGCGTGCA;
	reverse primer: TTAACATGGATCCTGCGCAC
Gene CG14688	forward primer: CATACTTTGACAGACGGCCG;
	reverse primer: CGGCTACATTGTCATCGAGG

Statistical analyses

All statistical analyses were performed with R Statistical Package v 3.1.1 (R Development Core Team 2014). We checked assumptions of parametric test by using Shapiro test for normality and Bartlett test of homocedasticity. For each body part, we used linear models to test for the effect of genotype (model lm (*Size ~ DGRP*)) or the interaction between genotype and temperature (model lm (*Size ~ DGRP*Temperature*)) on size. Reaction

norms for each DGRP line were calculated by using the regression model lm (*Size ~ Temperature*). From that model we extracted two properties of the reaction norms per DGRP line and body part: the absolute value of the slope as a measurement of thermal sensitivity, describing only the magnitude of the response to temperature, and the raw value of the slope as a measurement which describes also the direction of that response. Linear mixed models were calculated using *lme4* R package.

We used Pearson correlations (α=0.99) to test for linear correlation in size between body parts, controlling for DGRP lines. We also used Pearson correlations to test for linear correlations among our measured traits and between those and other available datasets for the DGRPs. For this, we used the mean value per DGRP line for each trait and the *corrplot* R package. We report both correlation coefficient and significance levels (α=0.99). Available DGRP phenotypes that were used to correlate with our traits were: size measurements at 25℃ (Vonesch *et al.* 2016), longevity (Ivanov *et al.* 2015), starvation resistance, chill coma recovery (Mackay *et al.* 2012), tolerance to infection with *Providencia rettgeri* bacteria (Howick & Lazzaro 2017) and resistance to infection with *Metarhizium anisopliae* fungi or with *Pseudomonas aeruginosa* bacteria (Wang *et al.* 2017). We also looked at the genes that contained SNPs significantly appeared under selection during evolution in fluctuating environments (Tobler *et al.* 2015).

Broad sense heritability for size at each temperature was estimated as $H^2 = \sigma^2_A/(\sigma^2_A + \sigma^2_W)$ where σ^2_A and σ^2_W are the among-line and within-line variance components, respectively. Heritability of plasticity was calculate as $H^2 = \sigma^2_{G*E}/\sigma^2_{TOTAL}$ where σ^2_{G*E} and σ^2_{TOTAL} are the variance associated with the genotype by environment interaction and total variance components, respectively, as proposed in Scheider and Lyman (1989). Variance components were extracted using *varcomp* R package.

For the functional validations of within-environment SNPs and genes we tested the model lm (*Size ~ Allele)* and lm (*Size ~ Genotype)*, respectively. For the validations of plasticity SNPs and genes we tested the

model we tested the model lm (*Size ~ Genotype*Temperature)* and lm (*Size ~ Allele*Temperature)*, respectively. In all cases, significant differences among groups were estimated by post hoc comparisons (Tukey's honest significant differences).

AUTHOR CONTRIBUTIONS

Elvira Lafuente and Patrícia Beldade conceived and designed the experiments; Elvira Lafuente performed the experiments, Elvira Lafuente and David Duneau analyzed the data. Elvira Lafuente and Patrícia Beldade wrote the manuscript.

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SUPPLEMENTARY MATERIAL

Figure 4.S1. Variation in size plasticity in the DGRP lines.

Figure 4.S2. Phenotypic co-variation in size and size plasticity with fitness and genetic distance.

Figure 4.S3. GWAS for variation in size plasticity per chromosomal arm.

Figure 4.S4. GWAS for variation in size per chromosomal arm.

Figure 4.S5. Candidate genes underlying variation in size and size plasticity.

Figure 4.S6. Effect and frequency of candidate SNPs/InDels for variation in size plasticity.

Figure 4.S7. Effect and pleiotropy of validated SNPs/genes.

Figure 4.S8. Non-validated candidates

The following documents are included in the digital supplement that accompanies this thesis.

Table 4.S1. Phenotypic variation in size.

Raw data for size measurements in thoraxes and abdomens of flies from DGRP lines reared at 17°C and at 28°C.

Table 4.S2. Phenotypic variation in size and size plasticity.

Summary data from size measurements in thoraxes and abdomens of DGRP lines. Number of phenotyped flies (N), mean and starndard deviation (SD) per temperature and raw and absolute values of the slope of the reaction norms (calculated by using the regression model lm (Size ~ Temperature), per DGRP line and body part).

Table 4.S3. Heritability calculations.

Variance components and broad-sense heritability estimates for size at 17°C and at 28°C and for plasticity per body part.

Table 4.S4. GWAS for variation in size plasticity.

Nominally significant SNPs (p-value threshold of 10e-5) from GWAS for the raw slope of the reaction norms (Value=Raw) and absolute slope of the reaction norms (Value=Absolute) per body part. The genomic position (from Genome Releases v.5 and v.6), type of SNP/InDel, potential impact, associated gene name (Flybase Gene ID) and putative consequence are also shown.

Table 4.S5. GWAS for variation in size.

Nominally significant SNPs (p-value threshold of 10e-5) from GWAS for size variation at 17°C and at 28°C per body part. The genomic position (from Genome Releases v.5 and v.6), type of SNP/InDel, potential impact, associated gene name (Flybase Gene ID) and putative consequence are also shown.

Table 4.S6. Functional validations of GWAS candidates.

Raw data for size measurements in thoraxes and abdomens of flies reared at 17°C and at 28°C from the different genetic backgrounds corresponding to each validation.

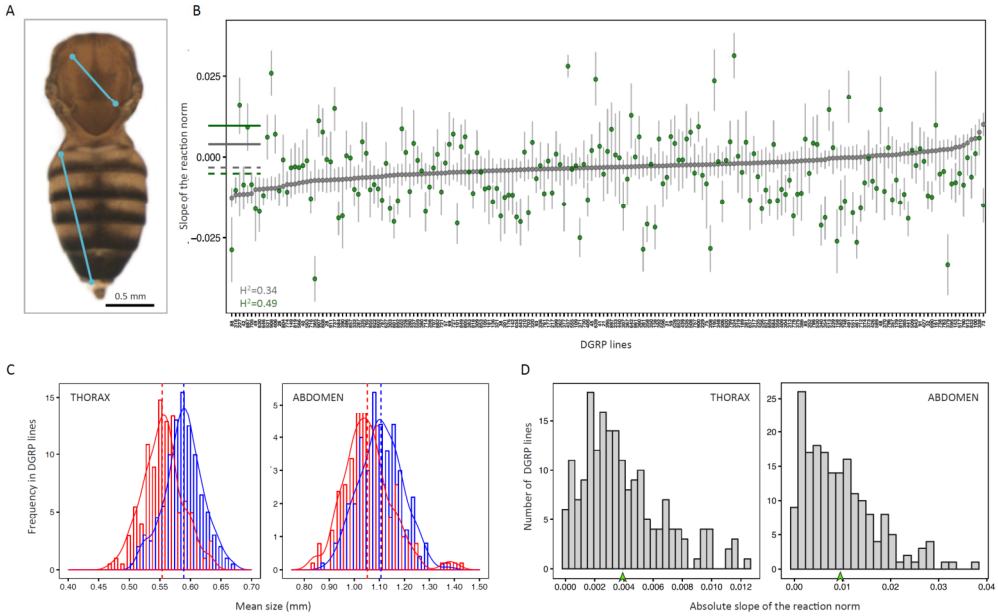


Figure 4.S1. Variation in size plasticity in the DGRP lines. A. Image of an adult female *D. melanogaster* fly showing the thoracic and abdominal transects. **B.** Slope and 95% confidence interval of the reaction norms in the DGRP lines, calculated as the regression model lm (*Size ~ Temperature*) in the thoraxes (grey) and abdomens (green) of each DGRP line (Y axis). Slopes are ranked by their value in the thorax. Horizontal bars represent the mean of all DGRP lines for the raw slope of the reaction norm (dashed bar) and the absolute slope of the reaction norm (solid bar) per body part. **C.** Histograms showing the frequency of the size values in thoraxes and abdomens of the DGRP lines reared at 17℃ (blue) and 28℃ (red). Dashed line represents the mean value for all DGRP lines at a given temperature. **D:** Histograms for the absolute slope of the reaction norms (calculated as the absolute value for the slope of the regression lm (*Size ~ Temperature*) in thoraxes and abdomens. Mean value for the absolute slope of all DGRP lines is indicated with a green arrowhead.

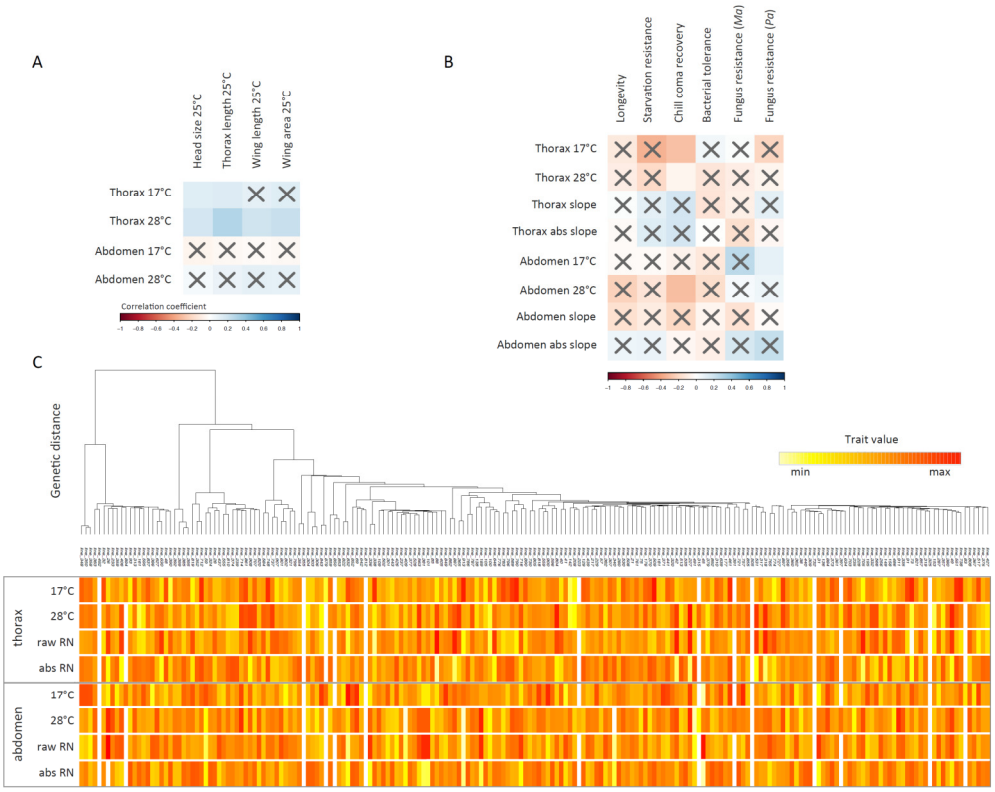


Figure 4.S2. Phenotypic co-variation in size and size plasticity with fitness and genetic distance. A. Heat map of Pearson's correlation coefficients between our within-environment size measurements (17℃ and at 28℃) and size measurements at 25℃. Positive correlations are denoted in blue and negative correlations in red. Non-significant correlations (p-value > 0.01) are indicated with an 'X'. **B.** Heat map of Pearson's correlation coefficients between our traits (mean size at each temperature and raw and absolute slopes of the reaction norms) and fitness-related traits. Positive correlations are denoted in blue and negative correlations in red. Non-significant correlations (p-value > 0.01) are indicated with an 'X'. **C.** Dendogram of the genetic distance between DGRP lines. Corresponding trait values are shown as a heat map and scaled for each trait independently. Coefficients of Blomberg's K phylogenetic signal were: K=0.24; p-value=0.17 (thorax at 17℃), K=0.23; p-value=0.37 (thorax at 28℃), K=0.22; p-value=0.78 (thorax raw slope), K=0.24; p-value=0.20 (thorax absolute slope), K=0.24; p-value=0.24 (abdomen at 17℃), K=0.22;p-value=0.67 (abdomen at 28℃), K=0.23; p-value=0.55 (abdomen raw slope) and K=0.21; p-value=0.89 (abdomen absolute slope). Pagel's λ coefficient of phylogenetic signal was λ =6.88e-05; p-value= 1, for all the traits in both body parts.

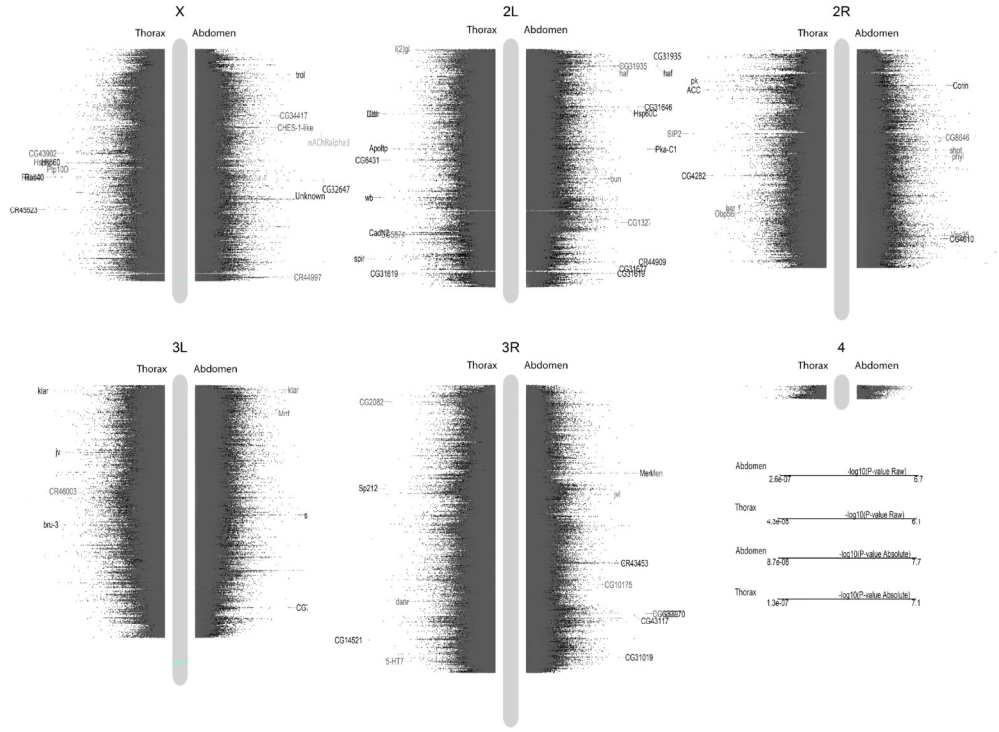


Figure 4.S3. GWAS for variation in size plasticity per chromosomal arm. Manhattan plots corresponding to the four GWAS performed for variation in size plasticity: raw slopes of the reaction norms (grey dots) and absolute slope of the reaction norms (black dots) for thorax (left side) and abdomen (right side) size. For each trait and body part, the GWAS was done testing the model lm (*Slope ~ Allele + (1|Wolb|DGRP)*). The significance level for each SNP along the chromosomal arms is shown as the $\log_{10}$ p-value. Some of the genes associated to SNPs/InDels with a p-value < 10e-5 are shown.

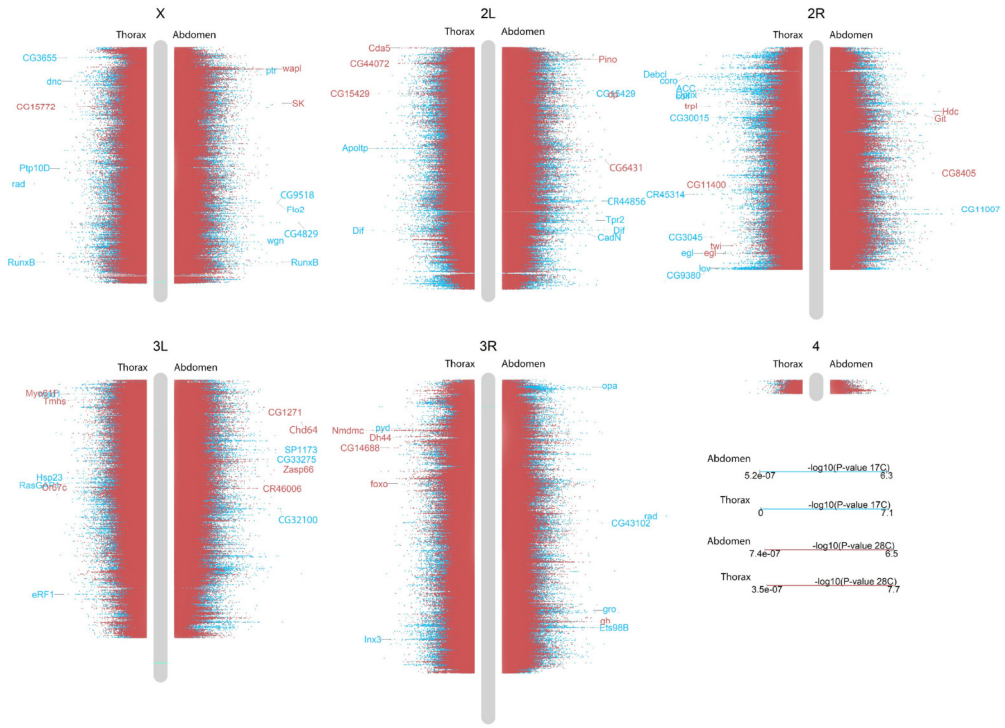
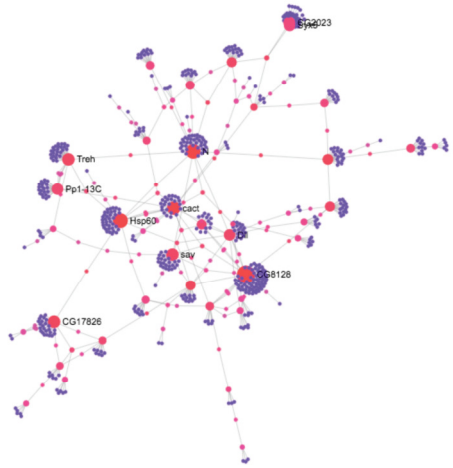


Figure 4.S4. GWAS for variation in size per chromosomal arm. Manhattan plots corresponding to the four GWAS performed for within-environment variation in size: at 17℃ (blue dots) and at 28℃ (red dots) for thorax (left side) and abdomen (right side) size. For each trait and body part, the GWAS was done testing the model lm (*Size ~ Allele + (1|Wolb|DGRP)*). The significance level for each SNP along the chromosomal arms is shown as the $\log_{10}$ p-value. Some of the genes associated to SNPs/InDels with a p-value < 10e-5 are shown.

A

Pathway (KEGG)	p-value	FDR
SNARE interactions in vesicular transport	2,33E-15	2,95E-13
Notch signaling pathway	1,96E-05	0,00125
Protein processing in endoplasmic reticulum	0,000141	0,00599
Wnt signaling pathway	0,000221	0,007
Hedgehog signaling pathway	0,000461	0,0117
Endocytosis	0,000562	0,0119
Ubiquitin mediated proteolysis	0,00309	0,056
Mismatch repair	0,0103	0,163
Progesterone-mediated oocyte maturation	0,0151	0,213
TGF-beta signaling pathway	0,0247	0,314
Phagosome	0,0297	0,325
Homologous recombination	0,0307	0,325
Ribosome	0,0426	0,416



B

Pathway (KEGG)	p-value	FDR
Protein processing in endoplasmic reticulum	2,71E-08	3,44E-06
Ribosome	1,12E-06	7,14E-05
Endocytosis	0,00324	0,137
RNA transport	0,0198	0,569
Wnt signaling pathway	0,0224	0,569
Propanoate metabolism	0,0352	0,672
TGF-beta signaling pathway	0,037	0,672
Citrate cycle (TCA cycle)	0,0758	1
Notch signaling pathway	0,0883	1
Protein export	0,0883	1

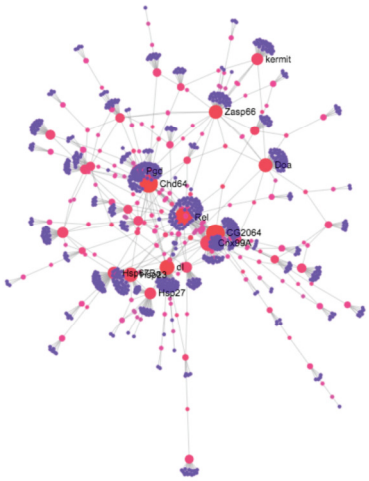
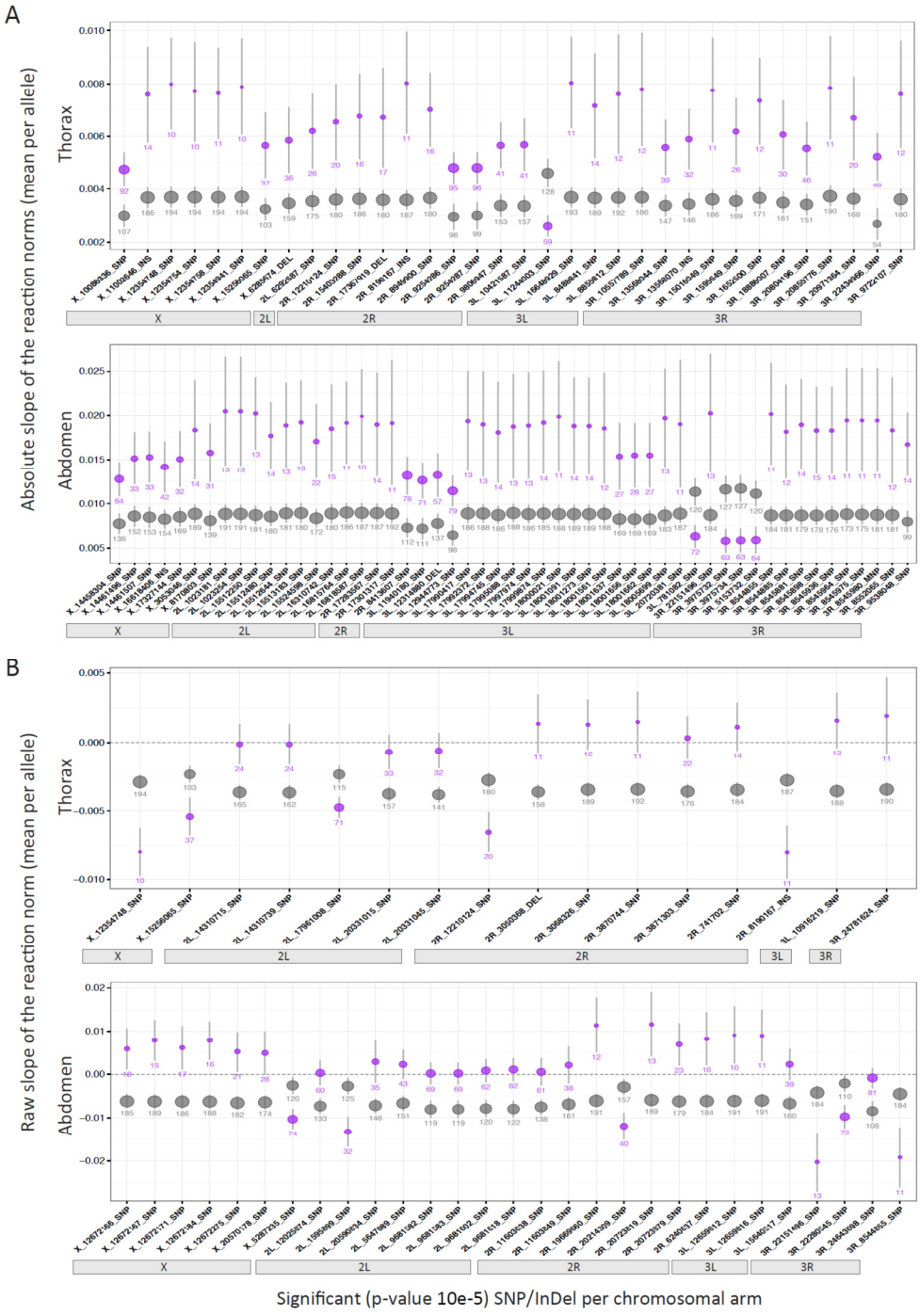


Figure 4.S5. Candidate genes underlying variation in size and size plasticity. **A.** KEGG gene enrichment analyses with associated p-value and false discovery rate (FDR) (upper panel) and gene network analyses (lower panel) for the putative genes associated with size plasticity. SNPs with p-value < 10e-5 from the GWAS for the raw and absolute slopes of the reaction norms were pooled to perform this analysis. **B.** KEGG gene enrichment analyses with associated p-value and false discovery rate (FDR) (upper panel) and gene network analyses (lower panel) for the putative genes associated with within-environment variation in size. SNPs with p-value < 10e-5 from the GWAS for variation at 17℃ and for variation at 28℃ were pooled to perform this analysis.

Figure 4.S6. Effect and frequency of candidate SNPs/InDels for variation in size plasticity. **A**. Mean and confidence interval of the absolute slope of the reaction norms (Y axis) per allele (minor in grey, minor in magenta) for each candidate plasticity SNP/InDel along the chromosomal arms (X axis) per body part. **B.** Mean and confidence interval of the raw slope of the reaction norms (Y axis) per allele (minor in grey, minor in magenta) for each candidate plasticity SNP/InDel along the chromosomal arms (X axis) per body part.



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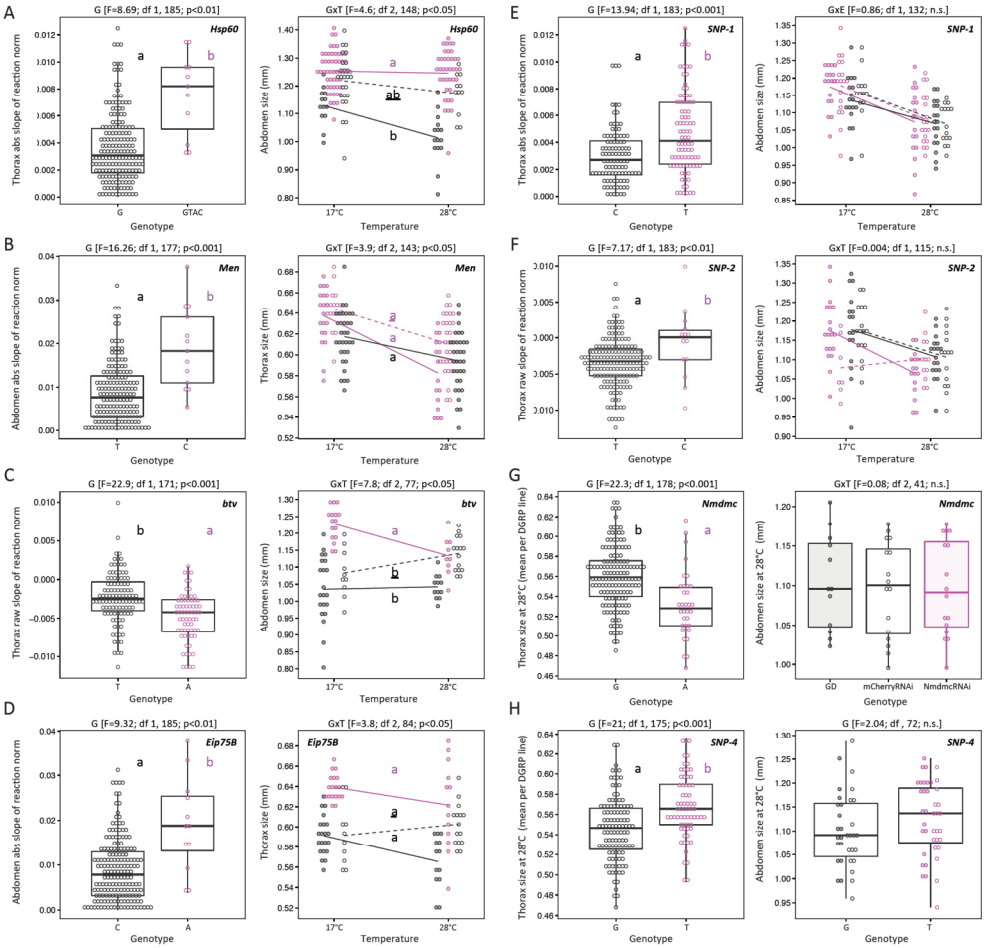

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Figure 4.S7. Effect and pleiotropy of validated SNPs/genes. For each SNP/gene the effect of the minor and major alleles in the DGRPs are shown in the left panels and the pleitropic effect is shown in the right panels. **A.** Left panel: slope of the reaction norms for thorax size in the DGRP lines with the major (black) and the minor (magenta) alleles for insertion X:11108613_INS within gene *Hsp60*. Right panel: Abdominal reaction norms for size in mutant *Hsp60A/+* (magenta) and controls *Canton-S* (filled circles, solid line) and *Fm7a/Canton-S* (empty circles, dashed line). **B**. Left panel: slope of the reaction norms for abdomen size in the DGRP lines with the major (black) and the minor (magenta) alleles for SNP 3R:12720159 within gene *Men*. Right panel: thoracic reaction norms for size in *Men* mutants *MenEx3/+* (magenta; filled circles, solid line) and *MenEx55/+* (magenta; empty circles, dashed line) and control line *w1118* (black). **C.** Left panel: slope of the reaction norms for thorax size in the DGRP lines with the major (black) and the minor (magenta) alleles for SNP 2L:17961008 within gene *btv*. Right panel: abdominal reaction norms for size in *btv-RNAi/bab-Gal4* (magenta) and control lines *KK* (black; filled circles, solid line) and *mCherry-RNAi/bab-Gal4* (black; empty circles, dashed line). **D.** Left panel: slope of the reaction norms for thorax size in the DGRP lines with the major (black) and the minor (magenta) alleles for SNP 3L:17999272 within gene *Eip75B*. Right panel: thoracic reaction norms for size in *Eip75B-RNAi/bab-Gal4* (magenta) and control lines *KK* (black; filled circles, solid line) and mCherry-RNAi/bab-Gal4 (black; empty circles, dashed line). **E.** Left panel: slope of the reaction norms for thorax size in the DGRP lines with the major (black) and the minor (magenta) alleles for SNP-1 (X:1019230). Right panel: abdominal reaction norms for size in the four Mendelian Randomization populations corresponding to SNP-1. The two populations fixed for the major allele are shown in black and the two populations for the minor allele are shown in magenta. **F.** Left panel: slope of the reaction norms for thorax size in the DGRP lines with the major (black) and the minor (magenta) alleles for SNP-2 (2R:7983239). Right panel: abdominal reaction norms for size in the four Mendelian Randomization populations corresponding to SNP-2. The two populations fixed for the major allele are shown in black and the two populations for the minor allele are shown in magenta. **G.** Left panel: thorax size at 28°C in the DGRP lines with the major (black) and the minor (magenta) alleles for SNP 3R:9049187 within gene *Nmdmc*. Right panel: abdomen size at 28°C in *Nmdmc-RNAi/tub-Gal4* (magenta) and control lines GD (black; filled circles) and *mCherry-RNAi/tub-Gal4* (black; filled circles). **H.** Left panel: thorax size at 28°C in the DGRP lines with the major (black) and the minor (magenta) alleles for SNP-4 (genomic position 3R:2645482). Right panel: abdomen size at 28°C in the four Mendelian Randomization populations corresponding to SNP-4. For the validations of plasticity SNPs/genes (panels A-F), we tested the model lm (*Trait ~ Genotype*Temperature).* For the validations of within-environment SNPs/genes (panels G and H), we tested the model lm (*Trait ~ Genotype).* Results from the models are shown above each plot. In all cases, significant differences among groups were estimated by post hoc comparisons (Tukey's honest significant differences) and are indicated by different letters in each plot.

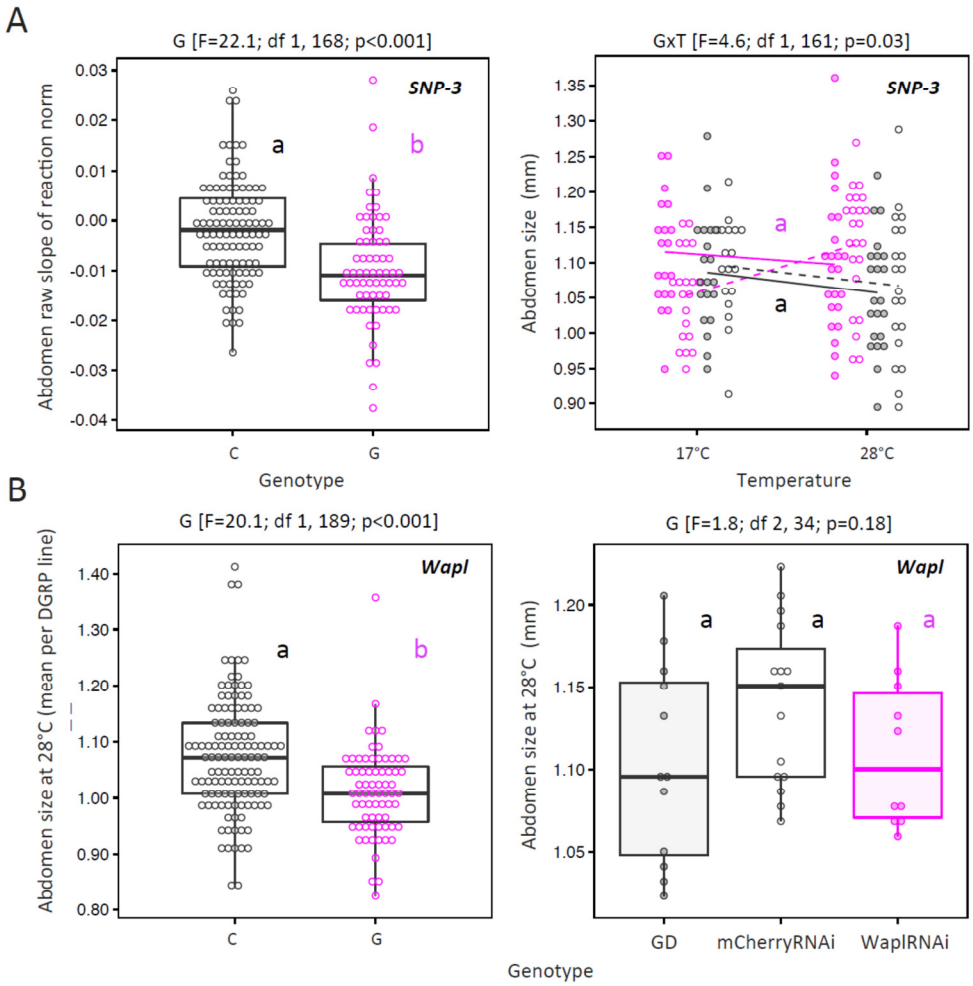
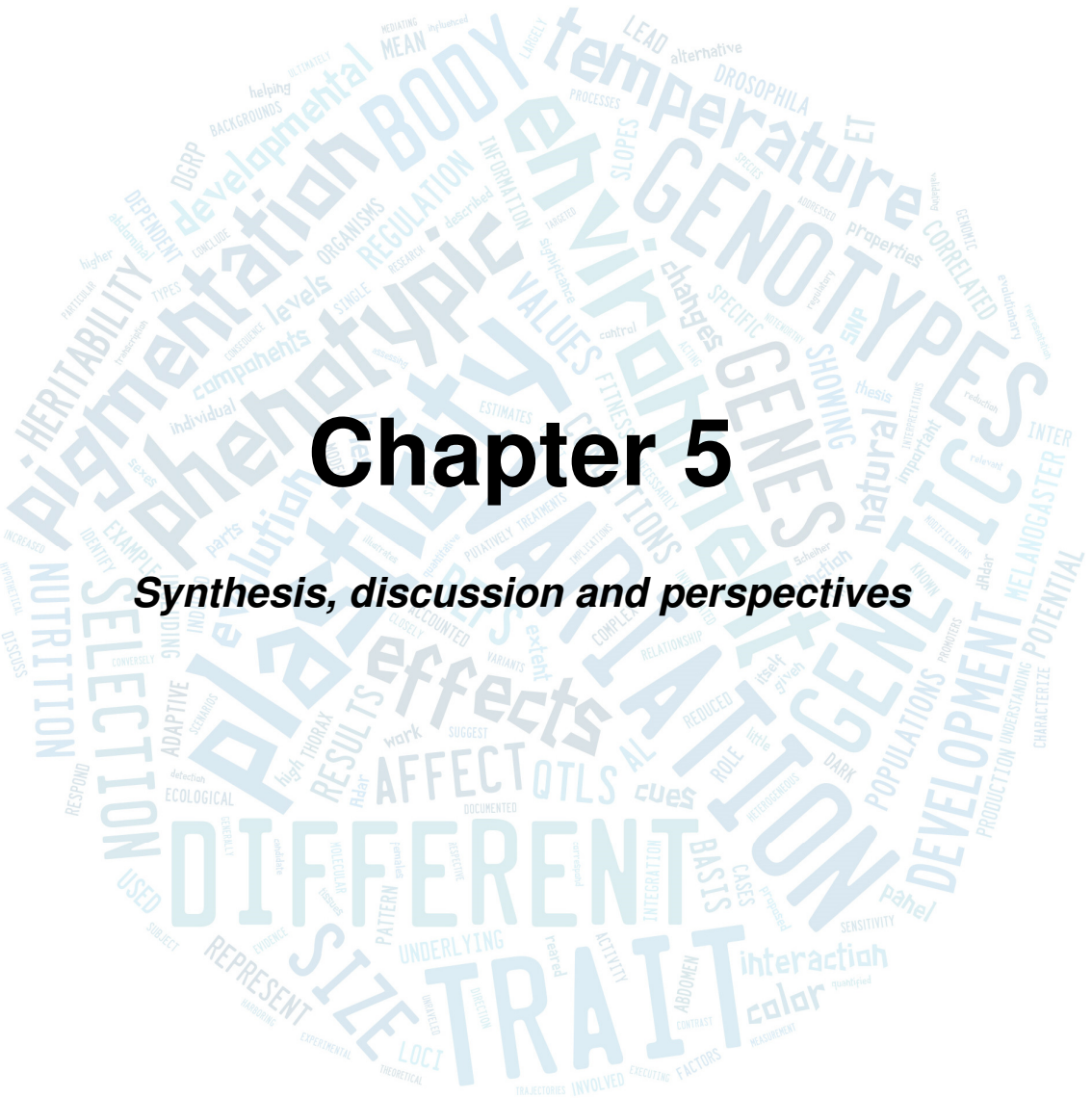


Figure 4.S8. Non-validated candidates. A. Left panel: slope of the reaction norm for abdomen size in the DGRP lines with the major (black) and the minor (magenta) alleles for SNP-3 (in gene *CG43117,* position X:1019230). Right panel: Abdominal reaction norms for size in the four Mendelian Randomization populations corresponding to SNP-3 The two populations fixed for the major allele are shown in black and the two populations for the minor allele are shown in magenta. The result of the model lm (*Trait ~ Genotype*Temperature)* is shown above the plot. The significant differences among groups were estimated by post hoc comparisons (Tukey's honest significant differences) and are indicated by different letters in each plot. **B.** Left panel: slope of the reaction norms for abdomen size in the DGRP lines with the major (black) and the minor (magenta) alleles for SNP X:2152985 within gene Wapl. Right panel: abdominal reaction norms for size in *Wapl-RNAi/bab-Gal4* (magenta) and control lines *KK* (black; filled circles, solid line) and *mCherry-RNAi/bab-Gal4* (black; empty circles, dashed line). The result from the model lm (*Trait ~ Genotype)* is shown above the plot. The significant differences among groups were estimated by post hoc comparisons (Tukey's honest significant differences) and are indicated by different letters in each plot.


Chapter 5
Synthesis, discussion and perspectives

ABSTRACT

External environmental cues can influence development, leading to the production of different phenotypes from the same genotype. This plasticity can result in a better match between the adult phenotype and the selective environment, thus helping organisms to cope with environmental heterogeneity. Plasticity can, itself, be thought of as a complex trait that is heritable, subject to selection and therefore can evolve. However, little is known about the loci contributing to natural variation in plasticity. In this thesis we have focused on body size and pigmentation in *Drosophila* to explore the developmental and genetic mechanisms underlying inter-genotypic variation in thermal plasticity. In this concluding chapter, I summarize our main findings, discuss them in a broader perspective, and comment on some of the limitations of this work. In addition, throughout my PhD project, I conducted several pilot studies and complementary experiments which will add to our understanding of how developing organisms integrate different environmental cues and of the potential role of RNA editing in thermal plasticity. Some of these the preliminary results are presented and discussed in this chapter, and hopefully can inspire future research.

BACKGROUND AND OPEN QUESTIONS

Environmental conditions can work as selective agents by filtering phenotypic variation across generations and can induce the production of phenotypic variation through the environmental regulation of development (i.e. developmental plasticity) (Bradshaw 1965). This developmental plasticity is ubiquitous and diverse in nature, as is demonstrated by a vast number of insightful studies that have reported environmentally induced changes in many organisms (e.g. plant and animal kingdoms; vertebrates and invertebrates), for a wide variety of traits (e.g. behavioral, morphological, life-histories), with very diverse inductive (and selective) environmental cues (biotic and abiotic) (Schlichting & Pigliucci 1998; Nijhout 2003; Beldade, Mateus & Keller 2011; Shingleton & Tang 2012; Huang *et al.* 2016). Genetic variation for plasticity, affecting different properties of the reaction norms, has also been documented in different systems (e.g. Robinson & Wilson 1996; Smekens & van Tienderen 2001; Crispo & Chapman 2010), showing that plasticity is heritable and can be subjected to selection. Despite the prevalence of plasticity in nature and its potential consequences for the evolution and ecology of populations, several key questions regarding the evolution and regulation of developmental plasticity remain unresolved, including some addressed in this thesis. We have used two iconic traits closely related to organismal fitness, *Drosophila* body size and pigmentation, as a model to explore the genetic basis and regulation of developmental plasticity. For example, we analyzed how genetic and environmental factors may affect the integration of suites of "related" plastic traits, such as color and color pattern components of body pigmentation or the sizes of different body parts within the same organism. Moreover, we explored the genetic basis for thermal plasticity in body size and body pigmentation by identifying (and validating) loci contributing to variation in plasticity and by addressing their potential function (i.e. sensing, modulation and/or executing environmental inputs; Figure 1.2) in the regulation of plasticity. Noteworthy, the molecular mechanism underlying plasticity include genes bearing allelic

variants that are responsible for inter-genotype differences in plasticity (e.g. accounting for genotype-by-environment interaction effects) and genes whose expression and/or function is environmentally-dependent. This distinction is crucial in the light of our results and we have used different approaches to explore both. Here, we discuss our results in the context of how organisms integrate complex environmental information to cope with environmental change and how this may affect the evolution of plasticity.

PARTITIONING COMPONENTS OF PHENOTYPIC VARIANCE

Phenotypic variation is the result of a complex integration of different levels of information, where the developmental environment includes more than one single inductive cue, the phenotype is more than one particular trait, and the selective environment presents more than one ecological challenge. Thus, the establishment of general principles about how genotypes are translated into phenotypes is a particularly challenging problem in biology. This situation is aggravated by the limited number of methods available to accurately quantify phenotypes, in contrast to the very sophisticated analytical tools available for genomic data. In classical evolutionary genetics, phenotypic variation is often partitioned into different components (and the interactions among them), thus helping to assess them individually (Figure 1.3A). The effect of the external environment on phenotypic variation, partly accounted for by the environment and the genetic-by-environment components (Figure 1.3), is one of the recurrent topics of this thesis and is further discussed in this section.

Effects of genotype, environment, and genotype-by- environment

In *Chapter 2* we developed a method to quantify body color and color pattern components and we showed how these traits vary between body parts, sexes, genotypes and temperatures in *D. melanogaster*. We expand some of our findings to natural populations of *D. melanogaster* that were collected along a latitudinal cline and to closely related *Drosophila* species. In

Chapters 3 and *4* we characterized the genetic variation for pigmentation components and size, respectively, in a different wild-caught *D. melanogaster* population: the Drosophila Genetic Reference Panel (DGRP) (Mackay *et al.* 2012; Huang *et al.* 2014). These data allowed us to characterize genetic correlations across traits, body parts and environments for a series of plastic phenotypes.

Taken together, we show that the effects of genotype (G) and environment (E) differ across body parts within an organism (e.g. size of the thorax and of the abdomen in Chapter 4; Figure 4.1A) and across related traits for a given body part (e.g. different pigmentation components in Chapters 2 and 3; e.g. Figures 2.2A and 3.2). Moreover, the associations between traits are themselves dependent on genetic and environmental conditions (e.g. Figures 2.2A, 4.2 and 3.2B). We reported cases in which traits showed similar responses to genetic or environmental effects. The size of the two body parts can be taken as an illustration of the latter. Regardless of the environment at which flies were reared, thoracic and abdominal sizes were always positively correlated (Figure 4.2B). This implies that selection for a bigger abdomen will lead to a correlated increased thorax size (Chapter 4; Figure 4.2) and presumably reflects constraints associated with maintaining body proportions (Mirth & Shingleton 2012). Similarly, we highlighted how related traits (e.g. color traits in Chapter 3) behave in a modular way, with tight correlations across genetic backgrounds or rearing temperatures. These results suggest that, for example, selection on the background color of the abdomen will lead to correlated responses of the color, but not the width of the abdominal bands (Chapter 3; Figure 3.2B). However, we also reported cases in which related traits show divergent and independent responses to either genetic or environmental factors. For instance, correlations between some pigmentation components were different in females and males of a given genetic backgrounds and so was the association between traits across environments (e.g. darkness in relation to other pigmentation traits; Figure 2.2A).

Notably, the genotype-by-environment effect also differed among related plastic traits. The extent and properties of the plastic responses (raw and absolute slopes of reaction norms) for various traits revealed different types of associations, some of which reflect a tight integration among traits while others showed greater independence between traits. For instance, plastic responses of abdominal pigmentation components were positively correlated (Figure 3.3B), while plasticity in pigmentation traits across body parts was not (Figure 3.3B). In summary, we showed that the effects of genotype (G), environment (E), and genotype-by-environment (GxE) differ among related plastic traits (Chapters 3 and 4).

The extent of integration or independence among traits has important implications for phenotypic variation and diversification as it affects how individual traits respond to selection. For example, plastic traits that are integrated into functional suites, thus enabling a concerted response to local environmental variability, may not be able to respond independently to the selective forces experienced in novel environments. Thus, the plastic responses of some of the integrated traits may be adaptive while responses of others may be maladaptive (e.g. van Bergen *et al.* 2017). A classical example of correlated plastic responses is the effect of temperature on different phenotypes, such as development time (e.g. diapause), body size, and other life-history traits in many arthropods. While diapause is thought to be an adaptive plastic response, this may not be true for correlated traits whose developmental rates are affected by availability of energy resources (Gotthard, Nylin & Nylin 1995). Our data suggest that thermally plastic traits in *Drosophila,* such as pigmentation components, might be able to respond independently to the selective forces experienced in nature.

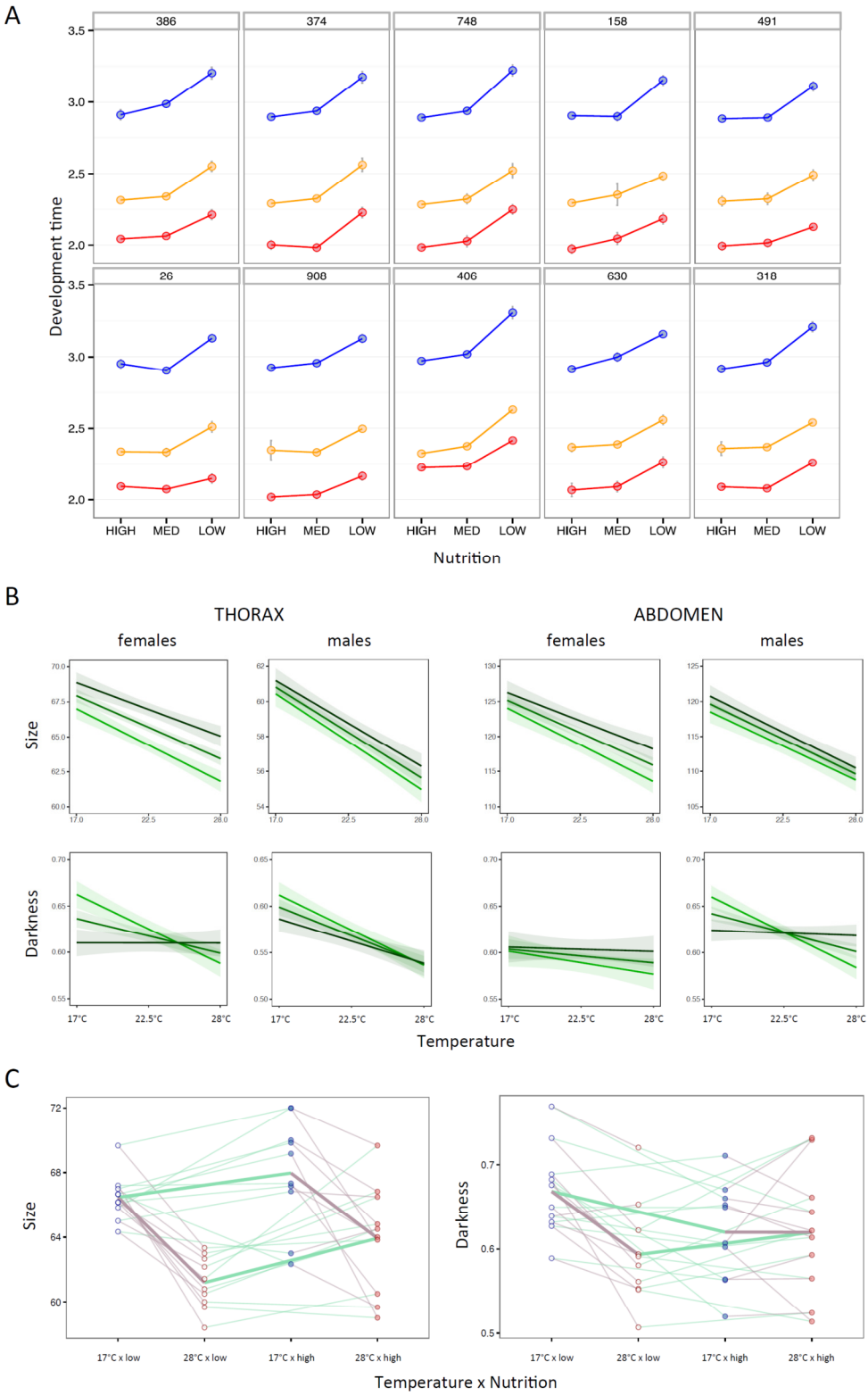
We also documented and characterized inter-individual variation presumably un-accountable by the effects of G, E or GxE. This corresponds to phenotypic variation for individuals of the same genotype and reared under the same environmental conditions. We saw intra-genotype and intra-environmental variation for some of our traits (e.g. coefficient of variation for

size in Chapter 4; Figure 4.2B). This component of phenotypic variance, that has often been overlooked but is getting increased attention (see Debat, Debelle & Dworkin 2009), could reflect small genetic differences between individuals (e.g. due to somatic mutation), micro-environmental variation (e.g. due to differences within a vial) or stochasticity in phenotype expression (e.g. via developmental noise). Notably, this component of phenotypic variance could have its own genetic basis, independent of the one controlling trait expression (Morgante *et al.* 2015), something that will, undoubtedly, be a topic of future research.

Effects of environment-by-environment interactions

Plastic responses can be triggered by different types of environmental cues, often in combination (Braendle & Félix 2008) and result in simultaneous changes in different traits. In fact, not only can the same cue affect different traits but also the same trait can be affected by several cues (Chevin 2013; Piggott, Townsend & Matthaei 2015). Most experimental studies of developmental plasticity have focused on the effects of single environmental cues (but Braendle & Félix 2008; Rodrigues *et al.* 2017). This is in contrast with what is the complexity of natural environments, with several environmental cues acting on multiple traits that might (or not) respond in the same way.

We have begun to explore environment-by-environment interaction effects on phenotypic variation by looking at the combined effects of variation in nutrition and in temperature in *D. melanogaster*. We know that each of these in isolation can affect body size and pigmentation as well as development time (Chapters 2-4 and e.g. David, Capy & Gauthier 1990; Nunney & Cheung 1997), but we know very little about their combined effects. Do the two factors in combination have redundant, additive or other types of interaction effects on phenotype expression? To investigate that, we reared a subset of ten DGRP genotypes under conditions representing a


A
386
374
748
158
491
26
908
406
630
318
Development time
HIGH MED LOW
Nutrition
B
THORAX
ABDOMEN
females
males
Size
Darkness
17°C
22.5°C
28°C
Temperature
C
Size
Darkness
17°C x low
28°C x low
17°C x high
28°C x high
Temperature x Nutrition

combination of low-medium-high nutrition and low-medium-high temperature ranges, and we quantified thorax and abdomen size and pigmentation in adults from both sexes. For each of the sexes and genotypes, we tested whether food levels, temperature and the interaction between them could account for variation in phenotype. We found evidence for environment-by-environment interaction effects for development time (Figure 5.1A) and pigmentation traits but not for size (Figure 5.1B,C). The combined effect of temperature and nutrition is largely additive for body size, with lower temperatures and poor nutritional conditions both reducing body size. In contrast, for overall darkness the effect of temperature was dependent on the nutritional environment. Hence, thermal plasticity for pigmentation is stronger in poor nutritional environments, and vice versa. We also found evidence for GxExE effects which implies that DGRP lines differed in their response to combinations of temperature and nutrition levels during development (Figure 5.1C). In fact, levels of thermal plasticity were not necessarily correlated with levels of nutritional plasticity; genotypes that were more plastic in relation to one environmental cue (e.g. temperature)

Figure 5.1. Combined effects of temperature and food levels on *D. melanogaster* body size and body pigmentation. A. Reaction norms for the mean development time of the 10 DGRP genotypes reared under low, medium and high nutrition (X axis) and at 17°C (blue), 23°C (yellow) or 28°C (red). Females and males are pooled in this analysis. Development time was affected by genotype, nutrition, temperature and the interaction between the two environments **(**model lm (*Development time ~ Genotype*Temperature*Nutrition);* p-value < 0.01). **B.** Reaction norms for thorax and abdomen size and overall darkness in females and males from 10 DGRP genotypes. The lines represent the best-fitted regression lines across temperatures for all DGRP genotypes reared at low (light green line), medium (green line) or high (dark green line) nutritional conditions. Shading represents the 95% confidence interval for each slope. **C.** Mean size and darkness of thorax (Y axis) produced by a given genotype in response to different combinations of the two environmental factors; temperature and nutrition. Variation in temperature is represented by red (28°C) and blue (17°C) symbols. Variation in nutrition is represented by closed (high) and open (low) symbols. Purple lines connect the different temperature treatments within the same nutrition, thus representing thermal plasticity. Green lines connect the different nutritional treatments within the same temperature, thus representing nutritional plasticity. The thicker lines connect the mean trait values, averaged over all DGRP lines, within each experimental treatment.

were not necessarily more plastic in relation the other environmental cue (e.g. nutrition).

Further analysis of these results and more studies in this area will help to elucidate the extent and the mechanisms by which environment-by-environment interactions contribute to phenotypic variation and might shed light onto how organisms deal with multi-factorial changes in their environments.

GENETIC BASIS OF PLASTICITY

Heritable phenotypic variation is the raw material for evolution by natural selection. We have great knowledge about the genetic architecture of many adaptive traits and of the genomic modifications that lead to inter- and intraspecific variation in many different organisms and for a variety of traits (e.g. Honěk & Honek 1993; Williams 1994; Enard *et al.* 2002; Greenwood *et al.* 2011). Even though it is known that plasticity can be heritable and subject to selection, like other quantitative traits, studies of the genetic basis of plasticity are relatively scarce (but see Scheiner & Callahan 1999; Brommer *et al.* 2005). The genetic basis of plasticity includes loci involved in environmental-responsiveness as well as loci responsible for variation therein. The latter corresponds to the genotype-by-environment component of phenotypic variance in a population which can be studied, within a classical quantitative genetics framework, by comparing reaction norms between genetic backgrounds (Figure 1.3), as has been done in this thesis. Genetic variation for plasticity can affect different aspects of the plastic response such as the extent of plasticity resulting from environmental heterogeneity (Lind & Johansson 2007), the environmental threshold that triggers phenotypic changes (Moczek & Nijhout 2003), or the period of development that is responsive to environmental variation (i.e. the window of environmental sensitivity). In Chapter 2, we showed that genetic backgrounds of D. melanogaster vary in their window of thermal sensitivity for pigmentation development. Moreover, we showed that (putatively)

functionally related traits, such as body color and pattern, are sensitive to developmental temperature during different periods of pre-adult development (Figure 3.3).

In Chapters 3 and 4, we unraveled the genetic basis of variation in thermal plasticity by taking advantage of the availability of the DGRP mapping panel. This panel consists of more than 200 inbred fully-sequenced lines derived from a natural population in Raleigh, USA (Mackay *et al.* 2012; Huang *et al.* 2014). The DGRP has been frequently used to characterize correlations between traits and to uncover the genetic basis of phenotypic variation in different quantitative traits (e.g. Vaisnav *et al.* 2014; Wang, Lu & St. Leger 2017), but had not been used before to identify loci underlying differences in plasticity. We documented genetic variation for plasticity in body pigmentation and in body size in the DGRP (Chapters 3 and 4). Most genotypes were thermally plastic and responded in the direction that had been previously reported in other studies (e.g. the body size of about 60% of the genotypes was larger when raised at low temperature; Figure 4.1B). However, we also found traits for which only few genotypes were plastic (e.g. only 19% of the genotypes showed differences in thoracic color between the two temperatures; Figure 3.3A) and traits for which the plastic response showed the opposite direction than what had been previously described (e.g. 8% of the genotypes had smaller thoraxes when raised at low temperatures; Figure 4.1B).

Upon characterization of variation in size and pigmentation plasticity in the DGRP, we performed a genome wide association study (GWAS) to unravel the genetic basis of variation in plasticity, using as quantitative traits of interest the raw and absolute values of the slopes of the thermal reaction norms. We identified a number of putative QTLs (and validated some of them) associated with variation in plasticity for body pigmentation (Chapter 3; e.g. Figure 3.7) and size in *D. melanogaster* females (Chapter 4; e.g. Figure 4.4). The changes in DNA sequences that underlie differences in these (and other) traits represent the targets of selection. Therefore,

determining the nature and identity of these changes is of key importance for a better understanding of the regulation and evolution of plasticity. This has been a central topic throughout this thesis and our major findings as well as the limitations of our approach will be discussed in the following subsections.

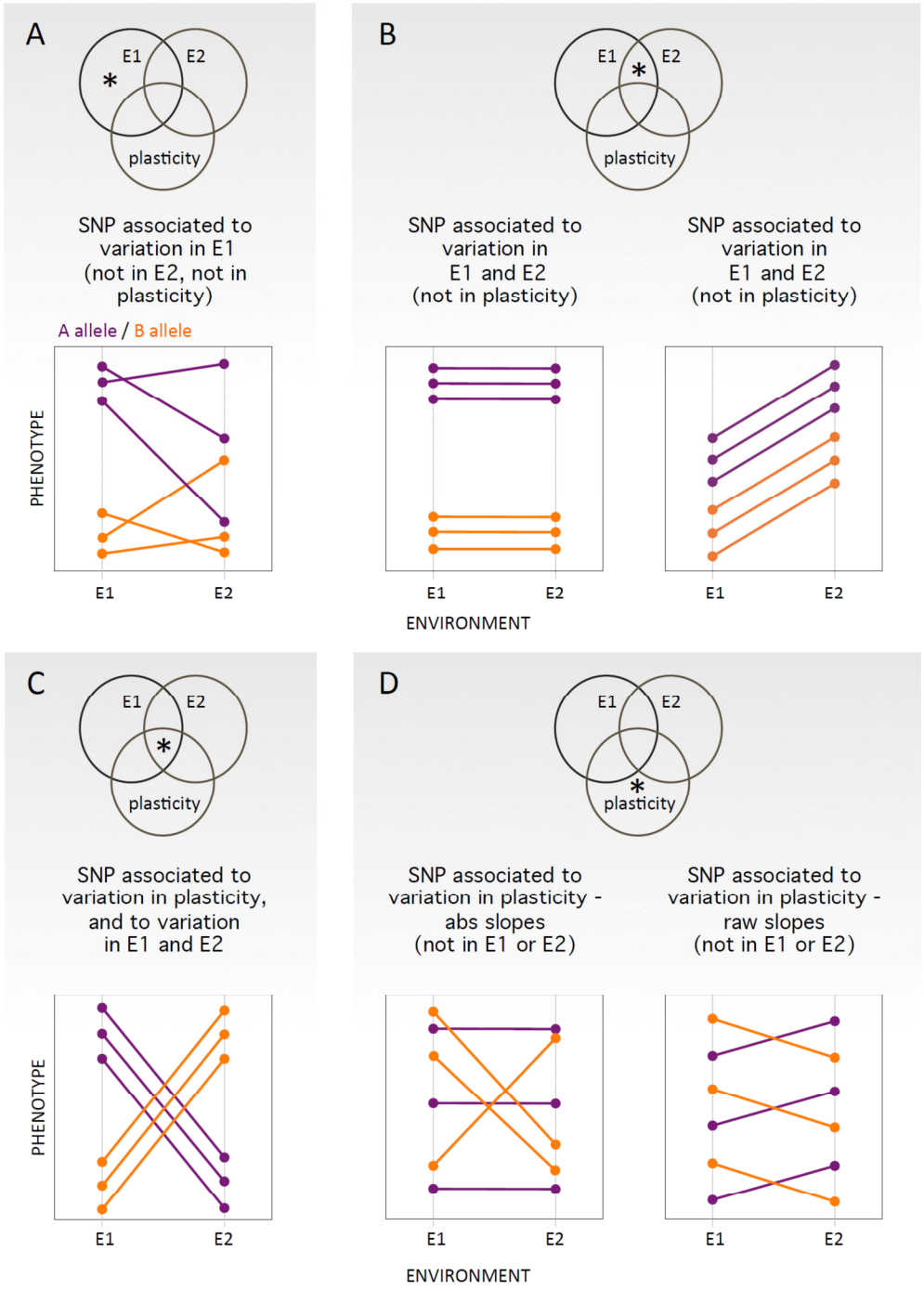
The genetic basis of variation in trait and in trait plasticity

The different models proposed to explain the genetic underpinnings of plasticity (Via et al 1995) either argue that plasticity itself has a genetic basis, independent of the one controlling the trait values themselves, or that plasticity is accounted for by alleles that differ in effect across environments: QTL-by-environment interactions (e.g. Via & Lande 1985; Scheiner 1993, Via *et al.* 1995; de Jong 2005). Most research on the genetic basis of plasticity has focused on the latter, identifying loci that showed QTL-by-environment interactions (but see Scheiner & Callahan 1999; Brommer *et al.* 2005). Examples of this include studies in *Drosophila* exploring the effects of temperature on bristle number (Gurganus *et al.* 1998), lifespan (Vieira *et al.* 2000) and other fitness related traits (Fry *et al.* 1998). Similarly to what we reported for variation in body pigmentation (Chapter 3) and in body size (Chapter 4), those studies found QTLs with environment-specific effects, as well as QTLs with fixed effects across environments. The implicit assumption made in some of those studies was that QTLs showing a QTL-by-environment interaction affect phenotypic plasticity and, therefore, could represent, at least partly, the genetic basis of plasticity. However, alleles whose effects vary across environments do not necessarily contribute to variation in plasticity (i.e. to differences in slope of reaction norms between genotypes; Figures 3.3A and 4.1B). Figure 5.2 illustrates a set of scenarios under which a particular QTL can contribute to variation in trait values and show QTL-by-environment interaction while not contributing to variation in plasticity (Figure 5.2A). Scenarios in which a QTL contributes to variation in plasticity, with or without affecting trait values within environments, are depicted in Figure 5.2C and 5.2D. In this thesis, we focused on loci carrying

allelic variants associated to inter-genotype differences in plasticity for body pigmentation (Chapter 3) and size in *D. melanogaster* females (Chapter 4). Our work, using the slopes of the reaction as a quantitative trait (Chapter 3 and 4), revealed that the genetic basis for trait plasticity, to a large extent, differs from the genetic basis for phenotypic variation in the trait itself (at any given environment). This finding holds for loci underlying variation in pigmentation plasticity (Chapter 3) and for loci affecting thermal plasticity in body size (Chapter 4) as well as for putative and validated QTLs. These loci are presumably the ones that provide the raw material for selection to act upon during the evolution of plasticity under heterogeneous environments.

Different loci contribute to variation in plasticity of different traits

Many studies have explored the mechanistic basis of trait variability (or its counterpart, canalization) and its relationship with environmental variability. Some of these studies hypothesized about the existence of a universal mechanism controlling whether phenotypes would respond to (or buffer) environmental variation (see Shingleton & Tang 2012). Correlated responses of groups of traits to different sources of environmental (or genetic) variation, have been taken as an indication for such a shared regulatory mechanism (e.g. Clarke 1998; Willmore, Klingenberg & Hallgrímsson 2005), while uncorrelated responses between traits have been taken as indication of the opposite (e.g. Debat, Debelle & Dworkin 2009; Pélabon *et al.* 2010). Our work intended to explore this matter beyond correlative inference, by comparing the genetic basis of plasticity of different traits. In the context of our data, it is conceivable, for instance, that genetic variation for thermal responses could be determined by allelic variants at the level of sensing (e.g. genetic polymorphisms leading to differential perception of temperature). In this situation we would expect to find a considerable overlap between QTLs contributing to variation in plasticity across traits. Instead, our data revealed mostly “private QTLs” (e.g. body


A
E1
E2
plasticity
SNP associated to variation in E1 (not in E2, not in plasticity)
A allele / B allele
PHENOTYPE
E1
E2
B
E1
E2
plasticity
SNP associated to variation in E1 and E2 (not in plasticity)
SNP associated to variation in E1 and E2 (not in plasticity)
E1
E2
E1
E2
ENVIRONMENT
C
E1
E2
plasticity
SNP associated to variation in plasticity, and to variation in E1 and E2
PHENOTYPE
E1
E2
D
E1
E2
plasticity
SNP associated to variation in plasticity - abs slopes (not in E1 or E2)
SNP associated to variation in plasticity - raw slopes (not in E1 or E2)
E1
E2
E1
E2
ENVIRONMENT

part and trait specific effects) but included also some shared QTLs contributing to plasticity in more than one single plastic trait. There was also little overlap between QTLs for different properties of the thermal response (i.e. raw versus absolute value of the slope of the reaction norm). These conclusions applied to QTLs underlying variation in pigmentation plasticity (Chapter 3) and to QTLs underlying variation in size plasticity (Chapter 4). For example, when looking at the loci underlying variation in plasticity for abdominal pigmentation components (Chapter 3), 2% of the genes were

Figure 5.2. The relationship between allelic effects on trait mean and on trait plasticity. Schematic representation of the ways in which SNPs can affect phenotypic values under fixed environmental conditions (E1 or E2) and/or plasticity in relation to those environmental values (reaction norms). Colored lines represent reaction norms for different genotypes. Purple and orange represent genotypes that differ in which allele they bear at a specific SNP (A or B) whose association to phenotypic variation is illustrated in the Venn diagram. The "*" represents which trait(s) the SNP could be significantly associated with. **A.** Genotypes with the A allele have higher trait values than those with the B allele in environment E1 but not in environment E2. Genotypes with A and B alleles have reaction norms that can be flat and steep as well as to go in any direction. Thus, this SNP is associated to variation in environment E1, not affecting variation in environment E2 nor variation in plasticity. **B.** Genotypes with the A allele have higher trait values than those with the B allele both in environment E1 and E2, but both alleles have reaction norms of the same slope. This SNP is associated to variation in both enviromnets (E1 and E2) but not to variation in plasticity. Panel on the right is a QTL showing QTL-by-environment interaction and not associated to variation in plasticity. **C.** Genotypes with the A allele have higher trait values than those with the B allele in environment E1 and lower trait values than those with the B allele in environment E2. Genotypes with the A allele have reaction norms with negative slopes while genotypes with the B allele have reaction norms with positive slopes. This SNP is associated to variation in both environment (E1 and E2) as well as to variation in variation in plasticity. **D.** Genotypes with the A allele and B alleles low or high trait values in both environments (E1 and E2) but different reaction norms. In left panel, genotypes with A allele have flat reaction norms while genotypes with B allele have steep reaction norms. In right panel genotypes with A allele have reaction norms with a positive slopes while genotypes with B allele have reaction norms with a negative slopes. This SNP is associated to variation in plasticity but not to within-environment variation (not to E1 or to E2). These type of QTL for variation in plasticity can affect different aspect of the thermal responses, such as the extent of plasticity (i.e. flat vs steep reacton norms on left panel) and/or the extent and direction of the plastic response (i.e. oppostite directions of the reaction norms on right panel).

common between darkness and pattern and 7% were common between color traits (Figure 3.6A). Al together the little degree of overlap between putative QTLs contributing to variation in plasticity of different body parts and/or different traits, is inconsistent with the idea of a universal mechanistic basis for plasticity and suggests the potential for independent evolution of plasticity of different traits (or of the same trait in two body parts).

The identity of the genes contributing to variation in plasticity

Studies in different model systems have taught us about the extent and magnitude of the effects that environmental variation can have on gene expression (Aubin-Horth & Renn 2009). However, whether specific gene classes and/or genomic regions are more likely to underlie environmentally-induced phenotypic differences is not known. Our analyses of the genes and genomic regions harboring SNPs involved in variation in thermal plasticity did not provide evidence for an overrepresentation of specific genomic locations (i.e. intronic, coding, regulatory), gene classes (e.g. regulatory and structural genes), gene functions or biological processes (Figures 3.S6 and 4.S5, Tables 3.S3 and 4.S4). Coming back to the example described before, if genetic variation for thermal plasticity would mostly occur at the level of sensing, one would expect to find, within our QTLs, an over-representation (e.g. in GO categories) of nervous system players. We found no such evidence of specific plasticity players (i.e. sensors, mediators, executors; Figures 3.S6 and 4.S5). Instead, we reported that genes influencing plasticity had a wide variety of known functions, potentially mediating environmental effects at different levels, such as the perception of the environmental cue (*e.g.* the gene *btv* for size plasticity), the transmission of that information to developing tissues (e.g. *Eip75B* for size plasticity; *gce* for pigmentation plasticity) and the execution of the information on those tissues (e.g. *Men* for size plasticity; *ebony* for pigmentation plasticity). Taken together, our results suggest that the environmental regulation of size and pigmentation is orchestrated by different molecular players, acting (and

possibly interacting) at several steps of development. This describes a scenario where variation in plasticity in different traits is not only controlled by different loci, but these can act at very different levels during the environmental regulation of development.

Trait regulation, trait variation and plasticity therein

One aim of this thesis was to shed light on the loci underlying differences in traits (and trait plasticity) between genotypes (Chapters 3 and 4). We used two iconic traits in *Drosophila*, for which there is extensive knowledge on their development, and this allowed us to explore the relationship between the genes underlying variation in trait (and trait plasticity) and the genes regulating trait development (or environmental responsiveness). Some of the genes we found to be underlying variation in plasticity are also known to play important roles in the development of the trait itself. These genes were a 'hit' in both the GWAS for variation in the trait at any given environment and in the GWAS for variation in trait plasticity. For instance, the development of pigmentation is likely to have many common players, if not most (e.g. melanogenesis genes). However, the differences between genotypes found in pigmentation traits, were due to allelic variants in those genes as well as other genes not previously associated with pigmentation. Gene *ebony* can be taken as an example; its function in the development of pigmentation, promoting the deposition of black pigments, is well-established (e.g. Wittkopp, True & Carroll 2002; Takahashi *et al.* 2007). Our analyses (including validations of the GWAS hits) confirmed that *ebony* is involved in variation in pigmentation as well as in variation in pigmentation plasticity (Figure 3.S10). Similarly, some of the genes we described to be underlying variation in plasticity are part of endocrine hormonal systems (e.g. gene *gce;* Figure 3.7), which had been described as intermediaries in linking external information with developmental trajectories (Nijhout 1998). In contrast, our analysis also identified putative QTLs, such as gene *sala* that, to our knowledge, have not been described as being involved in pigmentation

development or thermal plasticity in *Drosophila*. To sum up, our data on trait-associations (discussed earlier) and the little of overlap between loci underlying thermal plasticity suggest that the potential for independent development and evolution of plasticity across traits is strong in *D. melanogaster*.

Aside from unraveling the genetic basis of inter-genotype variation in plasticity, a better understanding of the molecular mechanisms of plasticity would greatly benefit from the identification of genes that are involved in producing alternative phenotypes and whose expression is affected by the environment. Researchers have explicitly analyzed the effect of different environmental cues on gene expression (e.g. Levine, Eckert & Begun 2011; Zhou *et al.* 2012), however many of these studies lack the association between differential gene expression and distinct phenotypic outcomes (i.e. differences in plasticity). A possible way of exploring the relationship between environmentally-induced molecular changes and environmentally-induced phenotypes, would be to characterize transcriptomic profiles in a stage and tissue specific manner (i.e. those relevant for the plastic phenotype under study). This will allow to identify candidate effector genes for plasticity; those whose expression varies between environments for lines with different reaction norms (e.g. flat *vs.* steep reaction norms). These genes, whose expression is regulated by the environment and show differential expression in plastic and robust genotypes, are expected to be the ones executing the environmental information during development.

A potential role for RNA editing in thermal plasticity

Aside our genome-wide search for genes putatively contributing to variation in plasticity, we also took a candidate gene approach in the search for molecular mechanisms involved in the regulation of the environmental sensitivity of development. A number of molecular mechanisms, such as methylation patterns and posttranscriptional modifications, have recently gained much attention in the context of plasticity, as their contribution to

inter-individual variation does not depend on variation in the nucleotide sequence of DNA. We investigated the potential role of one these mechanisms, RNA editing, in the regulation of developmental plasticity (Figure 5.1). RNA editing is a process that enables the production of different mRNA molecules from the same primary transcript, resulting in different peptide products (Bass 2002). This process is ubiquitous in metazoans and has been previously suggested to play an important role in thermal adaptation (see Garrett & Rosenthal 2012). In *D. melanogaster*, many transcripts are post-transcriptionally edited by the enzyme dAdar (which stands for *Drosophila adenosine deaminase acting on RNA*), and this editing occurs more frequently in mRNA molecules from genes related to immunity and the nervous system (see Stapleton, Carlson & Celniker 2006; Duan *et al.* 2017), both of which involve genes from melanogenesis biosynthesis. Importantly, it has been shown that the activity of dAdar is temperature-dependent (Rieder *et al.* 2015). We thus, set-out to investigate if Adar-mediated RNA editing could contribute to thermal plasticity in body pigmentation.

In a pilot study, we used a semi-quantitative PCR approach to verify that dAdar was expressed at the stages and tissues that could potentially be relevant for pigmentation development (Figure 5.3A). After establishing that, we investigated whether genotypes with reduced ability for RNA editing would differ in their levels of thermal plasticity. To assess that, we compared thermal reaction norms for pigmentation traits between genotypes with normal and altered Adar function. If, upon reduction of Adar activity, plasticity had increased (i.e. a steeper reaction norm), we would conclude that RNA editing helps buffering environmentally-induced variation. Conversely, if, upon reduction of Adar activity, plasticity had reduced (i.e. a flatter reaction norm), we would conclude that RNA editing promotes environmentally-induced variation (Figure 5.3B). Our results suggest that reduced editing results in reduced plasticity for some thermally plastic traits

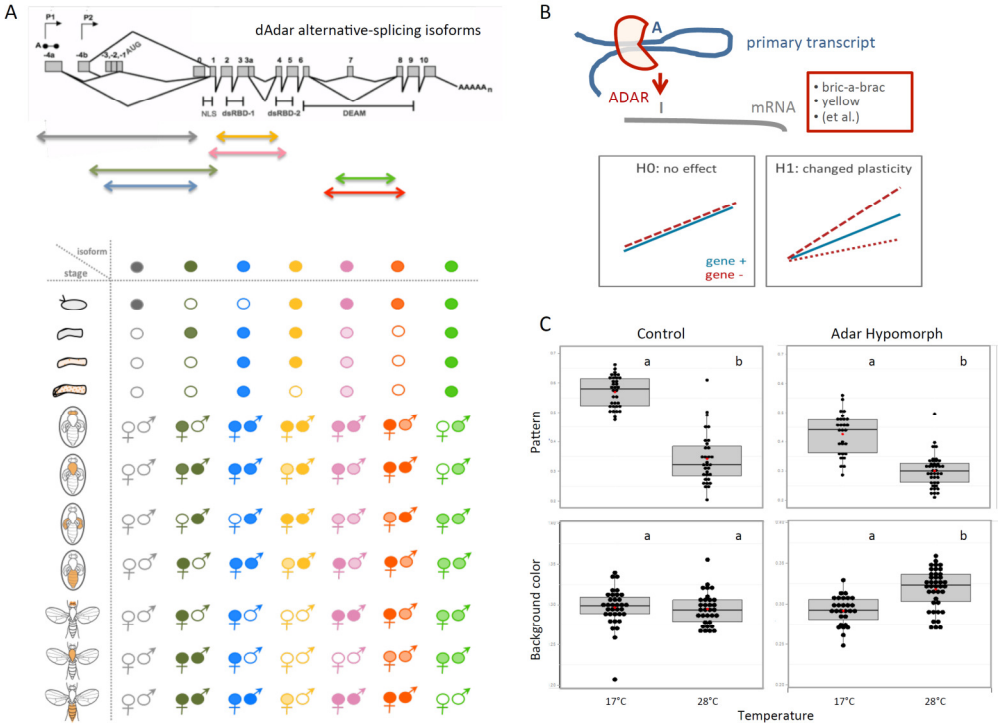


Figure 5.3. A potential role for RNA editing in thermal plasticity of *D. melanogaster* body pigmentation. A. dAdar gene structure showing: the exons (grey boxes) and the two alternative promoters (P1 and P2), as well as the nuclear localization signal (NLS), the two double stranded RNA binding domains (dsRBD-1 and dsRBD-2) and the deamination domain (DEAM) (Chen *et al.* 2009). Color arrows correspond to the regions amplified by the seven primer combinations we used to detect some of the alternative spliced isoforms of dAdar (Marcucci *et al.* 2009). **B.** Schematic representation summarizing the detection (filled circles) or no detection (empty circles) of each Adar isoform cDNA in different stages of development and in different body parts. Sexes were analyzed separately from pupal stage onwards. Different colors correspond to the different alternatively spliced isoforms on A. Stages depicted in the diagram are (from top to bottom): embryo, first instar larva, second instar larva, third instar larva, pupal head, thorax, wings, abdomen, and adult head, thorax, wings, and abdomen. Dark and light color intensities represent differences in expression levels (we use darker color circles when the PCR bands were at least 2 times thicker). **C.** Graph showing the quantification of pattern and color traits in an hypomorph Adar mutant with 80% reduction in expression and activity, and in its respective control. In each boxplot, black dots represent phenotypes of single individual females, red dots are the medians and the black lines are the means. Statistical significance for effect of temperature on pigmentation traits is indicated with letters above each boxplot (Pairwise Wilcoxon Rank Sum Test *** p <0.001, n.s: non-significant).

(notably, width of dark abdominal bands) and increased plasticity for others (notably, color of abdominal dark bands) (Figure 5.3C). Thus, the effect of dAdar in the regulation of plasticity seems to be largely trait-dependent.

These preliminary results suggest that RNA editing could play an import role in thermal plasticity in *D. melanogaster*. Further investigation of these results could involve more functional assays where the effect of RNA editing on plasticity could be tested on multiple genetic backgrounds with different levels of editing. Given that RNA editing is largely dependent on its enzymatic activity, it would be relevant to explore not only the effect of dAdar expression on plasticity but to also address its activity. Some well-known pigmentation genes, such as *yellow* and *bric-a-brac*, are putatively edited (Ramaswami *et al.* 2013). Exploring whether these and other candidate genes undergo temperature-dependent editing, ultimately leading to changes in reaction norms, would confirm the role of RNA editing in thermal plasticity for body pigmentation.

EVOLUTION OF PLASTICITY

Natural selection acting on genetic variation has led to differences between species (Scheiner 1993) and between populations (Crispo & Chapman 2010) in the way they respond to environmental inputs. Evolutionary processes can drive transitions to and from environmentally sensitive development as well as adjust the extent and properties of plastic responses (e.g. Aubret & Shine 2009; Schwander *et al.* 2010). While plasticity can hinder adaptation in some circumstances (e.g. Langerhans & Dewitt 2002), it is commonly recognized that a certain extent of phenotypic plasticity can lead to increased population persistence in novel environments, providing time for adaptive evolution to take place. Moreover, phenotypic plasticity may even facilitate local adaptation when environmentally induced phenotypic variants bring the population closer to a peak in the fitness landscape (see Moran 1992; Price, Qvarnström & Irwin 2003; Ghalambor *et al.* 2007).

It has been proposed that if heritability of plasticity for a given trait is higher than heritability of the trait itself, one could expect evolution in heterogeneous environments to occur via plasticity (Scheiner & Lyman 1989). Our estimates for heritability of plasticity in pigmentation (Chapter 3) and of plasticity in size (Chapter 4) were, in most cases, of the same order of magnitude as the heritability estimates of the trait itself. Noteworthy, estimates of heritability have clear limitations due to the fact that they can be dramatically influenced by environmental conditions in which data is collected (Chown *et al.* 2009). Therefore, comparisons across settings and, even more, comparisons between laboratory and natural conditions, can be arduous.

Plasticity genes and the raw material for the evolution of plasticity

The presence of genotype-by-environment interactions in a population ultimately implies that natural (or artificial) selection for a specific relationship between phenotypes and environments is possible (DeWitt & Scheiner 2004). Studying one population of naturally segregating alleles, the DGRP, we documented considerable genetic variation for plasticity and identified loci associated with variation in plasticity for different traits. These are presumably the loci that selection can target for the evolution of thermal plasticity in size and in pigmentation components. However, these loci identified in the DGRP can be, but are not necessarily, the same that will underlie plastic responses in other natural populations. Moreover, the DGRP represents naturally segregating alleles but due to severe inbreeding and the potential effects that this may have on genetic phenomena, such as linkage and/or epistasis, they may not truthfully represent genotypic diversity. Our finding that some of these loci appeared to have been targets of selection in populations evolved under fluctuating thermal regimes (e.g. allelic variants in genes *Men* and *btv;* Chapter 4) is encouraging. However, in order to make generalizations regarding these loci and their effects on plastic responses, more comprehensive characterizations in other natural populations would be

required. Our data (Chapters 3 and 4) suggests that the evolution of plasticity can happen, to a large extent, through genetic and molecular mechanisms that are independent of the evolution of the trait itself. Moreover, we showed that different properties of the thermal response (i.e. direction and extent of plasticity) can have their own distinct genetic control. This can have important implications for the evolution of environmentally-induced variation as plasticity, and plasticity properties, may be favored by selection independently (see Via & Lande 1985; Scheiner & Lyman 1991).

Ecological scenarios favoring plasticity

Environmental sensitivity of developmental processes is likely to be the ancestral state of most organisms, with selection acting on increased sensitivity or the ability to buffer environmental effects (Newman & Müller 2000; Nijhout 2003). Theoretical models have proposed a variety of conditions that could favor the evolution plasticity, including the predictability of the environment (e.g. Leimar, Hammerstein & van Dooren 2006), the reliability of the environmental cue (e.g. van den Heuvel *et al.* 2013) and the potential (low) costs of plasticity (e.g. Callahan, Maughan & Steiner 2008; Murren *et al.* 2015).

Some studies of developmental plasticity have shed light on the effects of seasonal environmental changes (e.g. Brakefield & Reitsma 1991) and the consequences of decoupling inductive cue and selective environment (e.g. Langerhans & Dewitt 2002). However, work that specifically tests which environmental conditions drive the evolution of plasticity is limited, possibly due to the challenge of designing appropriate experimental set-ups. The assumptions derived from theoretical models could potentially be tested using an experimental evolution approach, however, determining the exact conditions that should be used remains a contentious topic. Regarding the temporal component of environmental heterogeneity, for instance, it remains unclear whether the environment should fluctuate within a given generation (e.g. between different stages of

development; see Moran 1992) or between generations. Another challenge is finding the right match between an inductive environmental cue that accurately predicts the forthcoming environment and the factors that will act as selective agents therein. In some cases of adaptive plasticity, the inductive cue and the selective environment are different (e.g. photoperiod and reproductive opportunities in many species of butterfly) while in other cases, both environments are the same (e.g. predator presence and predation in *Daphnia*). Furthermore, the potential costs of plasticity can be difficult to assess (see Murren *et al.* 2015) and the extent to which these costs may constrain the evolution (and maintenance) of plasticity depend, to a large extent, on the interplay between fitness, phenotypes and environments (e.g. whether plasticity is beneficial or not).

While interpretations of the relationship between the proximate and ultimate mechanisms of phenotypic variation are crucial for our understanding biological phenomena (Mayr 1963; Tinbergen 1963), they can often be complicated to assess. This is, at least in part, due to of the inadequacy of proxy measures for fitness (Scott-Phillips, Dickins & West 2011). Assessing the adaptiveness of a trait may be particularly problematic when evaluating the adaptive significance of reaction norms (DeWitt & Scheiner 2004). In fact, with the exception of a few emblematic examples of adaptive developmental plasticity (e.g. Watt 1968; see Nijhout 2003), in many species little is known about the relationship between environmentally-induced phenotypes and fitness. In the case of the traits used in this study, body size and body pigmentation, the inductive cue and the selective environment are likely to be the same; both thermally induced phenotypes are presumably related to thermoregulation needs (i.e. thermal melanism hypothesis and temperature-size rule) (e.g. Clusella-Trullas *et al.* 2008; Ghosh, Testa & Shingleton 2013). Our analyses of correlations between fitness-related traits and levels of plasticity (Figure 4.S2) provided no evidence for a cost of plasticity. Moreover, we found that the alleles associated with higher plasticity (i.e. steeper reaction norms) occurred at

lower frequency in the DGRP (Figures 3.6C and 4.S6). However, the adaptive value of different reaction norms in the DGRP as well as the prevalence of the identified molecular mechanisms in other *Drosophila* populations remains to be tested. Future work studying variation in plasticity in natural (or experimental) populations, induced by different thermal regimes, could help elucidate the ecological relevance and adaptive significance of plasticity in body size and body pigmentation. This could greatly contribute to our understanding of the ecological conditions that favor plastic or non-plastic development and of the mechanisms underlying transitions between the two.

CONCLUDING REMARKS

Genotype and environment are intricately linked in the production and evolution of phenotypes. The environment beyond filtering phenotypic variation during evolution by natural selection, can lead to the production of new phenotypic variants. This plasticity can help organisms exploit novel environments, provide the means of rapidly adjusting to external change, and even promote adaptive evolution. By studying the patterns of variation in different phenotypic characters, we were able to disentangle important aspects on how genetic, environmental and genetic-by-environmental effects impact phenotypic variation. Moreover, we unraveled the genetic basis for inter-individual variation in thermal plasticity that is necessary for the evolution of plasticity under heterogeneous environments. Studies on plasticity, such as ours, teach us about the proximate mechanisms underlying differences between individuals and about how these might affect evolutionary trajectories. More empirical work is needed in this field of research to bridge the gap between hypothetical scenarios derived from theory and the precise interplay between genotypes, phenotypes and fitness that takes place in natural environments. In this respect, I hope that the work presented in this thesis is a small step towards a better understanding of the regulation and evolution of plasticity.

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