

In the heat of the moment

How *Drosophila melanogaster*'s response to temperature is modulated by sensory systems, social environment, development, and cognition

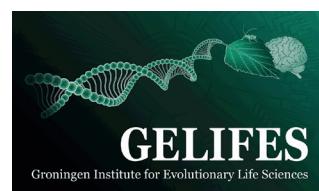


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university of
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This research was carried out in the Evolutionary Genetics, Behaviour and Development (EGDB) group at the Groningen Institute for Evolutionary Life Sciences (GELIFES) according to the requirements of the Graduate School of Science and Engineering (Faculty of Science and Engineering) and the Graduate School of Medical Sciences (University Medical Center Groningen) from the University of Groningen.

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General Introduction

Andrea Soto Padilla

One of the most fascinating endeavours of science is the unravelling and understanding of behaviour. The search for the reasons behind what we do, that incessant need to comprehend what determines our actions, has motivated countless research projects and forced us to look to diverse animal species as models of our own humanity. We know that organisms, just like us, do not simply behave as automatons that respond in a strict and predictable manner to specific stimuli; instead, they balance external stimuli from their environment with their own internal needs to determine how to behave (McFarland, 1977; Mowrey and Portman, 2012; Palmer and Kristan, 2011). For example, a thirsty animal might risk exposing itself to a threat, while without thirst the threat would be completely avoided. In this way, understanding behaviour requires understanding how external forces affect organisms and their reactions, as much as the mechanism that organisms use to cope with these effects.

Temperature as modulator of animal behaviour

Temperature is a main environmental force that all organisms have to cope with. Temperature can affect all levels of biological organization by directly influencing the rate of enzymatic reactions, which in turn affect metabolic processes, physiological responses and ultimately behaviour (Abram et al., 2017; Angilletta, 2006). Worldwide, climate change has produced an increase in mean temperature and a greater chance of extreme temperature events (Stocker et al., 2013), which are expected to produce detrimental effects on many species that regulate their behaviour based on how cold or hot the environment is (Kellermann et al., 2012). This has created a need to comprehend how species will respond to their future environmental reality, from the behaviours that might be modified by temperature and to the new behaviours that might emerge due to temperature effects (Abram et al., 2017; Dell et al., 2011). The reactions from different species will largely depend on their capacity to cope with warmer climates as endothermic or ectothermic creatures. Endotherms, such as mammals or birds, are organisms capable of controlling

their body temperature through metabolic processes (Lowell and Spiegelman, 2000). This endogenous homeostatic control is directly influenced by environmental temperature and depends on the interaction between perceived external temperature and functional body temperature range to activate the physiological responses that keep body temperature stable (Clarke and Rothery, 2008; Lowell and Spiegelman, 2000). As endotherms exhibit considerable phenotypic plasticity in their thermoregulatory response (Boyles et al., 2011), they could take advantage of this physiological flexibility to cope with climate change. Ectotherms, on the other hand, are organisms that depend on the environmental temperature to determine their body temperature (Purves et al., 2003). This implies that they cannot rely on a physiological adjustment of body temperature, but rather must perform thermoregulatory behaviours, such as seeking areas under a shade, to control their body warmth (Abram et al., 2017; Huey et al., 2003). The changes produced by climate change will then constrain when and where ectotherms can develop (Stevenson, 1985), as increasing temperatures might motivate ectothermic species to avoid entire regions or lead some others to extinction by limiting the times of day they can be active.

Ectotherms are affected differently by temperature depending on their body size (Huey, 1979; Stevenson, 1985). Large ectotherms, such as lizards, have a body that requires time for heat to transfer throughout. This slow heat conductance allows them to partially control their body temperature by regulating blood flow, which prevents fast and extreme heat increases (McNab and Auffenberg, 1976; Spotila, 1980; Stevenson, 1985). This permits them to freely move in space and time without being majorly affected by the small temperature changes that occur through any given day (May, 1976; Stevenson, 1985; Woods et al., 2015). On the other hand, small ectotherms such as insects have large body areas with small volumes that allow for a fast heat conductance, which forces them to quickly adopt the environmental temperature in which they are immersed (Barlett and Gates, 1967; Tracy, 1982). This implies that the best strategy for small ectotherms is to select ideal times of day or particular microsites in which the temperature permits them to maximize performance (Abram et al., 2017). As temperature changes around the world, these ideal times are shifting and directly affecting insects' distribution, survival, and population dynamics (Mowrey and Portman, 2012).

Temperature as driver of change in insects' dynamics

Insects' lack of control over their own body temperature implies that their basic enzymatic processes are under direct influence of the environmental temperature. Warming speeds up the rate at which enzymatic reactions occur (Angilletta, 2006; Huey, 1979; Logan et al., 1976), which will consequently accelerate insect's development and performance rates. As a result, the most obvious change in insect's behaviour due to climate change is an advance in their typical periods of emergence and activity (Forrest, 2016). However, this change is not just a simple forward shift in calendar activity throughout the insect world. Different insect populations possess different thermal tolerance and phenotypic plasticity to cope with changing temperatures (Bowler et al., 2015; Estay et al., 2014; Hodgson

et al., 2015), which has led to a diverse set of responses between insects. For example, multiple grasshopper species living on elevated areas have prolonged their development and increased their body size due to climate change, while grasshopper species living in lower areas have shortened their development and become smaller (Buckley et al., 2015). Increasing temperatures in Greenland have augmented the growth rates of mosquitoes more than of beetles that prey on them, which has led to an overall increase in the mosquito population (Culler et al., 2015). In Australia, warmer temperatures have favoured the numbers of the diamondback moth over their parasitic wasp, *Diadegma semiclausum*, facilitating moth invasion of agricultural regions (Furlong and Zalucki, 2017). To further complicate this issue, changing temperatures can have a different impact on each performance measurement within the same insect species. For example, female fecundity of the mosquito *Aedes aegypti*, vector of Denge, yellow fever, and chikungunya viruses, increases at high temperature while larvae development is reduced. This leads to more eggs being deposited than the ones that get to eclose. As temperatures decrease later in the year, these excess eggs are allowed to develop and eclose, producing an untimely mosquito outbreak (Chaves et al., 2014). For the butterflies *Lasiommata megera* and *Agrotis segentum*, warming temperatures have stimulated an extra generation before the typical winter break through which larva would have typically developed (Dyck et al., 2015; Esbjerg and Søgaard, 2014). This added generation is smaller and its larvae are unsuccessful in completing their own development, causing decay in population numbers the year after. One main concern in temperature studies then, is to understand how specific insects respond to temperature changes to predict how our warming environment may affect them and the larger ecosystems that depend on them.

Temperature as constant factor in *Drosophila*'s life

One insect of particular interest is the fruit fly *Drosophila melanogaster*. This fly has been a fundamental model organism for our understanding of development, genetics, neural circuits, and complex behaviours. For over 100 years, flies have allowed us to explore the underlying basis of anatomical traits (Williams and Sehgal, 2001), learning and memory (Mao and Davis, 2009; Waddell, 2010), place-learning (Ofstad et al., 2011), sleep cycles (Donlea et al., 2014), circadian rhythms (Sehgal, 2017; Yao and Shafer, 2014), maternal effects (Mohan et al., 2018), female reproductive behaviours (Gorter et al., 2016; Luturney and Billeter, 2016), and social behaviour (Ramdyia et al., 2015; Schneider et al., 2012). The approximate 100 000 neurons of the fly brain (Pandey and Nichols, 2011) have been extensively explored using advanced genetic techniques (Bader et al., 2007; Chiang et al., 2011; Donlea et al., 2014; Guven-Ozkan and Davis, 2014; Hampel et al., 2015; Zwarts et al., 2012) to create a detailed map of the circuitry controlling flies' actions, providing neuroscientists all over the world with a detailed research model. *Drosophila* have also proved to be a useful model to understand the effects of temperature over the evolution, development, and behaviour of small ectotherms. Evolutionarily, temperature has played a constraining role, leading fruit fly species from different climates to develop distinct coping mechanisms. For example, the climate in tropical areas allows fly species to be active throughout the day, while the cold nights followed by hot days of the desert favour fly activity mostly at dawn, when temperature is in the preferred range. This

implies that tropical species are exposed to well-lit environments, while desert species typically encounter dimmer light conditions. In consequence, tropical species are more likely to use visual cues displayed by the male for successful mating, while desert species take advantage of cues that function without proper vision (Jezovit et al., 2017). These alternative cues are most likely sexually dimorphic cuticular hydrocarbons (CHs), the main chemical components linked to communication between flies (Billeter et al., 2009; Vosshall and Stocker, 2007). The size and amount of a species CHs can be affected by social environment, photoperiod, humidity and temperature (Frentiu and Chenoweth, 2010; Krupp et al., 2008). In fact, the length of a species CHs can be predicted by temperature, as CHs are fundamental to a species resistance to thermal stress and desiccation (Jezovit et al., 2017). Fly species in temperate and tropical climates present longer CHs than species in arid areas. It is possible that the development of shorter CHs, combined with the low light settings of the desert at dawn, led species in these areas to a sexually dimorphic CHs pattern. Meanwhile, the luminosity around flies in tropical zones favoured the development of visual cues instead of sexually differentiated CHs. These conclusions demonstrate how temperature, an environmental variable, can affect the evolution of diverse physiological adaptations and communication mechanisms within closely related organisms.

Temperature is also a fundamental component of every aspect of the life of a single fly. During the larval stage, sensation of temperature will condition where a larva moves to and which areas are avoided. First instar larvae will seek temperatures under 23°C (Rosenzweig et al., 2008), while third instar larvae will move to areas under 18°C (Liu et al., 2003), probably to reduce the risk of exposure to extremely high temperatures during pupation. Larvae will also pupate closer to food substrates when environmental temperature is lower, although humidity and food moisture influence this tendency (Pandey and Singh, 1993; Schnabel and Grossfield, 1992). Rearing flies in restricted temperature environments influences flies' developmental rate, body size, and adult temperature preference. Flies raised at high temperatures (~27-30°C) have a shorter developmental time, a smaller body size, and prefer higher temperatures when compared to flies raised between 18°C and 25°C (Good, 1993; Wang et al., 2008). Flies exposed to warmer climates also have a higher resistance to experimental temperature extremes than flies from colder environments (Kellermann et al., 2012; Krstevska and Hoffmann, 1994). Differences in the production and efficiency of heat-shock proteins, which are expressed under high temperatures to aid in preventing protein misfolding, might be the underlying basis for this diverse tolerance (Feder et al., 1996; Kjærsgaard et al., 2010; Welte et al., 1993). Flies from the same population separated into cold (13°C) and hot environment (29°C) after two days of life showed a differentiated gene expression profile during adulthood, including genes for diverse heat-shock proteins (Chen et al., 2015). This suggests that flies are capable of coping with temperature changes as they occur, without necessarily requiring a long acclimatization process. In consequence, research has focused on understanding how flies cope with fast and dynamic thermal challenges during their daily life to unravel the mechanisms behind temperature sensing and processing. It is possible that by understanding these, it would be feasible to predict how fly behaviour will be altered by the large-scale challenges presented by climate change.

Temperature methods to study *Drosophila*'s thermal response

How flies respond to their immediate temperature environment has been mainly investigated in adult fly behaviour in three main experimental paradigms: preference in a fixed temperature gradient (Hamada et al., 2008; Hong et al., 2008; Rajpurohit and Schmidt, 2016); preference when presented with two temperature choices (Galili et al., 2014; Gallio et al., 2011; Kim et al., 2010; Ni et al., 2013); or climbing speed after being introduced to a fixed high temperature for a short period (Dell et al., 2011; Kjærsgaard et al., 2010; Latimer et al., 2011; Latimer et al., 2015). These methods have allowed elucidating much of the functional mechanisms behind flies' temperature perception and processing (Gallio et al., 2011; Hamada et al., 2008; Ni et al., 2013). Flies have multiple thermosensory receptors distributed peripherally and in their brain (Barbagallo and Garrity, 2015; Bellemere, 2015). These thermosensors are specialized in sensing cold or warm temperatures (Gallio et al., 2011; Hamada et al., 2008), and fast or slow and large or small temperature changes (Frank et al., 2015; Liu et al., 2015; Ni et al., 2013). The peripheral receptors, located in the second and third antennal segments, connect to the thermosensors in the brain, which in turn project to higher brain centres linked to behavioural regulation (Gallio et al., 2011; Tang et al., 2013). The thermosensory cells

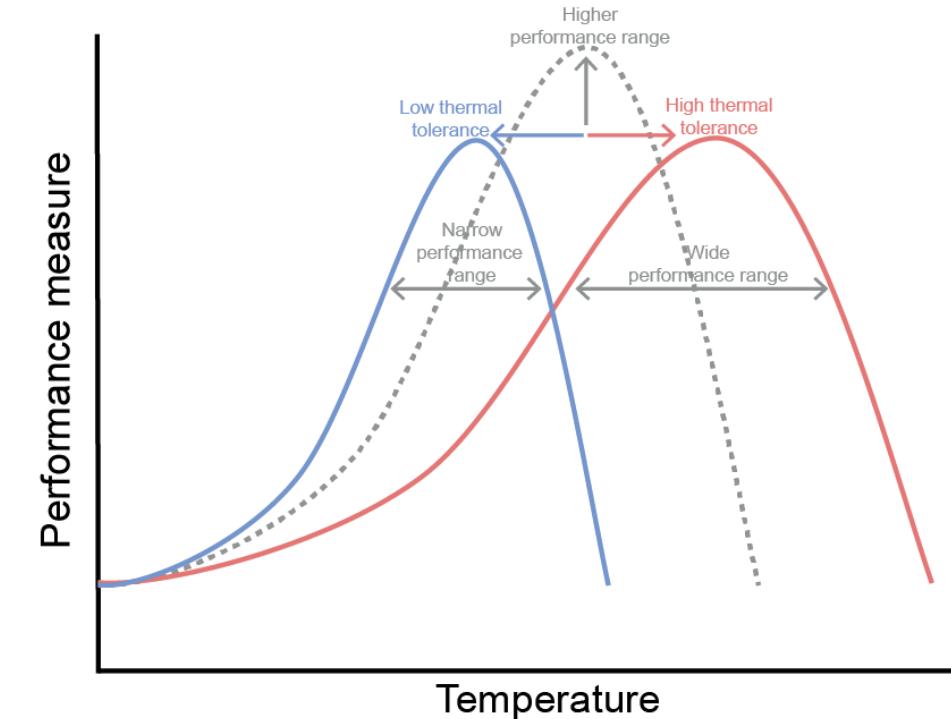


Figure 1 Examples of thermal performance curves. Displacement to the right (red) indicates higher temperature tolerance than displacement to the left (blue). A wider area under the curve (red) indicates a wider range of temperatures at which an organism can perform. A higher maximum point (gray), indicates a higher performance.

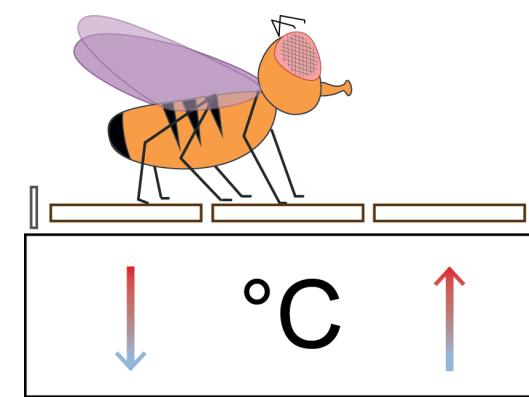
in the brain express the Transient Receptor Potential protein A1 (*TrpA1*; Hamada et al., 2008). *Trps* are a family of nociceptive cation channels found from flies to humans (Cao et al., 2013; Wu et al., 2010); *TrpA1* in particular, functions as a nociceptor in vertebrates, sensing chemical, mechanical and cold stimuli (Bandell et al., 2004; Kwan et al., 2006), and mediating pain after damage and inflammation (Bautista et al., 2006; Obata et al., 2005). Likewise, fly larvae lacking *TrpA1* show defects when responding to noxious mechanical or thermal stimulation (Neely et al., 2011; Zhong et al., 2012) and adult flies without this receptor stop avoiding harmful temperatures (Hamada et al., 2008). These suggest a close similarity between *Drosophila* and vertebrate *TrpA1*, which implies that elucidating how flies avoid dangerous temperatures could have implications for understanding nociception in mammalian species.

Fixed temperature gradients have helped determine how flies prefer temperature at $\sim 25^{\circ}\text{C}$ (Sayeed and Benzer, 1996). Temperature gradients are created by heating and cooling opposite ends of a conductive base on which a group of flies would be placed and allowed to move freely. After certain amount of time, flies distribution along the gradient is used to assess if certain temperatures are preferred or avoided. However, this method presents two main problems. First, if a lamp is used as a heat source, the phototactic tendencies of flies (Markow, 1979) might favour selecting an area due to illumination and not temperature preference. Second, thermal gradients cannot account for the effect of temperature over the metabolic rate and speed of movement of small ectotherms. As small ectotherms quickly adopt the temperature of the environment around them, their enzymatic systems gain and loose kinetic energy as they move through the temperature gradient (Dillon et al., 2012; Stevenson, 1985). It is possible that the areas at which flies stop moving represent temperatures that do not provide sufficient energy to continue locomotion instead of being temperatures actively preferred.

The effect of temperature over flies' locomotion has been explored by generating thermal performance curves after exposing flies to diverse fix temperatures (Dillon et al., 2012; Klepsat et al., 2013; Latimer et al., 2011; Latimer et al., 2014; Latimer et al., 2015). A thermal performance curve (Fig. 1) is a nonlinear continuous reaction norm that links values of performance traits to a range of environmental temperatures (Huey, 1979; Izem et al., 2005). The thermal performance curves of ectotherms typically follow a path of continuous increase as temperature rises until a maximum point is reached and a quick decline follows (Angilletta, 2006; Dillon et al., 2012; Huey, 1979; Huey and Kingsolver, 1989). Variations in the curve can indicate an organism's maximum temperature tolerance, the temperature range at which such organism can respond, and the rate at which a behaviour can be performed. For example, a curve displaced to the right indicates an organism with a higher temperature tolerance than one with a curve to the left; a wider area under a curve of a similar height as another indicates an organism can perform at a wider range of temperatures; and a higher curve points towards a creature that can reach a faster performance rate. Traditionally, the thermal performance curve of small ectotherms to increasing temperatures has been seen as a reflection of the increased rates of their underlying biochemical reactions that lead to faster behavioural responses (Abram et al., 2017; Dillon et al., 2012). Models based on enzyme kinetics predict the response of small ectotherms to rising temperatures (Huey and Kingsolver, 1989; Klepsat et al., 2013; Logan et al., 1976), supporting the conclusion that flies'

motility over a thermal gradient might be the simple reflection of the energy provided by the different temperatures. Nevertheless, as flies possess a complex thermosensors system, thermal performance curves based on reactions to fixed temperatures might not be able to encompass the whole behavioural response of flies to changing environments.

Temperature arena to produce dynamic thermal changes



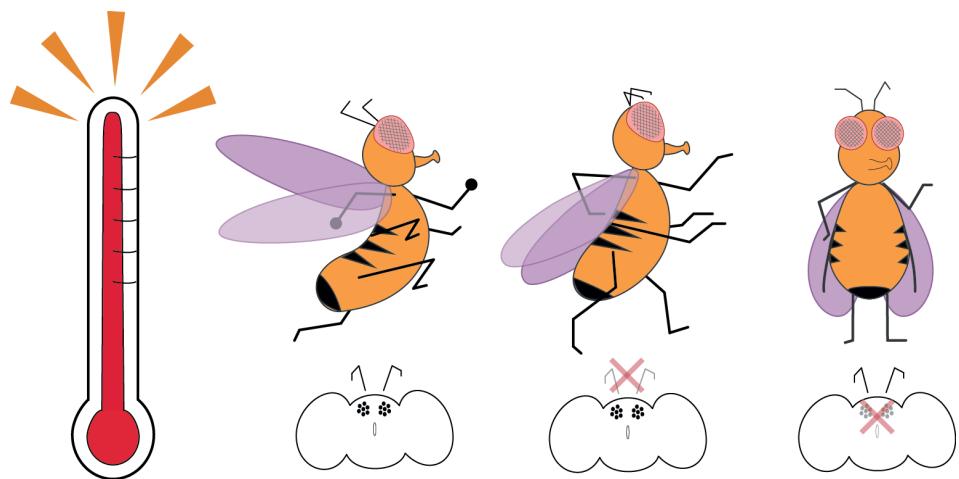
Flies thermosensors activate at different rates of temperature change. *Drosophila* larvae possess multiple isoforms of *TrpA1*, some of which activate at fast temperature changes and produce an intense avoidance response, while others activate at slower temperature increases and produce a milder behavioural reaction (Luo et al., 2017). The combination of these diverse *TrpA1* isoforms ensures that larvae efficiently avoid sudden dangerous

temperatures, while allowing further exploration if the rate of temperature change is less hazardous. Similarly, the peripheral thermosensors of adult flies respond to fast and large temperature changes, while the brain thermosensors activate with slower and less steep increases in thermal gradient (Ni et al., 2013). This suggests that flies respond differently to sudden high temperatures than to gradually increasing thermal gradients, which implies that the biochemical effect of heat might not be the only determinant of flies' behaviour.

Exploring flies' responses to different types of temperature increases required an approach that allowed dynamic thermal changes, unlike the fixed conditions presented in a thermal gradient or during sudden exposures to warmer environments. To achieve this, the work presented here used a new automated temperature-controlled arena that permits precise and fast temperature changes in time and space, introduced and discussed in **Chapter 2**. The arena consists of three copper tiles whose temperatures are independently controlled by a low voltage power supply coordinated by a programmable circuit that receives real-time feedback from thermal sensors under the tiles. The programmable circuit can be instructed to make specific temperature changes at determinate times, creating precise thermal shifts that do not require human intervention beyond placing the fly in the arena. The copper tiles are surrounded by an aluminium ring constantly heated to 50°C to prevent flies from escaping through the sides. A glass plate placed on top of the aluminium ring, a few millimetres above the copper tiles, stops flies from flying away and forces them to walk from tile to tile. The arena is coupled to a camera that constantly records fly movements, which can later be tracked using custom made fruit-fly tracking software to explore how flies respond to diverse temperature challenges. For example,

the reaction of flies to gradually increasing temperatures was observed by heating the three copper tiles to the same temperature at the same time. As reported in **Chapter 2**, this technique showed that *Drosophila* from multiple species responded with different locomotion rates according to their own thermal tolerance, which led to diverse thermal performance curves. The independent control of the three tiles also allowed for more dynamic temperature combinations at a given moment. For example, a tile could be kept at a comfortable temperature for flies (22°C), while the other two tiles were heated up. This led flies to remain in the comfortable tile, even after the other two tiles were cooled down to a comfortable temperature, as shown in the conditioning experiment explained in **Chapter 2**. Changing which tile was at 22°C while the remaining two tiles were heated, logically forced flies to constantly shift location. Interestingly, the hotter the other two tiles, the faster the flies moved to the new 22°C site, implying that the intensity of the temperature contrast was a relevant factor guiding flies' behaviour. These dynamic temperature set-ups positioned the new temperature-controlled arena in an ideal place to test both flies' response to gradually raising temperature and flies' reaction to sudden temperature increases. To understand if the difference in these depended on a mere biochemical effect or required flies' to process temperature information, we compared the thermal reaction curves of wild-type flies with that of temperature mutants, as shown in **Chapter 3**.

Temperature processing as basic element of *Drosophila*'s thermal response



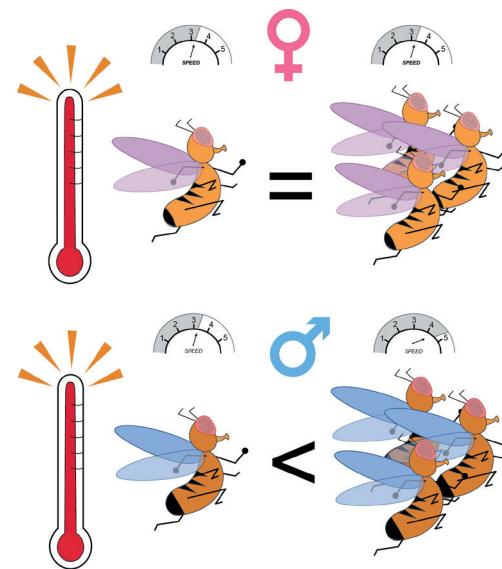
In nature, organisms cope with heterogeneous thermal landscapes and a variety of other external stimuli to satisfy their physiological needs. It would be expected that creatures developed the capacity to integrate the thermal information with other internal and external inputs to coordinate their behavioural responses and ensure the most beneficial course of action (Abram et al., 2017; Woods et al., 2015). For example,

the locust *Locusta migratoria* nymphs choose colder areas in a thermal gradient after starvation. This increases the efficiency of nutrient assimilation and chance of survival, although diminishing developmental rate and growth (Coggan et al., 2011). The main effect of colder temperature depends on its kinetic effect over metabolic process as lower temperature diminishes metabolic rate and increases the efficiency of some processes, such as protein and carbohydrate integration. Interestingly, the behavioural driver for *Locusta* is malnourishment instead of the direct effect of temperature, even though physiologically the benefits emerge from temperature's enzymatic consequences. Similarly, species of ants, beetles and blood-feeding insects use their peripheral thermosensors to detect temperature changes, which guides their path towards suitable food sources (Breugel et al., 2105; Evans, 1966; Kleineidam et al., 2007; Lazzari and Núñez, 1989), seems to also depend on nutritional status, and suggests that at least some insects integrate multiple types of internal information with information about the temperature of their environment to regulate their behavioural response.

Although not many modifiers of the response to temperature have been explored in *Drosophila*, flies temperature preference changes according to humidity (Prince and Parsons, 1977) and flies possess a complex temperature sensing system based on diverse peripheral and brain thermosensors (Gallio et al., 2011; Hamada et al., 2008; Lee et al., 2005; Ni et al., 2013; Zhong et al., 2012), which suggests that flies are also capable of integrating temperature information with other stimuli to coordinate their behaviour. In **Chapter 3**, wild type and thermosensory mutant flies were exposed to gradually increasing thermal gradients and sudden changes in temperature to test if temperature processing was necessary to react to dynamic thermal changes. Being small ectotherms, one potential result was that flies' locomotion at different temperatures would be regulated by the energy from the kinetic effect of heating and the processing of thermal information. However, results demonstrated that flies lacking thermosensors expressing *TvpA1* in the brain do not respond to either gradual or dramatic temperature shifts. Meanwhile, flies lacking peripheral thermal receptor *Gr28b(D)* in the antennae responded to temperature in a similar fashion as controls, except that their performance was reduced. This suggests that flies must process temperature information to be able to respond to dynamic temperature environments and that different thermosensors are responsible for different aspects of this response to temperature.

Flies are likely still affected by the kinetic effect of temperature. Daily peak activity patterns, for example, are dependent on temperature cycles (Lee and Montell, 2013), suggesting that the amount of energy provided by temperature directly influences how active flies are. However, for temperature cycles to influence daily locomotor activity, brain thermosensors must also be intact (Das et al., 2015; Roessingh et al., 2015), further reinforcing that flies final behavioural output is dependent on their processing of temperature information and not only on a direct kinetic response to thermal input.

Temperature response as a plastic reaction to social interactions



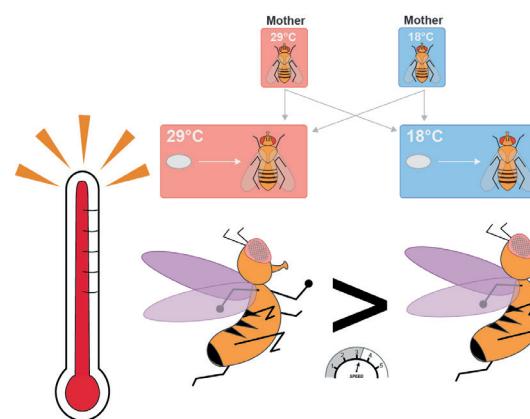
Flies' temperature processing suggests that temperature information could be combined with other internal and external inputs to regulate flies' behavioural output. It is possible that flies would override their typical thermal response if faced with other more salient stimuli, such as hunger, reproductive drive, or predatory threat, as is the case for other ectotherms (Regal, 1966). Some of these modifiers could control flies' response to increasing temperatures and reduce the thermal stress produced by warmer environments, ensuring survival and prolonging survival (Fischer et al., 2010). Understanding these factors

will allow comprehending what aspects of a fly life could affect its resilience to climate change and the more frequent extreme weather events.

High temperatures are considered a stressor for small ectotherms due to their negative metabolic and developmental effects, such as the stimulation of heat-shock proteins production or reduced lifespan (Neven, 2000; Tomanek and Sanford, 2003). To determine which factors could immediately affect thermal behaviour, it is important to consider elements that directly influence the stress response. One particular element that is intertwined with the stress response is the interaction with others of the same species. The effect of others over individual stress has been characterized in multiple species, particularly rodents, showing a wide range of effects linked to the type and intensity of interactions (Beery and Kaufer, 2015): in general, aggression, crowding, and social isolation act as promoters of stress, while healthy social connections act as a powerful buffer to resist stress exposure. For example, humans, non-human primates, and rodents constantly living in a subordinate position have reduced health and well-being (Kirschbaum et al., 1995; Lupien et al., 2009; Sapolsky, 2005), while stable and non-subordinate social relationships in humans and baboons increase lifespan and reduce stress hormones (Holt-Lunstad et al., 2010; Silk et al., 2010). *Drosophila* males kept in isolation post eclosion accentuate their sexual and aggressive behaviour once placed with peers (Bastock and Manning, 1955; Sene, 1977), while flies' that interact with each other when exposed to an aversive odour escape the dangerous areas more efficiently (Ramdyia et al., 2015). It is possible then that the thermal response of *Drosophila* at harmful temperatures will change according to the social environment around them.

The social effect over the stress response is further complicated by sexual differences found in many species. For example, crowding is stressful for male rats, while calming for females (Brown and Grunberg, 1995), and only female prairie voles, not males, suffer from the separation from a same-sex companion (Carter et al., 1995), and isolation has a larger effect on female mice (Senst et al., 2016). Physiological differences explain, at least partially, these contrasting behaviours: while the first phases of the stress response are identical between females and males, females produce larger amounts and possess more receptors for affiliative hormones liberated after the acute stress reaction (Taylor et al., 2000). This favours bonding between females when exposed to stress instead of the typically described fight or flight response commonly seen in males. Interestingly, *Drosophila* also possesses a sexually dimorphic stress pathway (Neckameyer and Nieto, 2015), which suggests that groups of female and groups of male flies might react differently to increasing temperatures. In fact, as demonstrated in **Chapter 4**, female *Drosophila* consistently interact with each other as temperatures increase, while male flies have an inconsistent number of interactions; this leads to a slower average locomotion at high temperatures of females in comparison to males when temperature is high, which suggests lower stress in the group of females than in the group of males. Flies kept in a group before the start of experiments show the same sexually dimorphic trend as flies kept in isolation, even though the effect was larger for those with previous social experience. In contrast, sudden isolation seemed to have a similar effect for females and males. Flies from both sexes grown in a group and suddenly isolated before being exposed to increasing temperatures had a lower performance that grouped flies or flies raised and tested alone. These data suggest that social stressors, such as isolation, could be equally deleterious for all individuals, independent of their sex. Taken together, these results imply that the social condition in which a fly is in, added to its own sex, will affect its behavioural response to temperature.

Temperature response as a plastic feature of *Drosophila* history



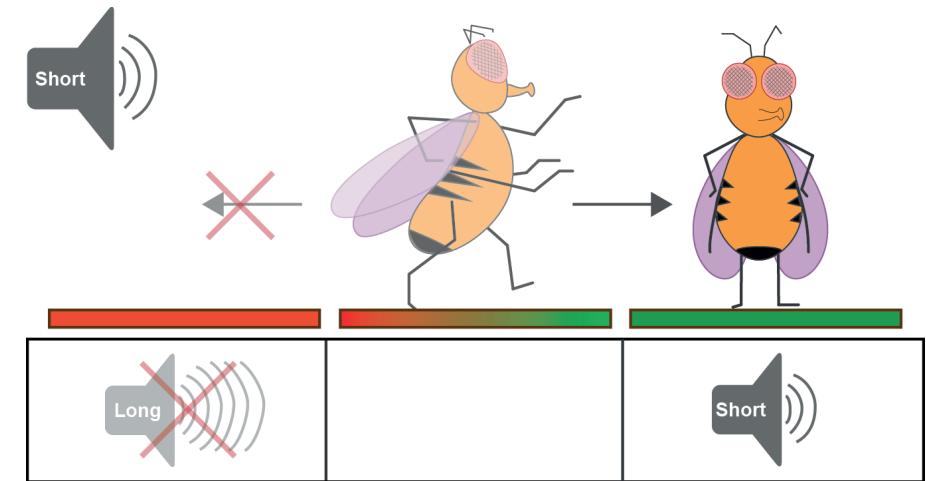
The capacity of a fly to respond to a variable thermal environment is constricted by genetic changes produced through multiple generations and by the individual's own phenotypic plasticity (Mathur and Schmidt, 2017). *Drosophila* species adapted to different climates throughout the world demonstrate the genetic effects of environmental selection (Jezovit et al., 2017). *Drosophila*'s plastic response can be observed when comparing flies of the same species reared at

different temperatures or when exposing flies to extreme temperatures for a short period (Hoffmann et al., 2003). For example, flies raised in cold temperatures will perform better when tested at cold temperatures than flies from the same species reared in hot areas; meanwhile, flies from tropical areas will tolerate higher temperatures better than flies from cold zones (Gibert et al., 2001). Similarly, flies from multiple species of *Drosophila* exposed to 32°C, 33°C or 35°C for just one hour and then placed at 37°C will take longer to be knocked down than flies kept at 25°C (Kellett et al., 2005). The most salient mechanism related to these flexible reactions is the change in production of specific heat-shock proteins (Hoffmann et al., 2003). Flies with a non-functioning heat-shock protein factor move faster at high temperatures (28–34°C) than flies with normal heat-shock protein production (Kjærsgaard et al., 2010), suggesting that heat-shock proteins reduce stress as temperatures increase (Hoffmann et al., 2003).

The capacity to produce heat-shock proteins to face thermal stress could be influenced by flies' maternal experience. *Drosophila melanogaster* mothers provide mRNA coding for small heat-shock protein to their fertilized eggs, partially determining their future offspring capacity to tolerate thermal-stress (Brown et al., 2014; Morrow and Tanguay, 2015). Overexpression of a heat-shock protein gene in female ovaries led to larvae with higher thermal tolerance (Lockwood et al., 2017), suggesting a maternal effect over flies' temperature resistance. In fact, mothers kept at high temperatures (36–38°C) influence the phenotype of their offspring, which develops larger wings, even if the offspring is kept at lower temperatures after eggs deposition (Andersen et al., 2005). It is thus possible that if mothers experience an extreme environment, they will also affect their offspring's behavioural response to temperature to better face such an environment. To test this, mother flies were kept at two extreme temperatures (18°C and 29°) and their offspring were raised at that same temperature or at the opposite temperature, as described in the design presented in **Chapter 5**. The newly eclosed adult flies were exposed to multiple thermal challenges to observe the significance of the maternal environment to restrict offspring's response. Noticeably, the most influential component in offspring's reaction was the temperature at which they have been reared, and not the environment experience by the mothers. It is possible that the maternal influence on thermal resistance, such as the effect the production of heat-shock proteins, is only relevant during the first stages of life. In fact, embryonic stages are more thermally sensitive than posterior larvae development (Welte et al., 1993), suggesting that mothers' influence dissipates as the fly experiences its own environment. Considering the variability of temperatures that an adult fly would encounter in a natural setting, it is logical to assume that fast adaptations to its own experience are the most relevant component of their response to temperature.

Temperature as a salient stimulus for *Drosophila*

The relevance of temperature over the behavioural response of *Drosophila* indicates that thermal information is a salient stimulus that coordinates fly's actions. This suggests that flies could use temperature information to make decisions as much as they are directly affected by the physiological effect of temperature. For example, flies can be conditioned to prefer certain areas of an arena using temperature to produce avoidance of other



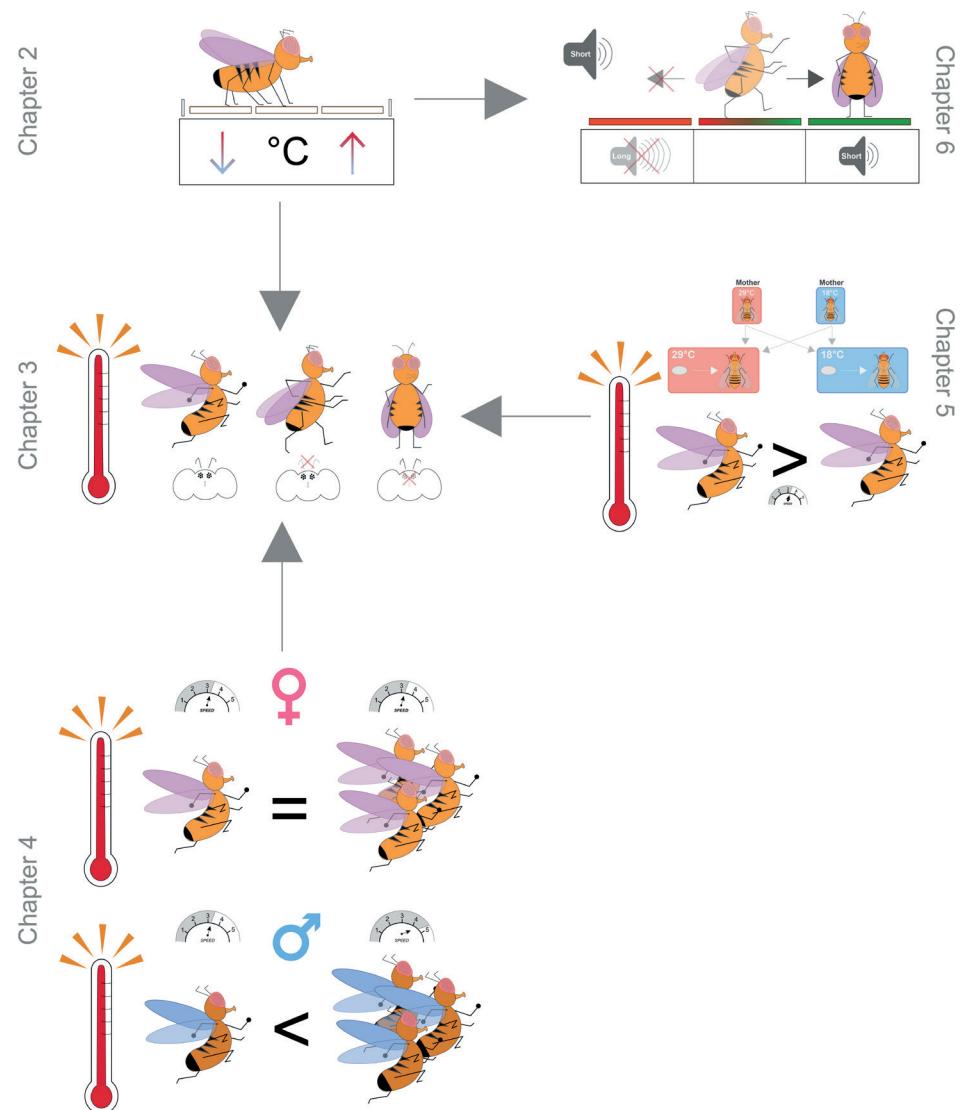
zones (Ofstad et al., 2011; Putz and Heisenberg, 2002; Wustmann and Heisenberg, 1997; Wustmann et al., 1996). In its simplest form, these experiments have conditioned flies to prefer one side of the arena based only the thermal information, which suggests that temperature processing is what is guiding flies' behaviour. These experiments led to the description of multiple characteristics and genes associated to fly learning and memory (Diegelmann et al., 2006; Putz and Heisenberg, 2002; Wustmann et al., 1996; Zars and Zars, 2006; Zars et al., 2000).

Flies are also capable of associating temperature and other stimulus to select comfortable areas and avoid damaging heat. For example, flies placed in a heated arena (36°C) with one restricted comfortable area (25°C) were capable of associating the comfortable area with a particular figure presented to them (Ofstad et al., 2011). This demonstrated that flies' spatial memory emerges from the integration of multiple types of information, including thermal conditions. It is possible then that other types of information are also associated with temperature data to guide fly behaviour. For example, flies could use the temporal information of changing temperatures to displace to a more comfortable area when their location is about to become too cold or too hot. The use of temporal information in a range of seconds to minutes is known as interval timing (Tucci et al., 2014). Interval timing perception permits predicting events in the near future in consistently changing environments, which allows organisms to adjust their behaviour and be better prepared for what is to come (Reilly, 2013). Although studies of interval timing are typically performed in mammals or birds (Buhusi and Meck, 2005; Tucci et al., 2014), insects have also shown to use this time range. For example, ants trained to feed in a specific location for a specific duration will wait approximately the same amount of time if food is no longer presented (Schatz et al., 1999; Schilman and Roces, 2003); parasitic wasps estimate the amount of time walked over a host to calculate the amount of eggs to be deposited (Schmidt and Smith, 1985; Schmidt and Smith, 1987); and bumblebees can be trained to wait a specific amount of time after a light cue before extending their proboscis to receive sugary water (Boisvert and Sherry, 2006). To test if *Drosophila melanogaster* were also capable of using time information in the interval timing range, we exposed flies to short and long cues that indicated specific areas of the temperature-controlled arena that would

become comfortable, while the rest of the arena increased temperature, as described in **Chapter 6**. Flies were to associate auditory or visual cues of short and long duration with specific arena areas in which temperature would remain comfortable. We found that flies, unfortunately, used only the temperature information to guide their behaviour, instead of taking advantage of the timed cues. Probably, the temperature information was too salient in comparison to the timed information, complicating creating an association between the two. Nonetheless, we encourage others to continue exploring flies' interval timing perception, as *Drosophila* could be an excellent model to unravel the neural basis underlying this process.

Thesis at a glance

The work presented here demonstrates that temperature is a fundamental component of every aspect of a fly's life. Since development, the temperature at which a fly grows will determine how well it can cope with the climate challenges of its adult life. Once in adulthood, an intricate system of peripheral and brain thermosensors coordinates how flies respond to dynamic temperature changes. This response is not just a predictable reaction; it is a complex process that can be affected by other internal and external features of the fly, such as its own sex and the sex of surrounding flies. Considering the relevance of *Drosophila* as a model organism, it is fundamental to continue exploring how temperature interacts with the other features of fly's existence, as it will help us predict how small ectotherms might be affected by climate change, while also answering basic neuroscience questions, such as how a brain integrates temperature information.



Thesis Diagram: *Chapter 2* describes the temperature-controlled arena in which all main experiments presented here are based. *Chapter 3* explores temperature perception of flies lacking antennal or brain thermosensors and demonstrates that they are necessary for a normal locomotor response to increasing temperature. *Chapter 4* demonstrates that social interactions affect this locomotor response, and that this effect is sexually dimorphic. *Chapter 5* shows that developmental temperature, despite maternal condition, is the main determinant of the locomotor response to increasing temperature of adult flies. *Chapter 6* uses the temperature-controlled arena to explore complex cognitive skills of *Drosophila* and demonstrates that, in this particular experimental setting, flies use temperature information and not temporal cues to guide their behaviour.

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An automated method to determine the performance of *Drosophila* in response to temperature changes in space and time

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Abstract

Temperature is a ubiquitous environmental factor that affects how species distribute and behave. Different species of *Drosophila* fruit flies have specific responses to changing temperatures according to their physiological tolerance and adaptability. *Drosophila* flies also possess a temperature sensing system that has become fundamental to understanding the neural basis of temperature processing in ectotherms. We present here a temperature-controlled arena that permits fast and precise temperature changes with temporal and spatial control to explore the response of individual flies to changing temperatures. Individual flies are placed in the arena and exposed to pre-programmed temperature challenges, such as uniform gradual increases in

temperature to determine reaction norms or spatially distributed temperatures at the same time to determine preferences. Individuals are automatically tracked, allowing the quantification of speed or location preference. This method can be used to rapidly quantify the response over a large range of temperatures to determine temperature performance curves in *Drosophila* or other insects of similar size. In addition, it can be used for genetic studies to quantify temperature preferences and reactions of mutants or wild-type flies. This method can help uncover the basis of thermal speciation and adaptation, as well as the neural mechanisms behind temperature processing.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58350/>

Keywords

Behavior, Issue 140, Temperature-controlled arena, locomotor behavior, *Drosophila*, temperature performance, automatic heating mechanism, positional tracking

Introduction

Temperature is a constant environmental factor that affects how organisms function and behave (Abram et al., 2016). Differences in latitude and altitude lead to differences in the type of climates organism are exposed to, which results in evolutionary selection for their response to temperature (Jezovit et al., 2017; Rajpurohit and Schmidt, 2016). Organisms respond to different temperatures through morphological, physiological, and behavioral adaptations that maximize performance under their particular environments (Sinclair et al., 2012). For instance, in the fruit fly *Drosophila melanogaster*, populations from different regions have different temperature preferences, body sizes, developmental times, longevity, fecundity, and walking performance at different temperatures (Gibert et al., 2001; Klepsat et al., 2013; Rajpurohit and Schmidt, 2016; Trotta et al., 2006). The diversity observed between flies of different origins is explained in part by genetic variation and plastic gene expression (Chen et al., 2015a; Latimer et al., 2011). Similarly, *Drosophila* species from different areas distribute differently among temperature gradients and show differences in resistance to extreme heat and cold tests (Andersen et al., 2015; Kellermann et al., 2012; Krstevska and Hoffmann, 1994).

Drosophila has also recently become the model of choice to understand the genetic and neural basis of temperature perception (Frank et al., 2015; Gallio et al., 2011; Hamada et al., 2008; Liu et al., 2015; Ni et al., 2013). Broadly, adult flies perceive temperature through cold and hot peripheral temperature sensors in the antennae and through temperature sensors in the brain (Barbagallo and Garrity, 2015; Frank et al., 2015; Gallio et al., 2011; Hamada et al., 2008; Liu et al., 2015; Neely et al., 2011; Ni et al., 2013; Zhong et al., 2012). The periphery receptors for hot temperatures express *Gr28bd* (Ni et al., 2013) or *Pyrexia* (Tang et al., 2013), while the periphery cold receptors are characterized by *Briviso* (Gallio et al., 2011). In the brain, temperature is processed by neurons expressing *TtipA1* (Hamada et al., 2008). Behavioral studies on mutants of these pathways is improving our understanding of how temperature is processed and give insights into mechanisms that vary among populations of *Drosophila* from different regions.

Here we describe a temperature-controlled arena that produces fast and precise temperature changes. Investigators can pre-program these changes, which allows for standardized and repeatable temperature manipulations without human intervention. Flies are recorded and tracked with specialized software to determine their position and speed at different phases of an experiment. The main measurement presented in this protocol is the walking speed at different temperatures, because it is an ecologically relevant index of physiological performance that can identify individual thermal adaptability (Gibert et al., 2001). Together with temperature receptor mutants, this technique can help reveal the mechanisms of thermal adaptation at a cellular and biochemical levels.

Protocol

1. Preparation of Fly Food Medium

- Pour 1 L of tap water into a 2 L glass beaker and add a magnetic stir bar. Put the beaker on a magnetic hot plate at 300 °C until boiling temperature is reached.
- Stir at 500 rounds/min and add the following: 10 g of agar, 30 g of glucose, 15 g of sucrose, 15 g of cornmeal, 10 g of wheat germ, 10 g of soy flour, 30 g of molasses, and 35 g of active dry yeast.
- When the mix foams vigorously, turn down the hot plate temperature to 120 °C while continuing stirring.
- Turn the hot plate temperature further down to 30 °C after 10 min and continue stirring until the mix cools to 48 °C. Measure the temperature by inserting a thermometer directly into the food without touching the walls of the beaker.
- Dissolve 2 g of p-hydroxy-benzoic acid methyl ester into 10 mL of 96% ethanol and add it to the mix, together with 5 mL of 1 M propionic acid. Continue stirring for 3 min.
- Turn the hot plate off and pour 45 mL of food into the rearing bottles and 6.5 mL of food into the collection vials.

2. Preparation of Flies

- Place 20 male and 20 female flies in the rearing bottles containing 45 mL of fly food medium. Transfer the flies to new bottles after 3 to 4 days by tapping them down and then tapping them into the fresh bottles. Discard flies after three changes.
 - Place the bottles inside the incubator under 12-h/light 12-h/dark cycles with a constant temperature of 25 °C.

Note: A new generation of flies will eclose after 10 days.
- Anesthetize newly eclosed flies on carbon dioxide pads for a maximum of 4 min and collect them in 2.5 cm x 9.5 cm fly rearing vials with 6.5 mL of fly food medium using a paintbrush.
 - Collect only virgin flies and separate them by sex into groups of 20 flies per rearing vial.
 - Place the vials inside incubators for 5-7 days, changing the flies to new vials every 2-3 days and on the day before experiments.

3. Frame of Lights

1. Make a wooden frame of 10 cm length, 4 cm width, 4 cm height, and 0.5 cm thick.
 2. On each of the short edges create a border of 4 cm length, 4 cm height, and 1.5 cm width towards the inside area of the wooden frame. Leave the internal face of the border open.
 3. Drill two holes of 0.5 cm diameter at the intersection of one of the long edges of the wooden frame and at each of the borders at the short edges.
 4. Place 10 cm of a warm white LED strip inside each of the borders on the short edges. Peel the back of the LED strip to immediately glue it in place.
- Note:** For experiments in which illumination needs to be eliminated, the warm white LED strip can be substituted for infrared LED strips.
5. Connect one end of the LED strip in one of the borders to the switching power supply and its other end to the LED strip on the opposite border.
 6. Turn the switching power supply on to verify that both LED strips turn on.
 7. Cover the open side of each border with a white piece of paper.
 8. Glue another piece of paper to each of the internal phases of the long edges.

4. Temperature-Controlled Arena

1. Turn on the temperature-controlled arena (Fig. 1A and 1C). Ensure that the fan starts running and the aluminum ring starts warming up.
 2. Use a USB cable to connect the temperature-controlled arena to the control computer running the *TemperaturePhases* script with the temperature sequences.
 3. Open the *TemperaturePhases* script in the control computer and verify that the temperature sequence is properly set up (Video 1).
 1. Check that the duration of each experimental phase is set to 60 s by verifying that “par.StimulusDur” is equal to 60 s.
 2. Check that the 1) number equal to desired number of phases, 2) iterative ON/OFF set-up of the indicative red light emitting diodes (LEDs), 3) 2 °C temperature increase per phase, and 4) 16 °C as the starting temperature are all correct under the “Start the experimental block” section.
- Note:** Allow the flies to acclimate to the Fly Arena for 7 min at 16 °C to avoid an artificial increase of speed during the first experimental phases (Fig. 2).
3. Run the *TemperaturePhases* script. The software will initialize for 5 seconds as determined in “arena.Wait” and then stop.
 4. Press the spacebar of the keyboard to begin running the experimental phases once a fly has been blown into the Fly Arena (step 5.3).
- Note:** The *TemperaturePhases* is the current script controlling the box;

however, it is possible to create other custom scripts to use this device that adjust to the requirements of different experiments.

4. Connect the camera on top of the arena to the recording computer using the camera’s USB cable.
5. Open the video recording program (see Table of Materials) in the recording computer by selecting “File | New Movie Recording”. A screen showing the image from the camera will open.
 1. Ensure that the camera image captures all edges of the arena and the indicative red LEDs
 2. Start recording by pressing the red button in the middle of the screen’s bottom edge showing the camera image once the frame of lights is set around the arena (step 5.4). NOTE: Small changes in lighting can affect accuracy of the tracking. It is recommended to keep the illumination of the temperature- controlled arena constant by fixing the location of the apparatus.

5. Temperature Behavioral Experiments

1. Prepare the Fly Arena (Fig. 1C).
 1. Place a strand of white conductive tape on the top of the copper tiles, ensuring all edges are covered.
 2. Place the heated aluminum ring around the copper tiles. The edge of the ring fits perfectly around the copper tiles so it is always placed in the same location.
 3. Clean the glass cover with a clean tissue and place it on the top of the aluminum ring, leaving a gap through which a fly can be blown in.

Note: Before the experiments, coat the glass cover with the siliconizing agent to create a slippery surface. Apply the siliconizing agent for 24 h and rinse it with water before use.
2. Run the *TemperaturePhases* script (step 4.3.3) and open the video recording program (step 4.5).
3. Blow the fly from a rearing vial (step 2.2.2) into the Fly Arena (e.g., 1 male fly in Fig. 3).
 1. Take a vial of flies from the incubator, tap it twice to force them to go to the bottom, trap one fly with a mouth aspirator, and close the vial and put it back into the incubator.
 2. Place the fly in the arena through the gap that has been left between the glass cover and aluminum ring (step 5.1.3).
 3. Close the gap between the glass cover and aluminum ring by pushing the glass cover until it reaches the edge of the aluminum ring as soon as the fly is introduced to the Fly Arena.
4. Place the frame of lights around the arena to ensure symmetric illumination.

1. Mark the location (*e.g.*, using a permanent marker) of the frame of lights around the Fly Arena (Fig. 1C) to ensure that the frame is always placed in the same location.
5. Start recording with the video recording program (step 4.5.2) and press the spacebar on the keyboard of the control computer to begin running the experimental phases (step 4.3.4).
6. After all experimental phases are done, save the video in .mp4 or .avi format and remove the fly from the Fly Arena with the mouth aspirator.

Note: The end of the experimental phases can be determined by both indicative red LEDs being turned off or by the *TemperaturePhases* script stopping.

1. Stop the video recording by pressing the stop button in the middle of the screen's bottom edge in the recording program. Press "File | Save as" to save the video.

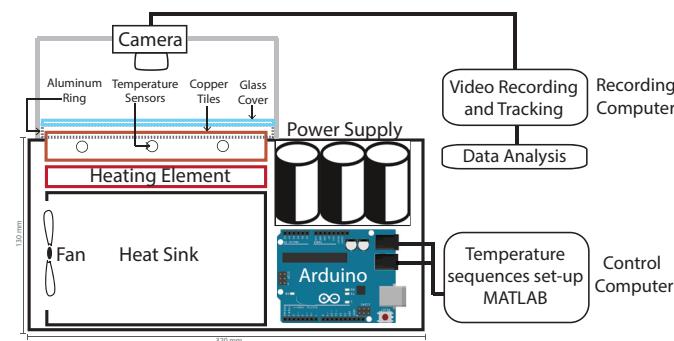
6. Video Tracking and Data Analysis

1. Use the *FlySteps* tracking software (Video 2) to track the videos.
 1. Open the "configuration_file.ini" inside the "FlyTracker" folder.
 2. Set the location of the videos in "video_folder" and the names of the videos in "video_files".
 3. Specify the borders of the Fly Arena in "arena_settings" based on (x, y) pixel coordinates of multiple points at the edge of the arena.
 4. Specify the location of the indicative red LEDs in "led_settings" based on (x, y) pixel coordinates of the location of the LEDs.
 5. Check the location of the borders of the Fly Arena by setting "debug" to "true" in "arena_settings", clicking "Save", and running the script in the terminal. A screen capture of the video will appear with a blue square formed by the coordinates inputted in "arena_settings".
- Note:** This square surrounds the area to be tracked.
6. Change "debug" in "arena_settings" to "false", click "Save", and run the screen in the terminal once more.
- Note:** This will start the tracking process.
- Note:** Flies can walk out of the tracking area onto the heated aluminum ring. This happens during the first seconds of an experiment, after which flies stop touching the heated ring and remain inside the tracking area.
- Note:** Videos can be tracked with other tracking software according to the experimenter's preferences.
2. Use the (x,y) location of each fly provided by the tracking software to calculate the measure of interest for the temperature performance. Custom scripts (*e.g.*, *FlyStepsAnalysis* in Supplementary) can be used.
3. Compare the temperature performance curves of different fly groups using repeated measurements (RM) analysis of variance (ANOVA) and *post-hoc* multiple

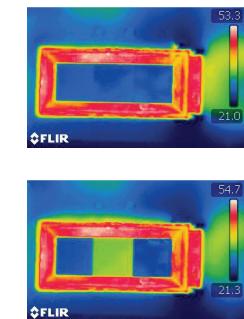
comparisons using statistical software (see Table of Materials).

Representative Results

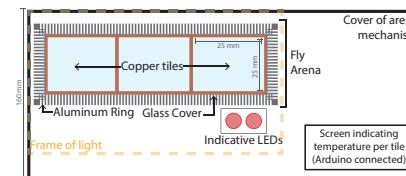
A. Lateral View



B. Thermal Images



C. Top View



D. Frame of Lights

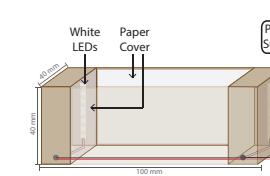


Figure 1 Diagram of temperature controlled-arena. A. A lateral view of the temperature-controlled arena. A programmable circuit connects a power supply and temperature sensors to heating elements under copper tiles to control their temperature. Tiles are constantly cooled down through a heat sink connected to a fan. A heated aluminum ring over which a glass cover rests surrounds the tiles. B. Thermal imaging showing the tiles set at 24 °C (top) and side tiles at 24 °C with a middle tile at 30 °C (bottom). C. A top view of the arena. A camera records the copper tiles, aluminum ring, and red LEDs, then automatically determines experimental phases. A screen in the corner of the box, not recorded by the camera, displays the current tile temperature. D. Ring of light: two warm white LED strips inside a wooden box covered in white paper ensure constant and symmetric illumination of the whole arena.

The temperature-controlled arena (Fig. 1A) consists of three copper tiles whose temperature can be individually controlled through a programmable circuit. Each copper tile possesses a temperature sensor that gives feedback to the programmable circuit. The circuit activates a power supply to increase the temperature of each tile. Passive thermoelectric elements act as constant heating elements to maintain the desired temperature, while a heat sink cooled by a fan provides constant cooling. The magnitude of the temperature change determines the speed of the process in a non-linear manner. An increase of 2 °C requires only 0.1 s, and an increase of 18 °C requires 4 s. A screen connected to the programmable circuit (Fig. 1C) informs the user of the temperature measured by the temperature sensors in each of the tiles. The copper tiles are surrounded by an aluminum ring constantly heated to 50 °C (Fig. 1B and 1C) by semiconductors around the periphery. This ring forms the edges of the Fly Arena (Fig. 1C), the area in which flies are to be placed. The Fly Arena is covered by a siliconized glass cover (Fig.

1A & 1C), which provides a 3 mm high space, which ensures that flies can walk but not fly. Next to the Fly Arena are two red LEDs (Fig. 1C) that can be programmed to mark different experimental phases. For example, for the results shown in Fig. 2A, each LED is associated with a different temperature, while for Fig. 2B, each LED indicates 60 s. The *FlySteps* software can register when each of the indicative LEDs is on, and the researcher can then use this information to automatically determine the experimental phases based on temperature or time.

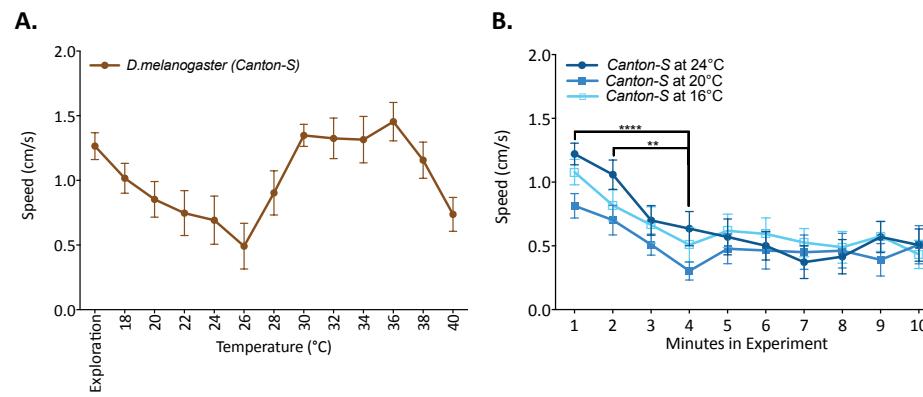
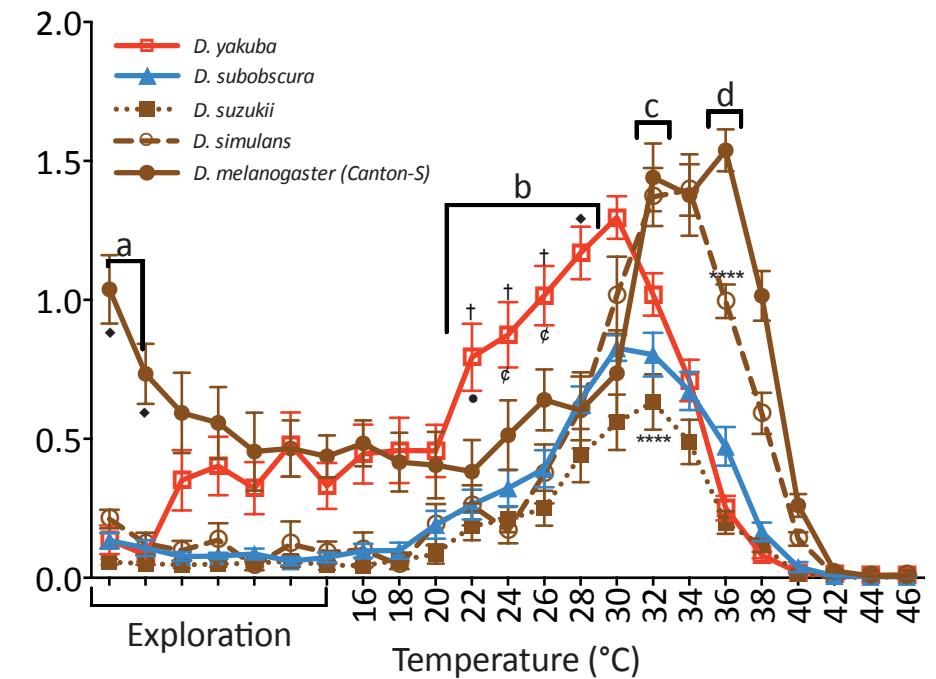


Figure 2 Flies must acclimate to the arena before starting the temperature protocol. A. Single male flies were introduced to the arena and allowed to explore at a constant 16 °C for 1 min, after which the temperature started increasing. B. Single flies exposed to 16 °C, 20 °C, or 24 °C (no group differences; two-way ANOVA $F_{(2,570)} = 4.156$, $p = 0.162$) have a higher locomotion at the beginning of the experiment than after 5 min (two-way RM ANOVA $F_{(9,570)} = 7.803$, $p < 0.0001$). Data are mean and standard error of the mean (\pm SEM) of 20 virgin female flies 5 to 7 days old tested over multiple days. Asterisk indicates significant difference among groups (**p < 0.0001; Tukey's multiple comparison test, p = 0.05)

The temperature-controlled arena can be used to compare the behavioral response of flies from different genetic backgrounds to dynamic temperature changes. For example, flies from different species can be exposed to gradually increasing temperatures (Fig. 3) to compare their differences in thermal performance. The speed of all species increases as temperature increases until reaching a point of maximum performance, after which it decayed and they perished. However, each species has a particular response curve with specific maximum response speeds and specific thermal tolerances. Previous reports have shown that *Drosophila* from different species differ among developmental timing, longevity, fecundity, body dimensions, sexual communication, and temperature tolerance (Jezovit et al., 2017; Klepsat et al., 2013; Latimer et al., 2011; Petavy et al., 2001; Trotta et al., 2006). Thus our description of species-specific locomotion in a temperature gradient adds to this body of work.

The temperature-controlled arena can also be used to explore the flies' response to conditioning experiments based on temperature. The simplest form of this approach is an operant conditioning paradigm in which flies are trained to prefer one side of the arena over the other, by warming up the side that will be avoided (Diegelmann et al., 2006; Zars and Zars, 2006; Zars et al., 2000). We exposed individual flies to 40 °C in the middle and one of the side tiles, while leaving the other side tile at a comfortable 22 °C (Fig. 4). Wild-type flies quickly stopped moving along the arena and remained in the comfortable



location. In contrast, the classic memory mutant *Dunce* kept exploring the arena and spent less time than controls in the comfortable location. The differences between performance of the wild-type flies and *Dunce* mutants became larger when all tiles were set to 22 °C and comparisons were made between the treatment groups. *Dunce* mutants also showed greater differences between their training and test phases in comparison to the wild-type flies (Fig. 4). These results suggest an effect of memory on remaining in the comfortable location.

Combinations of temperature and location are also useful to understand the function of different temperature receptors during dynamic temperature changes. We exposed individual *D. melanogaster* *Gr28b.d* and *TvpA1^{GAL4}* mutants to increasing temperatures (2 °C every 60 s) while providing a comfortable location at 22 °C (Fig. 5). The comfortable location shifted from left to right, and vice versa, per iteration. Results show that the

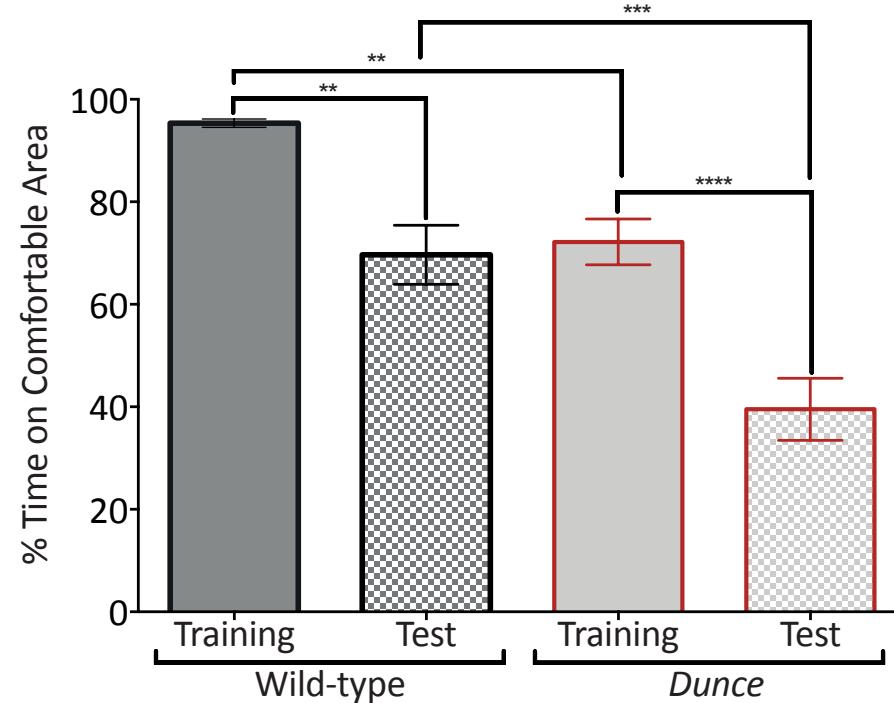


Figure 4 The temperature-controlled arena can be used for operant conditioning. *D. melanogaster* Canton-S strain (wild-type; black border) and *dnc^l* (*Dunce*; red border) mutants were trained to prefer a lateral tile at 22 °C after warming the middle and opposite lateral tiles to 40 °C for 4 min (training; no pattern). Memory of the heated areas is then tested by setting all tiles to 22 °C (test; grid pattern). Flies were conditioned to prefer tiles on the left in half of the experiments, then tiles on the right in the other half. The percentage of total time inside the tile at 22 °C during training and testing was measured to compare performances. Groups were significantly different (one-way ANOVA $F_{(3,76)} = 23.23$, $p < 0.0001$), with *Dunce* performing worse than wild-type overall. Data are mean (\pm SEM) of 20 virgin female flies 5 to 7 days old tested over several days. Asterisks indicate significance difference among groups (**p > 0.001; ***p > 0.001, ****p > 0.0001; Tukey's multiple comparison test, p = 0.05).

periphery temperature receptor *Gr28b.d* mutants behave as the control as they spend more time in the comfortable location as the temperature increases. However, brain temperature receptor *TlpA1^{GAL4}* mutants are not affected by the increasing temperatures and do not change their location in the arena. The increases and decrease in the curve of *TlpA1^{GAL4}* mutants show the effect in flies that were already sitting in the comfortable location before it became comfortable and remained there during that phase. The consistency of the peaks and valleys of the curve of *TlpA1^{GAL4}* suggest that these flies remained still for most of the experiment; hence, they were constantly counted when their location was the one considered comfortable. This conclusion was confirmed by visual inspection of the recorded videos. These results support previous physiological reports suggesting that periphery perception of fast and large changes does not depend on *Gr28b.d* (Liu et al., 2015), and reports that suggest that flies possess a main central mechanism to sense temperature based on *TlpA1* (Gallio et al., 2011; Tang et al., 2013).

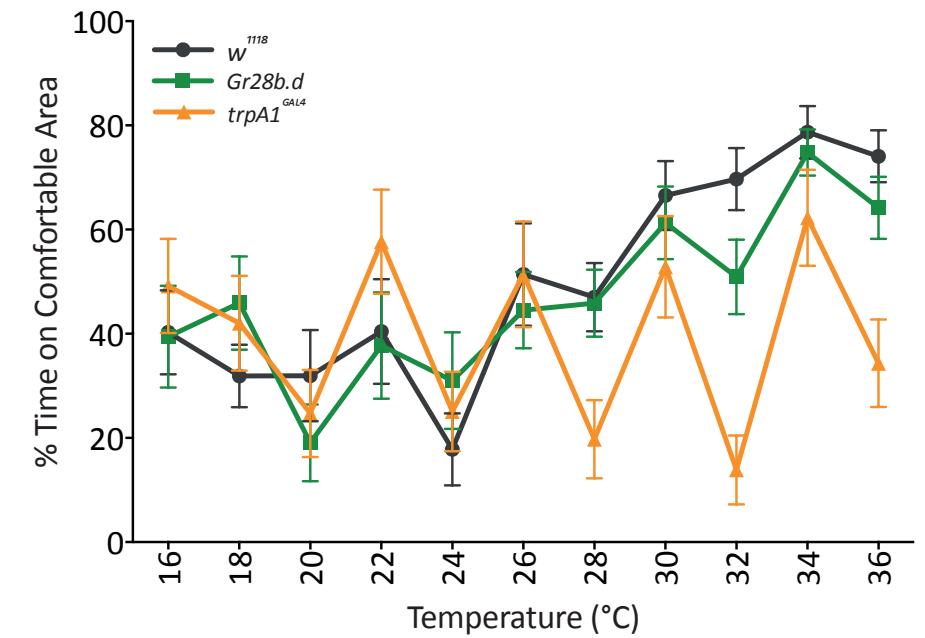


Figure 5 Response of temperature mutants to increasing temperature when a comfortable location is provided. Temperature mutants *Gr28b.d* (green; squares) respond as controls (*w¹¹¹⁸*; black; circles) by increasing the percentage of time in the comfortable area as temperature increases (two-way RM ANOVA $F_{(1,38)} = 0.5107$, $p = 0.479$). *TlpA1^{GAL4}* mutants (yellow; triangles) are different from controls (*w¹¹¹⁸*, black), as they do not increase the time in the comfortable area as temperature increases (two-way RM ANOVA $F_{(1,38)} = 1.670$, $p = 0.019$). Data are mean (\pm SEM) of 20 male flies 5 to 7 days old tested over several days. *TlpA1^{GAL4}* is significantly different from *Gr28b.d* and the control ($p < 0.05$; Tukey's multiple comparison test, $p = 0.05$).

Discussion

Here we have presented an automated temperature-controlled arena (Fig. 1) that produces precise temperature changes in time and space. This method allows exposure of individual *Drosophila* not only to pre-programmed gradual increases of temperature (Fig. 2 and 3) but also to dynamic temperature challenges in which each tile of the fly arena was heated independently to a different temperature (Fig. 4 and 5).

The temperature-controlled arena uses an innovative approach to the heating process. Instead of producing temperature in the tiles through thermoelectric Peltier heating elements used in traditional methods, the temperature-controlled arena uses current to warm up a copper mass with the copper tiles, and flies are placed at the top. The copper mass is constantly cooled down by a heat sink block connected to a fan. Peltier-like elements are used to maintain the desired temperature of the copper mass once it has

been warmed up. Because these elements are not the main temperature generators, they suffer less stress, which extends their life span and permits faster temperature changes. A programmable circuit that receives feedback from temperature sensors under each of the copper tile, which can also activate the low voltage power supply, coordinates the heating mechanism. Researchers can specify when and where temperature changes occur and determine the intensity and direction of such changes. Furthermore, coupling the method with specialized tracking software, such as *FlySteps*, permits analysis of all aspects relating to *Drosophila*'s movement, such as the overall speed at certain temperatures or time spent in a certain locations (Fig. 2, 3, 4 and 5). Nevertheless, all results must consider characteristics inherent to fly behavior that might affect their locomotion. For example, if flies are not allowed to explore the arena and settle before changing the temperature, speed measurements might be artificially high (Fig. 2). Flies can also leave odorants that affect subsequent flies; hence, the glass cover must be cleaned, and the tape covering the tiles must be changed between subjects. Given that locomotion declines as flies age (Jones and Grotewiel, 2011), it is important that flies are standardized for age to avoid variation in results. In our arena, flies have also shown centrophobism, preferring the edges over the middle area. Experimenters must control for this by changing the location of comfortable areas to prevent overestimating site preference.

The current characteristics of the arena and the requirements of the tracking process could limit some experimental procedures. For example, the close environment of the arena does not include access points through which odours could be introduced, which prevents studies in which this stimulus is important. Similarly, the *FlySteps* tracker necessitates videos with uniform backgrounds, which limits the possibility of adding food or other items to the fly's environment. The arena could be adapted to include a connection to a gas valve, and software developments exist that may allow for more objects to be present. Future projects may take advantage of these possibilities to adapt the temperature-controlled arena to specific experimental needs.

Finally, we have shown in the results that different species of *Drosophila* perform differently as temperature increases (Fig. 3) and that temperature mutants do not respond in the same way as controls (Fig. 5). This shows that this new method can be used to explore *Drosophila*'s thermal behavior and how it is affected by natural selection and functional characteristics. Finally, it illustrates that our method may help further understanding of thermal adaptation and speciation as well as the interactions of temperature receptors with other stimuli in future studies.

Disclosures

The authors declare that they have no competing financial interests.

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Scripts *TemperaturePhases*, *FlySteps* and *FlyStepAnalysis* can be found as supplementary information and in the following temporary and publicly available link:
<https://dataverse.nl/privateurl.xhtml?token=c70159ad-4d92-443d-8946-974140d2cb78>

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Thermosensory perception regulates speed of movement in response to temperature changes in *Drosophila melanogaster*

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Abstract

Temperature influences the physiology and behavior of all organisms. For ectotherms, which lack central temperature regulation, temperature adaptation requires sheltering from or moving to a heat source. As temperature constrains the rate of metabolic reactions, it can directly affect ectotherm physiology and thus behavioral performance. This direct effect is particularly relevant for insects, as their small bodies readily equilibrate with ambient temperature. In fact, models of enzyme kinetics applied to insect behavior predict performance at different temperatures suggesting that thermal physiology governs behavior. However, insects also possess thermosensory neurons critical for locating preferred temperatures, showing cognitive control. This suggests that temperature-related behavior can emerge directly from a physiological effect, indirectly as a consequence of thermosensory processing, or through a combination of both. To separate the roles of thermal physiology

and cognitive control, we developed an arena that allows fast temperature changes in time and space, and in which animals' movements are automatically quantified. We exposed wild-type *Drosophila melanogaster* and thermosensory receptor mutants to a dynamic temperature environment and tracked their movements. The locomotor speed of wild-type flies closely matched models of enzyme kinetics, but the behavior of thermosensory mutants did not. Mutations in thermosensory receptor gene *dTriP1* (Transient Receptor Potential A1) expressed in the brain resulted in a complete lack of response to temperature changes, while mutations in peripheral thermosensory receptor gene *Gr28b(D)* resulted in a diminished response. We conclude that flies react to temperature through cognitive control, informed by interactions between various thermosensory neurons, the behavioral output of which resembles models of enzyme kinetics.

Keywords

Fruit fly, Thermal performance, Enzyme kinetics, Locomotor activity, Thermosensory receptors

Introduction

Organisms are constantly exposed to environmental challenges over which they have no direct influence. One such challenge is temperature, a pervasive component that changes in time and space and directly influences biochemical processes (Abram et al., 2017), which in turn affects physiology (Kingsolver, 2009; Roberts et al., 2003; Soriano et al., 2002) and behavior (Crill et al., 1996; Ellison and Skinner, 1992; Gibert et al., 2001; Grigg et al., 2004; Klepsat et al., 2013; Latimer et al., 2015). Endothermic animals adapt to temperature through metabolic mechanisms that regulate their central temperature (Clarke and Rothery, 2008; Grigg et al., 2004). Ectothermic animals, in contrast, lack central temperature regulation and instead depend on behavioral strategies to find environments where the temperature meets their needs (Klein et al., 2014; Purves et al., 2003).

The capacity of ectotherms to tolerate temperature changes is influenced by their body size. The mass of large ectotherms reduces the rate at which their core heats in comparison to their surface area (Stevenson, 1985). This allows them to move freely through a wide temperature gradient without suffering physiological consequences. For small ectotherms, a large surface area to volume ratio means that their body temperature readily equilibrates with that of the environment (Garrity et al., 2010; Hong et al., 2008; Stevenson, 1985). As temperature directly affects the rate of biochemical reactions in enzymatic systems, the immediacy with which small ectotherms adopt the temperature around them could imply that their behavioral response closely tracks that of the physiological effect of temperature (Dillon et al., 2012). In fact, models of insect performance at different temperatures reflect the predicted response of enzymatic kinetics (Gilchrist, 1995; Huey and Kingsolver, 1989; Klepsat et al., 2013; Logan et al., 1976). However, small ectotherms such as the fruit fly *Drosophila melanogaster* possess central and peripheral thermosensory neurons relevant for their selection of preferred temperatures on fixed gradients (Barbagallo and Garrity, 2015; Frank et al., 2015; Gallio et al., 2011; Hamada et al., 2008; Liu et al., 2015; Ni et al., 2013). The fruit fly's thermosensory neurons express *Transient Receptor Potential A1* (*dTrpA1*), which influences temperature preference processes, temperature-dependent daily activity patterns and sleep regulation, as well as thermal nociception in both larvae and adults (Hamada et al., 2008; Lamaze et al., 2017; Luo et al., 2016; Neely et al., 2011; Roessingh and Stanewsky, 2017; Yoshii et al., 2009; Zhong et al., 2012). *dTrpA1* is expressed in the anterior cells (AC) of the adult fly central nervous system (Hamada et al., 2008), where it regulates the response to slow and shallow temperature changes (Ni et al., 2013). As these central neurons receive inputs from peripheral thermosensory neurons and project to multiple brain regions, they have also been suggested to be a site of regulation of temperature preference (Barbagallo and Garrity, 2015; Gallio et al., 2011; Tang et al., 2013). Flies also have other peripheral thermosensory neurons located in the second and third antennal segments. The second antennal segment produces a response to warming that projects to the AC (Tang et al., 2013). The third antennal segment harbors cold sensing neurons in the sacculus as well as hot and cold sensing neurons in the base of the arista (Gallio et al., 2011). Cold sensing neurons express the *Trp*-related channels *brivido1*, *brivido2*, and *brivido3*, while hot sensing neurons express the gustatory receptor *Gr28b(D)* (Fowler and Montell, 2013; Gallio et al., 2011; Ni et al., 2013). *Gr28b(D)* has been linked

to the response to fast and small temperature changes that do not require *dTrpA1* (Ni et al., 2013). The peripheral system also harbors secondary thermal projection neurons that respond to fast and large increases in temperature independent of *Gr28b(D)* (Frank et al., 2015; Liu et al., 2015). *Drosophila* thus possesses multiple systems to respond to temperatures, and both physiology and cognitive control may play a role in the resulting behavioral response (Abram et al., 2017).

Here, we set out to differentiate the contribution of the physiological effect of temperature from that of the sensory processing of thermal information in influencing the behavioral response of *Drosophila* to temperature changes. To do this, we developed a temperature-controlled arena that allows continuous tracking of the flies' movements in a spatially and temporally controlled thermal environment. Unlike approaches used in previous studies, this method does not require long exposure to fixed temperatures (Klepsat et al., 2013; Latimer et al., 2014) or human intervention during the experiment (Crill et al., 1996; Gibert et al., 2001). We quantified the locomotion of flies based on their speed, as previous studies have done in the context of testing the effect of age, geography, development and natural genetic variation on the behavioral performance of the flies at different temperatures (Crill et al., 1996; Gibert et al., 2001; Klepsat et al., 2013; Latimer et al., 2014). To clearly differentiate the contribution of the physiological effect and thermosensory processing, we compared the speed of wild type flies with that of *Gr28b(D)* and *dTrpA1* mutants over a large range of temperatures. The difference between the response of wild-type and mutant flies reveals how much of the speed at different temperatures depends on a direct physiological effect, which would affect the flies independent of the mutations, and how much it depends on the thermosensors. Our results demonstrate that the speed of the flies is comparable to enzyme kinetics-based models, but that flies do not increase speed in the absence of thermosensory processing, especially in *dTrpA1* mutants. This suggests that fruit flies, though directly affected physiologically by the increase of temperature, require thermosensory processing to produce a behavioral response to temperature. In addition, we show that both peripheral and central thermosensors are necessary for a normal response to changing external temperatures.

Materials and Methods

Drosophila rearing and stocks

Drosophila melanogaster flies were raised on a 12 h:12 h light-dark cycle at 25°C on fly food medium containing agar (10 g l⁻¹), glucose (167 mM l⁻¹), sucrose (44 mM l⁻¹), yeast (35 g l⁻¹), cornmeal (15 g/L), wheat germ (10 g/L), soya (10 g/L), molasses (30 g/L), propionic acid and Tegosept (for food medium preparation, see Gorter et al., 2016). All flies were collected using CO₂ anesthesia on the day of eclosion and aged in same-sex food vials of 20 flies each. Tests were done using 5-7 day old males with the exception of the wild-type test for which we also used virgin females.

Canton-S was used as the wild-type strain. Thermosensory mutants included *dTrpA1*^{GAL4}

(Kim et al., 2010), $w^{\circ};dTrpA1^{903w^*}/TM6b$ ($dTrpA1^{903w^*}$) (Zhong et al., 2012), and w^{1118} ; $Mi\{ET1\}Gr28b^{MB03888}$ [$Gr28b(D)$] (Ni et al., 2013). *UAS-dTrpA1 RNAi* and *dTrpA1^{SH}-GAL4* (Hamada et al., 2008) were used to create a *dTrpA1* knockdown in AC neurons. The *Gr28b(D)* line was obtained from the Bloomington Stock Center. The *dTrpA1^{GAL4}*, $w^{\circ};dTrpA1^{903w^*}/TM6b$, *UAS-dTrpA1 RNAi* and *dTrpA1^{SH}-GAL4* lines were a gift from Ralf Stanewsky (University of Münster, Institute of Neuro- and Behavioral Biology). Strain w^{1118} was used as control strain for *dTrpA1^{GAL4}* and *Gr28b(D)* mutants. The *UAS-dTrpA1 RNAi* and *dTrpA1^{SH}-GAL4* lines were crossed to y^-, w^- to generate controls for the knockdown. Third antennal segment removal was done using iridectomy scissors (Fine Science Tools No. 15000-03) on 0-1 day old flies under CO₂ anesthesia. These flies recovered for 4-5 days and were also tested on days 5-7.

Temperature-controlled arena

Flies were tested in an automated temperature-controlled arena (Fig. 1; for a detailed description see Appendix *Temperature-controlled arena*), the floor of which consisted of three adjacent copper tiles of 2.5 x 2.5 cm. The copper tiles are mounted on a 32 cm (L) x 16 cm (W) x 13 cm (H) box containing the heating and cooling elements of the thermal mechanism. Each tile presented temperature variation of ± 0.2 - 0.5°C around any given temperature between 15 and 50°C as measured by individual thermosensors connected to each tile. The heating rate varied according to the range of the temperature change: an increase of 2°C took ~ 100 ms and an increase of 18°C (from 22°C to 40°C) required ~ 4 s. Cooling took ~ 1 s for 2°C and ~ 6 s for 18°C (from 40°C to 22°C). Each tile could thus be independently and rapidly heated or cooled. The tiles were covered with white conducting tape (Tesa® 4104 white tape, 25 x 66 mm) and constantly illuminated with white light (Cold White 300x5050 SMD LED Flexible Light Strip providing 45 lx) to create a uniform and contrasting background surface that was replaced between experiments. There were no thermal gradients between the tiles that could influence the flies, as confirmed by thermal imaging (FLIR® T400sc, FLIR Systems Inc., Wilsonville, OR, USA; Fig. S1).

To confine flies to the arena, a 9.6x4.5 cm aluminium frame of 3 mm height and 1 cm width was placed around the copper tiles and covered by 3 mm-thick annealed glass plate of 9.3x4.2 cm, coated with siliconizing agent (Sigmacote®, Sigma-Aldrich, Darmstadt, Germany). The aluminium frame was constantly heated to 50°C using insulated resistors beneath the bottom surface of the frame.

Temperature protocols, Data Processing and Statistical Analysis

Individual flies were transferred to the temperature-controlled arena using a mouth aspirator. For the experiments involving temperature changes, flies were allowed to walk freely for 7 min at a constant temperature of 22°C to eliminate the natural exploratory phase in which flies walk faster and allow them to settle (Fig. S2). For experiments with

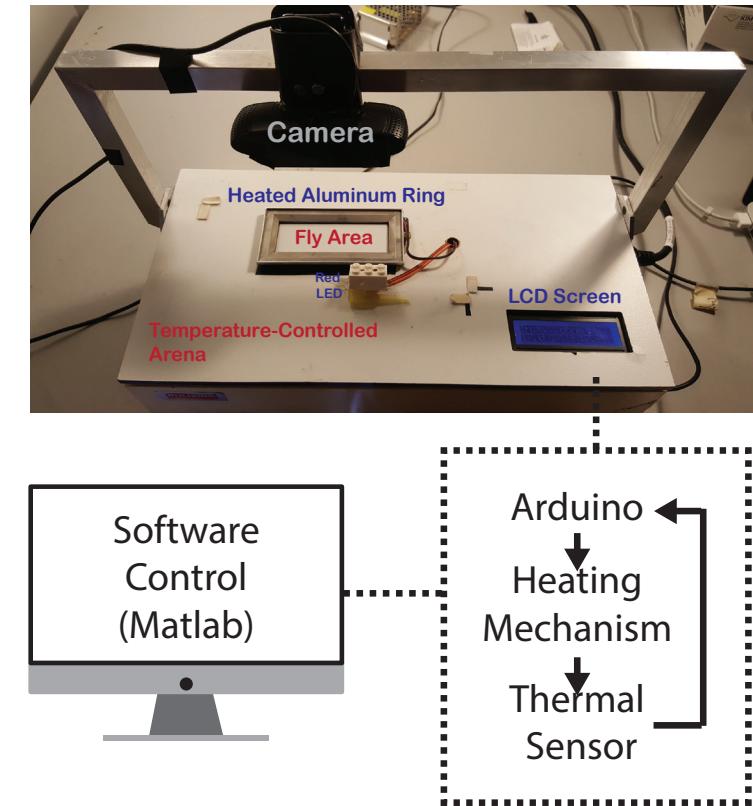


Figure 1 Temperature-controlled arena. Components of the temperature- controlled arena and diagram of the main control processes. Temperature changes are coordinated by a custom-written Matlab script through a programmable circuit (Arduino). The Arduino also receives feedback from thermal sensors under each tile to regulate and maintain the programmed temperature by controlling the heating mechanism.

a constant temperature, flies were introduced directly to the test temperature. Flies were video recorded with a high definition webcam (Logitech® c920, Logitech Europe S.A., Lausanne, Switzerland) and then tracked using software developed for this project in Python (Python Software Foundation v2.7.6, <http://www.python.org>) based on the Lucas-Kanade differential method for optical flow (see Appendix *Tracking Software*) and available on request. Fly centroid data were imported into Matlab (Matlab and Statistics Toolbox release 2014a, The Mathworks Inc., Natick, MA, US) and processed using custom-written scripts. The time on each tile, speed and path length were binned per minute. We considered a fly as being on a tile when it was across the border between tiles for at least 1 s and for a distance greater than the length of one fly (0.25 cm). Matlab output data were imported into GraphPad Prism (v6 for Mac OS Sierra, GraphPad Software Inc., www.graphpad.com) for statistical analysis of the effect of sex or genotype over the speed of flies at different temperatures using a two-way repeated-measurements (RM) analysis of variance (ANOVA) with Tukey's or Sidak's post hoc test for multiple comparisons.

A custom-written script in RStudio (RStudio Team 2016, v1.0.143) was used to model the speed of flies when exposed to increasing temperatures. As customary for modeling of performance at different temperatures (Angilletta, 2006; Gilchrist, 1995, 1996; Huey, 1979; Huey and Kingsolver, 1989; Klepsat et al., 2013; Latimer et al., 2014, 2015), we fitted multiple functions and polynomials to our data: Gaussian, modified Gaussian, quadratic, and eqns 6 and 10 from Logan et al. (1976). These last two equations are based on the rate of enzyme-catalyzed biochemical reactions and were designed to describe behavioral performance in arthropods at different temperatures. Eqn 6 of Logan et al. (1976) (Fig. 2) is represented by:

$$S(T) = \psi \{ \exp(\rho T) - \exp(\rho T_M m - \tau) \}$$

where the locomotion (S) is described as a function of temperature (T) that depends on ψ , a directly measurable process at the base temperature (such as speed in our study) dependent on temperature; r is interpreted as the composite of the Q_{10} value of enzyme-catalyzed biochemical reactions; and t which is defined as:

$$\tau = (T_M - T)/\Delta T$$

where T_M is the maximum lethal temperature; T denotes an experimental temperature; and ΔT is the width of the high-temperature boundary section.

Eqn 10 of Logan et al. (1976) (Fig. 2) derives from their eqn 6 and is sigmoidal in the first phase of the curve (ascent), which is considered a more accurate description of the phenomena than the straight line represented by eqn 6. Eqn 10 of Logan et al. (1976) is given as:

$$S(T) = \alpha \{ [1 + k \exp(-\rho T)]^{-1} - \exp(-\tau) \}$$

where T represents an experimental temperature; a , k and r are free parameters; and t is as described above.

Data were fitted to these models using the non-linear mixed effects function of RStudio (v1.0.143) and compared using the Akaike information criterion (AIC; as recommended by Angilletta, 2006). We confirmed these results by comparing our models under the Bayesian information criterion (BIC), which agreed with the AIC conclusion. We also calculated the residual sum of squares (r^2) and found an acceptable estimate for eqn 10 of Logan et al. (1976) even though model preference differed. This difference can be explained by the lack of capacity of the residual sum of squares to deal with model complexity, a problem fixed through the AIC (Symonds and Moussalli, 2011).

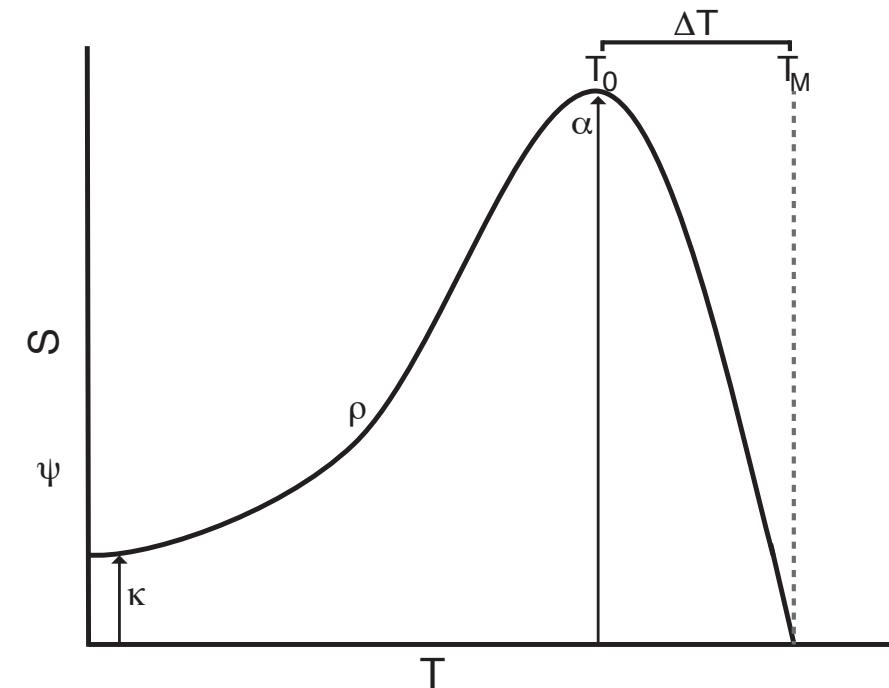


Figure 2 Representation of the parameters of Logan et al.'s (1976) equations. Temperature (T) affects a measurable process (ψ), such as locomotion (S), based on the rate of temperature change (ρ) and the decline in performance that occurs at high temperatures, represented by a high temperature boundary (ΔT) delimited by the temperature at which performance is maximum (T_0) and the maximum lethal temperature (T_M). Eqn 10 of Logan et al. (1976) determines a more sigmoid shape (α and κ) during the ascending part of the curve than their eqn 6.

Results

***Drosophila melanogaster* increases speed at increasing temperature following a model based on enzyme-catalyzed temperature performance**

In an automated temperature-controlled arena (Fig. 1), we exposed individual wild-type adult *D. melanogaster* to gradually increasing temperatures from 16°C to 46°C (2°C increase every minute) to experimentally determine their performance at different temperatures (Fig. 3A). Fly speed followed a skewed curve with a long tail in the cold part of the gradient, a gradual increase until a maximum temperature of 34°C, and then a rapid decay (Fig. 3B). This temperature-performance curve was observed in males and females, with no significant difference between the sexes (Fig. 3B). We therefore chose to only test males in further experiments. Model fitting showed that eqn 10 from Logan et al. (1976) best described the flies' performance when compared with other models commonly used to describe thermal response of ectotherms (Fig. 3C; Angilletta, 2006). Logan et al.'s

model is based on enzyme kinetics to predict temperature-related biological processes (AIC; Table S1). As the fly's speed increases with temperature with the same dynamic as enzymatic reactions, these data suggest that the influence of temperature on the fly's physiology may be directly regulating its speed.

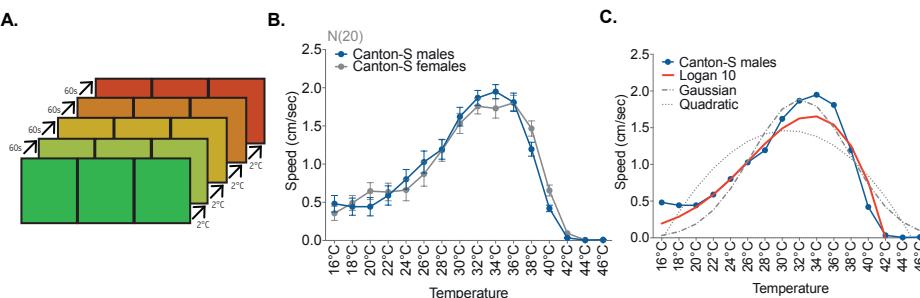


Figure 3 Models based on enzyme kinetics predict locomotor speed of *Drosophila melanogaster* at different temperatures. **A.** Increasing temperature performance procedure. The temperature of the tiles forming the floor of the temperature arena increases by 2°C every 60 s between 16 and 46°C. **B.** Speed of male and female Canton-S flies tested individually. The speed of male and female flies does not differ at any temperature (two-way RM ANOVA: $F_{DFn, DFd} = 0.0053_{1,38}$, $P=0.9537$). Each point represents the mean (\pm s.e.m.) of 20 flies tested over several days. **C.** Visualization of three models of temperature performance eqn 10 of Logan et al. (1976), Gaussian, quadratic] of male Canton-S flies. The model with the best fit is eqn 10 of Logan et al. (1976) (Table S2).

Thermosensory mutants do not follow the predictions of enzyme-catalyzed temperature-performance curves

We next determined the temperature-performance curves of thermosensory mutants in order to quantify the necessity of the thermosensory system for temperature response. Loss-of-function mutation in the gene *Gr28b(D)* gene, encoding a peripheral thermosensor, did not eliminate the locomotor response to temperature (Fig. 4B). *Gr28b(D)* mutants moved faster as temperature increased, but at a lesser rate than controls (Fig. 4B). The response of *Gr28b(D)* mutants is best described by eqn 6 from Logan et al. (1976) (Fig. S3A and Table S2), which is also considered to be a good representation of the performance at different temperatures based on enzyme kinetics. This suggests that *Gr28b(D)* mutants behave similar to wild-type flies but present a damped response to temperatures higher than 26°C.

The difference in speed between the *Gr28b(D)* mutant flies and wild-type flies at increasing temperatures may be because the mutant flies are not capable of walking faster as a result of a pleiotropic effect of *Gr28b(D)* on the locomotor system. We tested this possibility by surgically ablating the third antennal segment, which removed peripheral temperature sensors, including *Gr28b(D)*. We tested wild-type flies with partial (one antenna removed) or total (both antennas removed) third antennal segment ablation (Fig. 4C). Flies lacking the third antennal segment moved slower at warm temperatures than the controls, as *Gr28b(D)* mutants do. Moreover, flies in which the third antennal segment was removed from only one antenna move slower than controls but faster than flies lacking both third antennal segments, suggesting a complementary response between antennas. Together with our *Gr28b(D)* mutant results, these data demonstrate that *Gr28b(D)* must be functional

for flies to reach maximum speed when exposed to increasing temperatures outside of their comfortable temperature range. As ablation of the arista reproduses the *Gr28b(D)* null mutation, the mutant phenotype of *Gr28b(D)* is probably directly causally connected to the peripheral thermosensory system and not due to a pleiotropic function of this gene.

Flies with a loss-of-function allele of the central thermal receptor *dTrpA1* (*dTrpA1^{GAL4}*) did not change their speed as temperature increased (Fig. 4D). Model fitting showed that none of the models used to describe thermal reaction norms accurately described the response of these flies, leading us to conclude that thermal reaction is missing in these mutants (Fig. S3B and Table S2). To confirm the necessity of *dTrpA1* for temperature performance, we tested a second *dTrpA1* allele, *dTrpA1^{903w*}*, in a homozygous state as well as in transheterozygous combination with the *dTrpA1^{GAL4}* allele (Fig. 4E). Homozygous *dTrpA1^{903w*}* flies demonstrated a modest increase in speed at higher temperatures, suggesting that this allele is a hypomorph. Transheterozygous *dTrpA1^{903w*}/dTrpA1^{GAL4}* flies failed to increase speed at all temperatures, demonstrating lack of complementation between the two alleles, confirming that they are alleles of the same gene. These results confirm our initial observation that *dTrpA1* is necessary for the locomotor response to temperature increase and suggest that *dTrpA1^{903w*}* is a strong hypomorph of *dTrpA1*, while *dTrpA1^{GAL4}* is probably a null allele of *dTrpA1*.

As *dTrpA1* might have pleiotropic effect on locomotion, we tested its necessity for temperature performance specifically in AC neurons, the central sensor neurons for temperature preference (Hamada et al., 2008) and integrators of thermal information from the periphery (Tang et al., 2013). To do this, we created a *dTrpA1* knockdown in AC neurons by driving the expression of *UAS-dTrpA1 RNAi* using the *dTrpA1^{SH-GAL4}* driver, which is expressed in AC neurons (Hamada et al., 2008). We observed a dramatically reduced response to increasing temperatures when compared with controls (Fig 4F). This result further confirms the necessity of *dTrpA1* for locomotor performance and further indicates that this function is mediated by AC neurons.

Taken together, these results lead us to conclude that intact central thermal sensing is necessary for flies to increase speed according to temperature changes, and demonstrate that the direct effect of temperature on the fly's biochemical reactions is not sufficient to explain changes in speed in response to temperature changes.

GR28b(D) and dTrpA1 are necessary for a normal response to changing temperatures as well as constant temperatures

Gr28b(D) has been proposed to detect the process of temperature change or to be dedicated to perceiving temperature contrast (Gallio et al., 2011; Ni et al., 2013), both of which are relevant when reacting to an increasing temperature gradient. Similarly, *dTrpA1* has been reported to detect the rate of temperature change in *Drosophila* larvae (Luo et al., 2016), which could explain its necessity in our experiments for the response to increasing temperature (Fig. 4). Thus, it is possible that when flies are exposed to a constant temperature, neither of these receptors is fundamental for the response and flies can just react based on the direct physiological effect of temperature. We tested this possibility by

exposing flies to a constant temperature during 10 min within our temperature-controlled arena and quantifying their speed. Control flies increased their speed of movement when exposed to higher temperatures (Fig. 5B) in a comparable fashion to what was observed in the increasing thermal gradient (Fig. 3B). As with previous results, *Gr28b(D)* and *dTrpA1^{GAL4}* mutants did not follow a normal locomotor response to temperature: *Gr28b(D)* mutants increased locomotion at higher temperatures but were significantly slower at 32 and 36°C when compared with wild-type flies (Fig. 5B); *dTrpA1^{GAL4}* mutants maintained the same speed independently of the temperature they were exposed to (Fig. 5C). These results suggest that *Gr28b(D)* and *dTrpA1* are relevant for a normal response under conditions of constant as well as changing temperature.

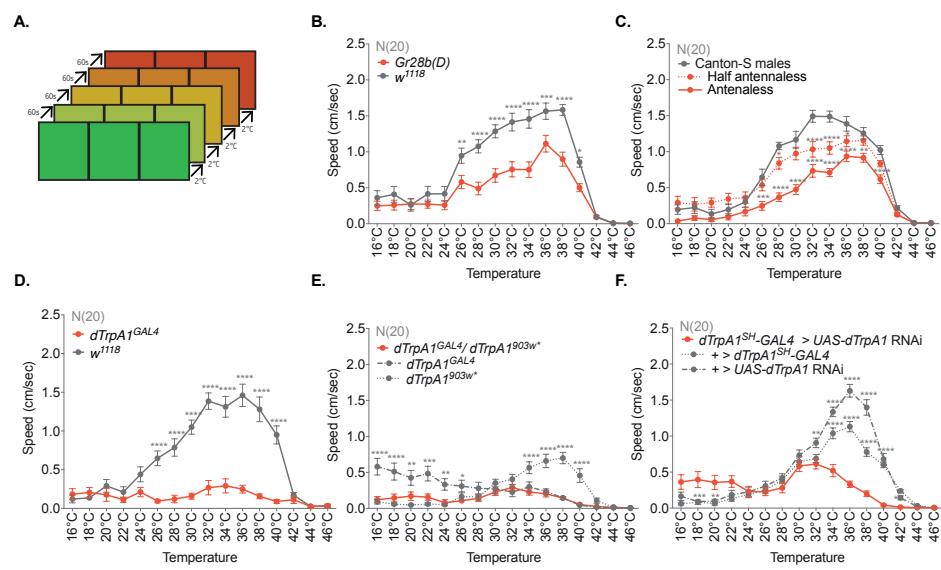


Figure 4 Locomotor speed of thermosensory mutant flies and flies without antennae at increasing temperature. **A.** Increasing temperature performance procedure. The temperature of the tiles forming the floor of the temperature arena increases by 2°C every 60 s between 16 and 46°C. **B.** Speed of control flies (w^{1118}) and *Gr28b(D)* mutant flies (two-way RM ANOVA: $F_{DFn, Dfd} = 19.53_{3,76}$, $P < 0.0001$). **C.** Speed of control flies (Canton-S males) and flies with one or both third antennal segments removed (two-way RM ANOVA: $F_{DFn, Dfd} = 27.38_{2,57}$, $P < 0.0001$). **D.** Speed of control flies (w^{1118}) and *dTrpA1* flies (two-way RM ANOVA: $F_{DFn, Dfd} = 82.01_{1,38}$, $P < 0.0001$). **E.** Speed of *dTrpA1^{GAL4}* flies, *dTrpA1^{903w*}* flies and the combination of both in trans (two-way RM ANOVA: $F_{DFn, Dfd} = 5.282_{2,57}$, $P < 0.0001$). **F.** Speed of flies with *dTrpA1* knockdown (*dTrpA1^{SH-GAL4>UAS-dTrpA1 RNAi}*) in anterior cell (AC) neurons and two controls (+>*dTrpA1^{-GAL4}*, +>UAS-*dTrpA1* RNAi; two-way RM ANOVA: $F_{DFn, Dfd} = 16.07_{2,57}$, $P < 0.0001$). Each point represents the mean (\pm s.e.m.) of 20 flies tested over several days. Asterisks indicate a significant difference between groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; Tukey's multiple comparison test, $\alpha = 0.05$).

***dTrpA1* but not *Gr28b(D)* is necessary or the response to large temperature changes**

In the previous sections, we have shown that *Gr28b(D)* and *dTrpA1* are necessary for the locomotor response to both constant and small increases in temperature. This suggests that these receptors are sufficient to regulate the locomotor response to temperature

changes. However, it has been suggested that flies possess different sensors adapted to different intensities of temperature change (Liu et al., 2015). Indeed, *Gr28b(D)* mutant neurons respond to small changes of temperature (~1°C per second) while larger and faster changes activate an excitatory pathway dependent on cold-sensing cells and not *Gr28b(D)* (Liu et al., 2015). To test the necessity of *Gr28b(D)* and *dTrpA1* in sensing larger temperature changes, we exposed flies to a temperature gradient between 16 and 36°C, increasing by 2°C every minute, while also providing a location heated to 22°C (Fig. 6A).

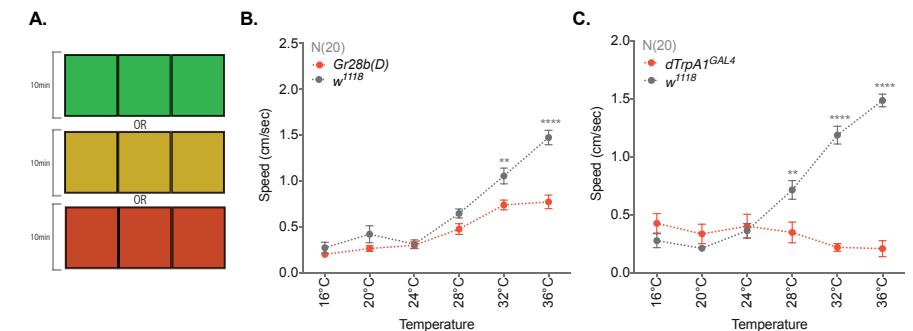


Figure 5 Locomotor speed of *Gr28b(D)* and *dTrpA1^{GAL4}* mutants at constant temperature. **A.** Constant temperature procedure. All floor tiles were kept at the same temperature of 16, 20, 24, 28, 32 or 36°C for 10 min. Each individual fly was exposed to only one of these temperatures per test. **B.** Speed of control (w^{1118}) and *Gr28b(D)* flies (two-way ANOVA: $F_{DFn, Dfd} = 45.35_{1,228}$, $P < 0.0001$). **C.** Speed of control (w^{1118}) and *dTrpA1^{GAL4}* flies (two-way ANOVA: $F_{DFn, Dfd} = 85.51_{1,228}$, $P < 0.0001$). Each point represents the mean (\pm s.e.m.) of 20 flies tested over several days. The connection between the data points is for illustration purposes only, as all the groups were independent. Asterisks indicate a significant difference among groups (** $P < 0.01$, *** $P < 0.0001$; Sidak's multiple comparison test, $\alpha = 0.05$).

This location was switched between left and right for each successive iteration. As flies always moved to this 22°C location irrespective of the arena temperature, they were exposed to a sudden temperature change ranging from 2 to 14°C (Fig. 6A). Control flies moved to the 22°C tile at each iteration, increasing their speed as temperature increased (Fig. 6B). *Gr28b(D)* mutants behaved in a similar manner to controls (Fig. 6B). *dTrpA1^{GAL4}*

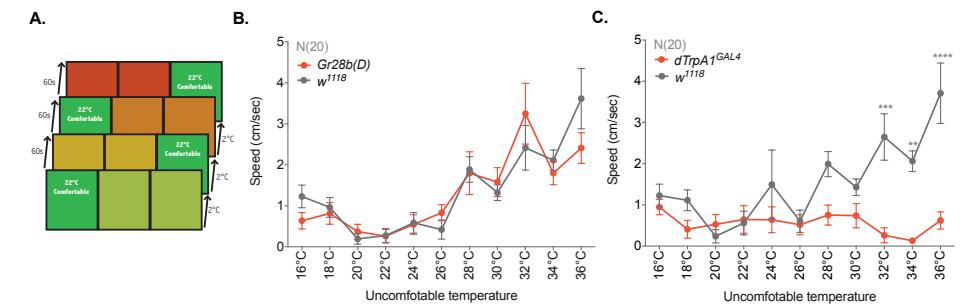


Figure 6 Performance of *Gr28b(D)* and *dTrpA1^{GAL4}* at increasing temperatures with changing location of the comfortable tile. **A.** Experimental protocol. Two tiles increase temperature by 2°C every 60 s, while one tile is kept at 22°C. The tile at 22°C shifts from left to right at every iteration (bright green). This forces flies to always cross the middle tile, the temperature of which gradually increases from 16 to 46°C. **B.** Speed on the middle tile of control (w^{1118}) and *Gr28b(D)* flies (two-way ANOVA: $F_{DFn, Dfd} = 0.1508_{1,299}$, $P = 0.6981$). **C.** Speed on the middle tile of control (w^{1118}) and *dTrpA1^{GAL4}* flies (two-way ANOVA: $F_{DFn, Dfd} = 32.74_{1,245}$, $P < 0.0001$). Each point represents the mean (\pm s.e.m.) of 20 flies tested over several days. Asterisks indicate a significant difference among groups (** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; Sidak's multiple comparison test, $\alpha = 0.05$).

mutants, however, did not increase their speed and did not seek the 22°C location (Fig. 6C). Our results suggest that flies respond to fast and large warming changes through a mechanism that requires *dTrpA1* but is independent of *Gr28b(D)*, as previously suggested (Liu et al., 2015).

Discussion

We quantified the contribution of the physiological effect and the thermosensory perception of temperature on the locomotor speed of *D. melanogaster*. We used a new temperature-controlled arena that allows dynamic temperature changes in time and space. As flies are small ectothermic animals, we expected a significant effect of warmer temperatures on metabolic reactions, resulting in faster movement speed even in the absence of functional thermosensory neurons. However, we found that the *dTrpA1* thermosensors are necessary for *D. melanogaster* to exhibit the locomotor reaction to temperature change (Figs. 4&6). We interpret this result as showing that thermosensation is the main component of the locomotor response to temperature. This does not imply that the direct physiological effects of temperature on the fly behavior can be neglected. What our results suggest is that the effect of temperature on biochemical reactions and on behavior are uncoupled. The fact that the best model representing the locomotor performance of *D. melanogaster* at different temperatures is based on enzyme kinetics probably means that the behavioral response to temperature mediated by the nervous system has been shaped by the rate of biochemical reactions (Fig. 3C; Table S1). One can imagine a scenario in which early unicellular organisms responded to temperature in an enzyme-based system. As multicellular organisms evolved, they required spatially separated enzyme systems to work together. These organisms, like modern ectotherms, needed to select environments in which they could be efficient and flexible, avoiding pushing their enzymatic systems over their maximum thermal tolerance by selecting environments below this range (Martin and Huey, 2008). Success in this process requires a central thermal processor that integrates the information from a peripheral thermosensory system that detects distinct thermal qualities of the current environment. In the case of *D. melanogaster*'s change in locomotion at different temperatures, the peripheral system seems to use different mechanisms according to the intensity of the thermal change: a *Gr28b(D)*-dependent mechanism that detects gradual and small temperature changes (Fig. 4A); and a *Gr28b(D)*-independent mechanism (Fig. 6B; also shown in larvae in Liu et al., 2015) that detects abrupt temperature variations. This is comparable to the findings on daily entrainment to temperature cycles, in which different thermal receptors in chordotonal organs detect a wide range (Sehadova et al., 2009; Wolfgang et al., 2013) or a small range of temperature changes (Chen et al., 2015).

Our results also suggest that *dTrpA1* is required for normal locomotor changes in response to any type of temperature change. This makes AC neurons, in which *dTrpA1* is necessary for the locomotor response to temperature changes (Fig. 4F), an enticing candidate for a role as central thermal processor. However, the studies on daily entrainment have not systematically concluded that either *dTrpA1* or AC neurons are fundamental for temperature entrainment (Das et al., 2015; Lee and Montell, 2013; Roessingh et al., 2015).

So far, *dTrpA1* expression has been observed only in subsets of clock neurons (Das et al., 2016; Lee and Montell, 2013; Yoshii et al., 2015), and AC neurons have been related to temperature preference before dawn but not to temperature entrainment (Tang et al., 2017). This suggests that the central system of *Drosophila* thermal behavior has a high level of complexity beyond AC neurons that allows an efficient and detailed detection of thermal stimuli and their integration with other internal states to coordinate the most efficient behavioral response.

In conclusion, this study adds to the body of work demonstrating that flies possess rich thermosensory mechanisms to respond to temperature and proves that they are not passive respondents, as could have been predicted by their lack of internal temperature regulation. Instead, *Drosophila* possess multiple thermosensors, located in both their central and peripheral nervous system, and the signals from these are integrated to respond to different types of thermal challenges. One output of this system we have measured here is an increase in locomotor speed with higher temperatures. It is likely that greater speed functions to escape damaging temperatures, thus allowing *Drosophila* to actively regulate temperature via positional avoidance or preference. Future studies could take advantage of this methodology, in combination with genetic and environmental manipulation, to illuminate the mechanisms that regulate the dynamic response to temperature observed in ectotherms.

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Competing Interests

The authors declare no competing of financial interests.

Author contributions

Conceptualization: A.S.-P., J.-C.B.; Methodology: A.S.-P., R.R., H.v.R.; Software: H.v.R.; Formal analysis: A.S.-P.; Investigation: A.S.-P., J.-C.B.; Resources: H.v.R., J.-C.B.; Data curation: A.S.-P.; Writing - original draft: A.S.-P., J.-C.B.; Writing - review & editing: A.S.-P., O.C.M.S., H.v.R., J.-C.B.; Visualization: A.S.-P., J.-C.B.; Supervision: O.C.M.S., H.v.R., J.-C.B.; Project administration: A.S.-P., O.C.M.S., H.v.R., J.-C.B.; Funding acquisition: A.S.-P., H.v.R., J.-C.B.

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Data availability

The datasets from the tracking process generated and analyzed for the current study are available from the DataverNL repository: <http://hdl.handle.net/10411/6CYOM9>

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.174151.supplemental>

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Appendices

Temperature-controlled arena

Current systems use Peltier elements to control the temperature of the relevant parts of research equipment because temperature can be controlled easily in a range suitable for most types of tests. However, because of the construction of Peltier elements based on a semiconductor material sandwiched between two conductors, they are prone to thermal stress that quickly destroys them when temperature is changed at a fast rate. Our temperature-controlled arena solves this problem by using a copper block and copper tiles system in which the temperature changes occur, leaving the Peltier elements to act only as constant heaters and diminishing their thermal stress.

The heart of the system is a copper block that acts as a well-controlled thermal mass, on which three tiles are glued to a heater element directly in contact with the tile and a thermal semi-insulator that sits between the heater element (printer circuit board of ~ 0.3 mm thickness with three fine meandering tracks that have a resistance of 5 Ohms each, one for each tile) and the copper block. The copper block is kept at a constant temperature, lower than the minimum desired temperature of the tiles, using Peltier elements clamped to a heat sink by thermally isolated bolts and spring washers. The heat sink is cooled with ambient air that is forced through it by a fan. The fan is isolated from the heat sink to avoid disturbance of the test specimen due to vibration. Each copper tile,

the copper block and the heat sink are equipped with thermal sensors that are used to control and monitor the complete system.

The temperature-controlled arena facilitates maintaining a constant temperature by heating each floor to the desired temperature through a controlled low-voltage power supply with feedback from their temperature sensor which is coordinated by a programmable circuit (Arduino UNO Atmel Atmega328). For increases in temperature, our arena permits heating individual tiles or multiple tiles together. To do this, a high voltage supply with a bank of boost capacitors of adequate capacity yields a high-power boost for a short duration of the order of 100 ms. It is possible to fix the voltage of the power boost and then use the time length of the boost to determine the value of the temperature increase. Other schemes, like a variable boost voltage, can lead to similar results. When the desired temperature is reached, the lower voltage supply takes over control and maintains the new constant temperature with a variability of less than 0.5°C. Cooling down is controlled by the heat flux through the semi-insulating substrate into the copper block. With the heat insulation properties of the semi-insulating substrate, the heat loss in the system can be balanced to the cool-down time. When the heat transfer to the copper block is higher, the heat loss and energy consumption of the system are higher, but the benefit is a faster cool down. To lower the energy consumption of a test cycle, a sophisticated control strategy for the copper block temperature is employed. In this case, the temperature of the copper block is kept at the temperature of the coolest tile, and only at a well-chosen time before one (or more) of the floors needs to go to a lower temperature is the block cooled down to ensure a fast cooling of the other tiles. The pre-cool time has to be experimentally determined and is dependent on the initial conditions, predominantly the temperature levels of all the floors.

Each experimental procedure is controlled using a Matlab (vR2014a MathWorks Inc,) script that coordinates the instructions of the programmable circuit to manage the heating and cooling of each cooper tile individually, and also to control the turning on and off of the red LED lights. While an experiment is running, a thermal sensor under each of the tiles continually measures tile's temperature. The programmable circuit processes this information and the temperature is constantly adjusted to maintain the desired level. We measured a constant variation of ±0.2–0.5°C around the goal temperature. The programmable circuit is also responsible for the time it takes to change the temperature in each individual tile. In our experimental design, this time range between 100 ms for an increase of 2°C and 4 s for an increase of 18°C (from 22 to 40°C).

Tracking software

We developed tracking software, as part of the temperature-performance paradigm, in which the user need only specify the location of the video files, the number of subjects to be tracked and the boundaries of the area of interest. Within the algorithm, the area of interest is represented as a series of (x,y) coordinates, which means that any arena shape can be selected, allowing the tracker to be applicable to multiple experimental set-ups. The individual subjects are detected through key points using the Minimum Eigenvalue Method (Shi and Tomasi, 1994) or the advanced features from the Accelerated

Segmentation Test (Rosten et al., 2010). These methods detect stable key points that are then tracked using optical flow based on the Kanade-Lucas-Tomasi (Lucas and Kanade, 1981; Shi and Tomasi, 1994; Tomasi and Kanade, 1991) algorithm.

The stable key points initially detected are clustered to represent the actual subjects of interest based on another algorithm called k-Means algorithm. k-Means uses Euclidian distances to accumulate clusters according to the initial number of subjects input by the user. We gave our tracker a choice between traditional k-Means, in which the clusters are determined in every time frame based on Euclidian distances, and k-Means++, in which the first detection creates a center in each cluster (seed), which is used in the second detection as the start point of a cluster. k-Means++ is in appearance more precise, but the seeds can drift away from the subjects, so we decided to give the option of traditional k-Means if the density of seeds is x times lower than that for k-Means++ in the first time frame. If k-Means is selected, the algorithm will use the Minimum Mean Square Error to calculate the values of all combinations for each cluster in the following time frame and select

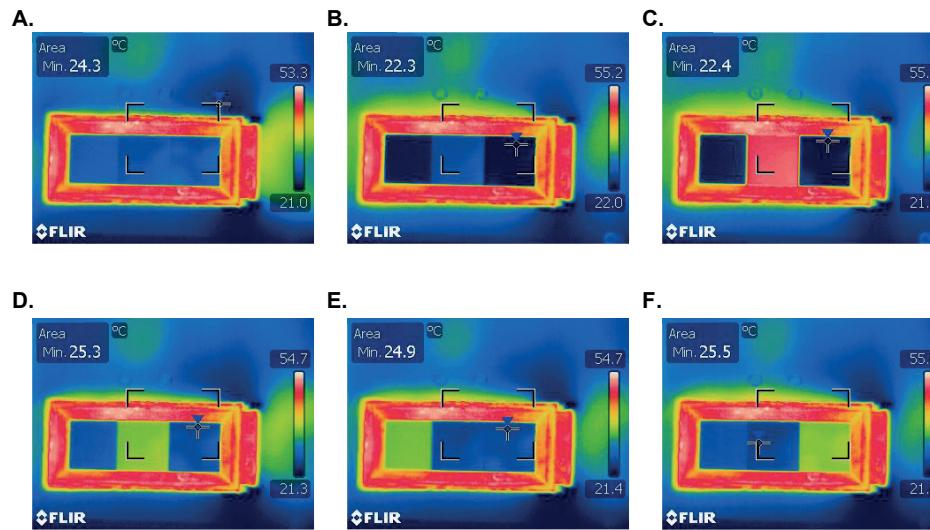


Figure S1 Thermal images of temperature-controlled arena. Thermal images demonstrate lack of heat diffusion between the tiles of the temperature-controlled arena. Images show the temperature detected in the focus point of the infrared camera (top left), the temperature gradient (right side), the temperature of each tile, and the temperature of the aluminum ring surrounding the arena (constant 50°C). **A.** All tiles are set to 24°C. **B.** Middle tile is set to 24°C and side tiles to 22°C. **C.** Middle tile is set to 40°C and side tiles to 22°C. **D.** Middle tile set to 30°C and side tiles to 24°C. **E.** Left tile set to 30°C and middle and right tiles to 24°C. **F.** Right tile set to 30°C and middle and left tiles to 24°C.

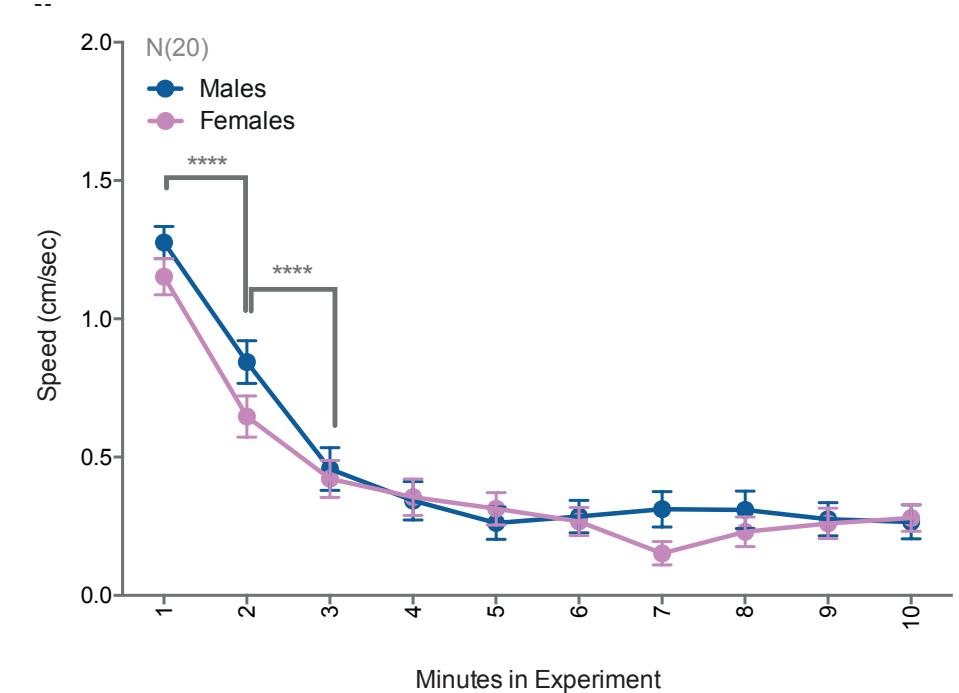


Figure S2 Performance of Canton-S males and females at a constant temperature of 22°C. Speed of individual male (blue) and female (pink) flies. There is a significant reduction in speed between minutes 1 and 2 and between minutes 2 and 3, but not between other time intervals (Two-way RM ANOVA: F(DFn, DFd)=80.56(9,882), p<0.0001). Males and females do not exhibit significant differences in speed (Two-way RM ANOVA: F(DFn, DFd)=0.8903(1,98), p<0.4377). *Significance difference among groups (Tukey's multiple comparison test, $\alpha=0.05$). Data are mean \pm s.e.m.

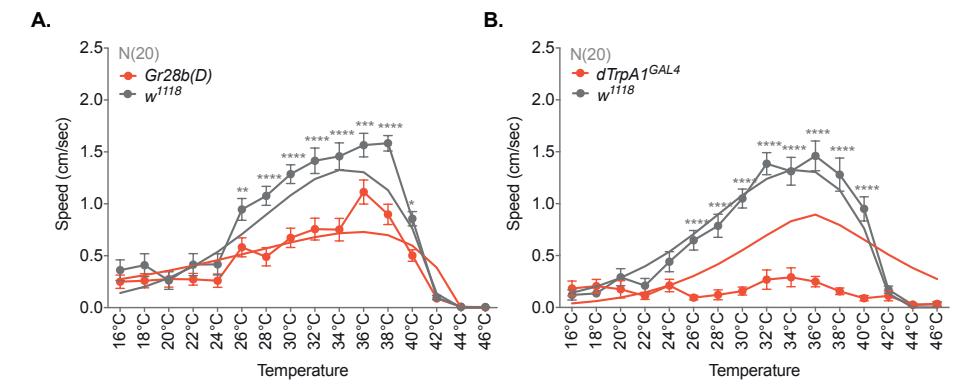


Figure S3 Model fitting of temperature performance of *Gr28b(D)* and *dTrpA1^{GAL4}*. Model fitting of previously shown (Fig. 4) temperature performance curves of *Gr28b(D)* mutant males **A.** (red), *dTrpA1^{GAL4}* mutant males **B.** (red), and *w¹¹¹⁸* males (grey). Data shows that control flies and *Gr28b(D)* mutants behave as predicted by Logan 10 and 6 models respectively while *dTrpA1^{GAL4}* mutants are not described by any of the models considered for temperature performance (Table S2). These suggest that *Gr28b(D)* mutants follow a pattern similar to that of control flies while *dTrpA1^{GAL4}* mutants have completely lost the capacity to respond as controls.

Models	K	AIC	Δ AIC	wAIC	Δ BIC	r^2
Logan 10	6	753.41	0	\approx1	0	0.67
Logan 6	5	848.40	94.98	2.37 e-21	86.059	0.54
Gaussian	4	903.17	149.75	3.03 e-33	131.905	0.85
Modified Gaussian	5	907.69	154.27	3.16 e-34	145.348	0.84
Quadratic	4	1079.43	326.07	1.56 e-71	308.227	0.70

Table S1 Predictive value of different temperature performance models against experimentally determined data. Data from temperature performance of males (Fig. 3B) were modeled using common functions used to describe temperature performance and compared using Akaike Information Criterion (AIC). K stands for number of parameters; Δ AIC reports the goodness to fit relative to the other models; wAIC (AIC weight) identifies the probability of that model representing a good approximation; Δ BIC reports the result of a Bayesian modelling comparison, which in our case favors the same model as the AIC; and r^2 is an additional check of the goodness to fit without the consideration of the models' complexity.

Group	Model	K	AIC	Δ AIC	wAIC	Δ BIC	r^2
Wild type	Logan 10	6	600.136	0	\approx 1	0	0.65
<i>Gr28b(D)</i>	Logan 6	5	312.516	0	0.77	0	0.59
<i>dTrpAI</i> ^{GAL4}	Modified Gaussian	5	-55.212	0	\approx 1	0	0.11

Table S2 AIC values of different thermal performance models compared to behavioural data of wild type, *Gr28b(D)* and *dTrpAI*^{GAL4} mutants. Wild type flies' response is best described by Logan 10 and *Gr28b(D)* mutants' response by Logan 6. In both cases, the r^2 results are acceptable. For *dTrpAI*^{GAL4} mutants, the best model according to the AIC is the Modified Gaussian model; however, the predictability of the r^2 is extremely low, which suggest that none of the models selected can properly described these data.



Qualitative differences between male and female social interactions modulate heat stress response in *Drosophila melanogaster*

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Abstract

Sex differences influence how organisms vary in their behaviour and relationship to others. During stressful events, males seek to escape dangerous zones or become aggressive toward other males while females favour social cohesion. This dimorphic response to stress has been suggested to emerge from sexually dimorphic investments in offspring caring. Females seek to protect their progeny, for which they create social networks, while males lack this motivation and favour individualistic responses. We investigated if these differences were present in *Drosophila melanogaster* by exposing flies to gradually increasing temperatures alone and in same-sex groups. At maximum stress temperature, female flies move with similar speed in both conditions while male flies moved faster in groups than alone. This emerged from females maintaining a consistent number of activity bouts and encounters while males actively avoided each other during stress, increasing the number of bouts and reducing their

number of encounters. The pheromone profile of the others contributed to this behaviour, as the response was inverted in females with a masculinized pheromone profile and males with a feminized pheromone profile. Different factors determined the motivation for interactions, as feminized males court and attempted mating when approaching each other, while mating was not involved between wild-type females. Female interactions depended on mechanosensory perception at comfortable temperature, as the mechanosensory mutants piezo showed less encounters than wild-type flies; however, at stressful temperatures, piezo mutants increased their number of encounters to that of wild-type flies, proposing a change in the mechanism controlling the search for others. These results indicate that *Drosophila* possess sexually dimorphic responses to stress when flies are grouped and suggest that the fly could be used to explore the mechanism behind sex differences in social behaviour under stress.

Keywords

Temperature performance, Social behaviour, Stress, Locomotor activity, Sexual dimorphism, Pheromones, Sensory systems.

Introduction

Organisms continuously process and integrate cues from their environment to produce adaptive behavioural responses. Individual characteristics, such as sex, determine how stimuli are processed, ultimately leading to behavioural differences. Being a female or a male causes differences in basic processes such as food preference (Manippa et al., 2017; Sinclair et al., 2017), olfactory ability (Kass et al., 2017; Oliviera-Pinto et al., 2014), and thermoregulation (Kaciuba-usciklo and Grucza, 2001; Kaikaew et al., 2017). Sex differences are also particularly evident during social interactions (Björkqvist, 2018; Gao et al., 2016; Leese, 2012; Nilsen et al., 2004). Women are more engaged in their social networks, have wider groups of friends, reciprocate friendships more readily, seek same-sex peers and suffer more from social exclusion than men do (for example, Seidel et al., 2013). In contrast, men have more restricted networks, cooperate less and respond less to others, engage more with peers of the opposite sex, and experience less pain when social excluded in comparison to women (Seidel et al., 2013; Szell and Thurner, 2013; Taylor et al., 2000). Animal studies have shown that being in a group is calming for female rodents (Brown and Grunberg, 1995; Kamakura et al., 2016), and leads them to become less fearful of open spaces (Westenbroek, 2004), while having the opposite effect on male rodents (Brown and Grunberg, 1995; Westenbroek, 2004). Female mice also appear to suffer more from social isolation (Senst et al., 2016) as shown by higher plasma levels of stress response factors and increased activation of brain areas related to stress (Grippo et al., 2007; Grippo et al., 2018; Kercmar et al., 2014; Mcneal et al., 2014), enhanced pain responsiveness (Martin et al., 2014), and greater learning deficiencies (Merz and Wolf, 2017; Mikosz et al., 2015), when compared to socially isolated males.

Sex differences in the effect of social interactions could be explained by the interaction of multiple sex-dependent physiological responses to social environment and stress that have evolved from the different reproductive roles of males and females. In most species, females are the main caregivers of their offspring, which has been proposed to have favoured the evolution of protection mechanisms that extend beyond the self to their progeny, including befriending peers to increase each other's offspring safety (Taylor et al., 2000). Functionally, this could be reflected in the positive functional response of females to others when stressed. For example, when reintegrated into a group, males increase testosterone production, a factor linked to competitiveness. In contrast, females increase progesterone production, a hormone linked to affiliative behaviour (Seidel et al., 2013). In a group, females also continuously produce pro-social and anti-stress peptides, such as oxytocin and endogenous opioids (review in Taylor et al., 2000), which further reinforces group relations and reduces stress. These suggest that the internal physiological mechanisms associating stress response and group behaviour differs between the sexes. However, the precise mechanisms that determine these differences remain largely unknown.

Drosophila melanogaster is an excellent animal model to elucidate the genetic, molecular and cellular mechanisms underlying social behaviours. Flies have a basic social behaviour repertoire: grouping promotes circadian synchrony (Krupp et al., 2008; Krupp et al., 2013; Levine et al., 2002), stimulates female sexual receptivity (Billeter et al., 2012; Gorter

et al., 2016; Latschev and Billeter, 2016) and increases lifespan (Gendron et al., 2013; Ruan and Wu, 2008). Encounters with their peers affect how flies choose food (Sarin and Dukas, 2009; Tinette et al., 2004), influence selection of oviposition site (Battesti et al., 2012; Battesti et al., 2015; Duménil et al., 2016; Keesey et al., 2016), and facilitate odour avoidance (Ramdyia et al., 2015). Interactions between flies are regulated by a combination of social recognition cues, such as pheromones, vibrations, and acoustic signals (Billeter and Wolfner, 2018; Fabre et al., 2012; Schneider et al., 2012; Versteven et al., 2017). Arguably, the best understood social cues are cuticular hydrocarbon (CH) pheromones produced in abdominal epidermal cells called oenocytes (Billeter et al., 2009). These CHs are sexually dimorphic. For instance, females express 7, 11-heptacosadiene in contrast to the elevated 7-tricosenes compound of males (Jallon, 1984). Genetically modifying or eliminating the expression of these pheromones affects flies' life span (Gendron et al., 2013), stimulates male-to-female aggression (Fernández et al., 2010), encourages male-to-male courtship and reduces male aggression (Billeter et al., 2009; Wang et al., 2011), eliminates species recognition (Billeter et al., 2009), and stops female communal egg-laying (Duménil et al., 2016). This indicates that CHs are fundamental for flies to recognize each other and display adequate social behaviours (reviewed in Billeter and Levine, 2015).

Like humans, flies exhibit a sexually dimorphic response to stress: males increase heart rate and locomotion more than females when exposed to starvation or oxidative stress (Neckameyer and Nieto, 2015). This dimorphic response correlates with the activation of sexually dimorphic brain areas related to locomotion (Belgacem and Martin, 2007) and production of dopamine (Argue and Neckameyer, 2013; Neckameyer and Weinstein, 2009; Rauschenbach et al., 2014). Dopamine has also been correlated with increased locomotion under stress in mice (Eells et al., 2002), and has been proposed to underlie a conserved network for social decision-making in insect, bird, and mammalian species including humans (Carp et al., 2018; Ebstein et al., 2010; Gunaydin and Deisseroth, 2014; Hall et al., 2015; Kamhi et al., 2017; Sasaki et al., 2006; Scheiner et al., 2006). It is therefore possible that flies possess a sexually dimorphic neural circuitry that integrates social interactions and the response to stress and hence they can be used as a suitable model to understand the underlying basis of this process that differs between females and males.

To test the hypothesis that social context modulates the stress response in a sexually dimorphic manner in *Drosophila*, we exposed single or grouped female and male *Drosophila melanogaster* to gradually increasing temperatures. We chose temperature as a stressor because flies have a predictable response to temperature that depends on dedicated receptors in the fly brain and the fly antennae (Gallio et al., 2011; Hamada et al., 2008; Soto-Padilla et al., 2018a). We report that the social interactions of males and females flies differently affect their response to temperature, due to qualitative differences in same-sex interactions, which depend on mechanosensory perception and chemical identification of the others.

Methods

***Drosophila* rearing and stocks**

Drosophila melanogaster flies were raised in LD 12:12 at 25°C on fly food medium containing agar (10 g/L), glucose (167 mM), sucrose (44 mM), yeast (35 g/L), cornmeal (15 g/L), wheat germ (10 g/L), soya (10 g/L), molasses (30 g/L), propionic acid and Tegosept (for food medium preparation see Gorter et al., 2016). Flies were collected using CO₂ anaesthesia on the day of eclosion and placed alone in individual glass vials of 40x8x0.8-1mm (VWR 212-0011P Test tube soda glass) filled with 0.3 ml of food and then tested when 5-7 day old.

Canton-S was used as the wild type strain. To masculinize the CH profile of female flies, +;PromE(800)-Gal4 males (Billeter et al., 2009) were crossed to +;+;UAS-Tra_{JR}/TM3,Sh_e (Fortier and Belote, 2000) females. To feminize the CH profile of male flies, +;PromE(800)-Gal4 males were crossed to w;UAS-TraF females (Ferveur et al., 1995). For controls, PromE(800)-Gal4 males and UAS-Tra_{JR}/TM3,Sh_e females were crossed to *Canton-S* females and males respectively, and UAS-TraF females were crossed to w¹¹¹⁸ males. w;Orco^l (Larsson 2004), w;Piezo^{ko} (Kim et al., 2012), w*,ppk23⁻ (Lu et al., 2012; Thistle et al., 2012), and Inactive (iav^l; O'Dell and Burnet, 1988) were used are odour, mechanosensory perception, contact CH, and auditory sensing mutants, respectively. *Canton-S* flies tested in the dark were used as visually impaired subjects. Mutants for both, odour and contact CH sensing, were generated by crossing w*,ppk23⁻ and w,ppk29⁻/CyO (Lu et al., 2012; Thistle et al., 2012) with w:+;Orco^l to obtain w*,ppk23⁻;+;Orco^l and w,ppk29⁻/CyO;+Orco^l/Tm3,Sh. Offspring were selected against CyO and Tm3,Sh. *Canton-S* (64349), PromE(800)-Gal4 (65405), UAS-TraF (4590), Orco^l (23129), Piezo^{ko} (59770), and iav^l (6029), are available from Bloomington Stock Centre; UAS-Tra2IR/TM3,Sh_e (8868) was obtained from the Vienna Drosophila Resource Centre; ppk23⁻ and ppk29⁻/CyO were a gift from Meghan Lurney and Kristin Scott.

Temperature controlled-area and temperature Protocol

Flies were tested in an automated temperature-controlled arena made of three adjacent copper tiles of 2.5 x 2.5 cm mounted on a thermal mechanism capable of stabilising the tiles between 15°C and 50°C with a variation of ±0.2-0.5°C as described in Soto-Padilla et al., 2018a; Soto-Padilla et al., 2018b. For experiments in the dark, red light at 650nm (LED Strip XL providing 4 lumen per LED) was used to allow monitoring fly movement. For all experiments, flies were reared singly and transferred to the temperature-controlled arena using a mouth aspirator to be tested alone or in a group of 3 flies. Flies were allowed to walk freely for 7 minutes at a constant temperature of 16°C to acclimatize to the arena, after which they were exposed to an increasing gradient (2°C every 60 seconds) between 16°C and 44°C.

Data Processing and Statistical Analysis

Flies were video recorded with a high definition webcam (Logitech® c920, Logitech Europe S.A., Lausanne, Switzerland) and then tracked using custom-made software (Python Software Foundation Version 2.7.6, <http://www.python.org>) as described in Soto-Padilla et al., 2018a; Soto-Padilla et al., 2018b. Fly centroid data were imported into a custom script in RStudio (RStudio Team 2016, Version 1.0.143; R Version 3.5.2) to calculate average locomotion and interaction measurements per each test temperature. Locomotion measurements were mean speed, number of activity bouts, and mean speed of activity bouts. A bout of activity was described as a fly walking faster than 0.5 cm/s for at least 0.5 seconds, detected as the minimum speed of wild-type flies at comfortable temperature (22°C). Social interaction measurements were: number of encounters and mean duration of encounters. An encounter was defined as two flies at a distance of 1 to 1.5 times the average size of a fly (2.5 mm) for at least 0.5 seconds. This was selected because manual measurements of the mean and median distance between the centroids of interacting flies from sample images of the different groups were between 1.2 and 1.3 times the average size of a fly, and the minimum duration of interactions was 0.5 seconds. Mean data of measurements was imported into Graph Pad Prism (v7 for Mac OS Sierra, GraphPad Software Inc., www.graphpad.com) for statistical analysis of the effect of sex or social condition (isolated or grouped) during the tests. Data distribution at 36°C was analysed with a D'Agostino-Pearson normality test using the omnibus K2 test variant. For the data that were not normally distributed (22.4% of all data sets) we first performed a log transformation and then tested again for normality. For the data sets that were still not normally distributed (14.4% of data sets) we checked for outliers using Prism's ROUT method at 1% and 5% and checked if eliminating these explained the deviation from normality. All data from mutants to both, odour and CH sensing, remained not normally distributed. These data were aligned and ranked transform in RStudio (RStudio Team 2016, Version 1.0.143, package ART) and then analysed using a two-way repeated-measurements (RM) analysis of variance (ANOVA). Data from all the other fly groups normalized once the outliers were eliminated. We nevertheless kept the outliers for the final statistical analysis because visual inspection of the video recordings confirmed that they were true representations of fly's behaviour. These groups were compared using a two-way repeated-measurements (RM) analysis of variance (ANOVA) with a Tukey's post hoc test for multiple comparisons.

Results

Social context and sex modulate the locomotor response to harmful temperatures

Female and male flies were exposed to gradually increasing temperatures (2°C every 60s) between 16°C and 44°C, either alone or in a group of three same-sex flies. All flies, alone or in groups, increased their speed as temperature augmented once the threshold of flies' comfortable temperatures was crossed (approximately 27°C; Sayeed and Benzer,

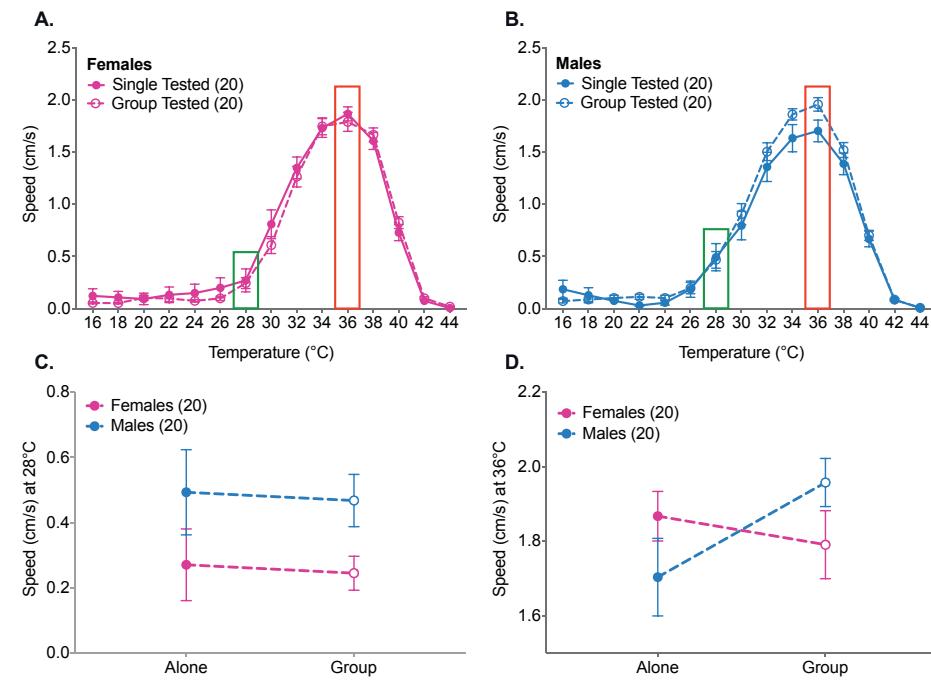


Figure 1 Speed of single and group tested female and male flies. **A.** Speed of single versus group tested female flies (Two-way RM ANOVA: $F_{1,38}=0.3941$, $p=0.5339$). **B.** Speed of single and group tested male flies (Two-way RM ANOVA: $F_{1,38}=1.294$, $p=0.2624$). **C.** Interaction of sex and test condition over speed at 28°C (Two-way RM ANOVA: $F_{1,76}=7.549$, $p=0.9978$). **D.** Interaction of sex and test condition over speed at 36°C (Two-way RM ANOVA: $F_{1,76}=3.937$, $p=0.0509$). Data are mean \pm s.e.m.

1996). Flies then reached a maximum speed at 36°C, after which speed quickly decayed until death at 44°C (Fig. 1A and B). In insects, stress has been associated with increased locomotion because it elevates the concentration of hormones, such as juvenile hormone, that stimulate locomotive behaviour (Johnson, 2017). Due to this association, 36°C was interpreted as the point of maximum stress within our experiments and therefore the point where we expected to find the largest effect of sex or social condition over the stress response. We found that while females do not differ in speed when alone compared to when grouped (Fig. 1A), males move faster at 36°C when tested in a group than when tested alone (Fig. 1B). This interaction between sex and social context is not seen at 28°C (Fig. 1C; $p=0.9978$), but grouped males become faster than grouped females and single males at 36°C (Fig. 1D; $p=0.0509$), suggesting that males and females experience and respond to stressful temperatures differently when alone or in groups.

Sex-specific differences in movement and social interactions explain the different speed of males and females in a group

We next considered two behaviours that could affect the observed interaction between sex and social context on speed at 36°C. First, we considered sex differences in number and speed of bouts of activity between single and grouped flies. Second, we explored sexually

dimorphic difference in the frequency and length of social interactions of grouped flies. The number of bouts of activity showed that females have no differences in the number of bouts when tested single or alone while males have more bouts when tested in a group than when tested alone (Fig. 2A). Grouping also changed the speed of the bouts in a sexually dimorphic manner as grouped females moved slower than single females and grouped males move faster than single ones (Fig. 2B). These results suggest that grouped female and male flies respond differently to harmful temperatures: in comparison to single flies, females maintain a consistent number of bouts and move slower when grouped while males react with more and faster bouts when in a group. The slower movement of grouped females is not sufficient to cause a significant difference in the comparison of overall speed between single and grouped flies in Figure 1A; however, it is possible that the higher speed of grouped males seen in Figure 1B is a result of the additive contribution of more bouts at a higher speed.

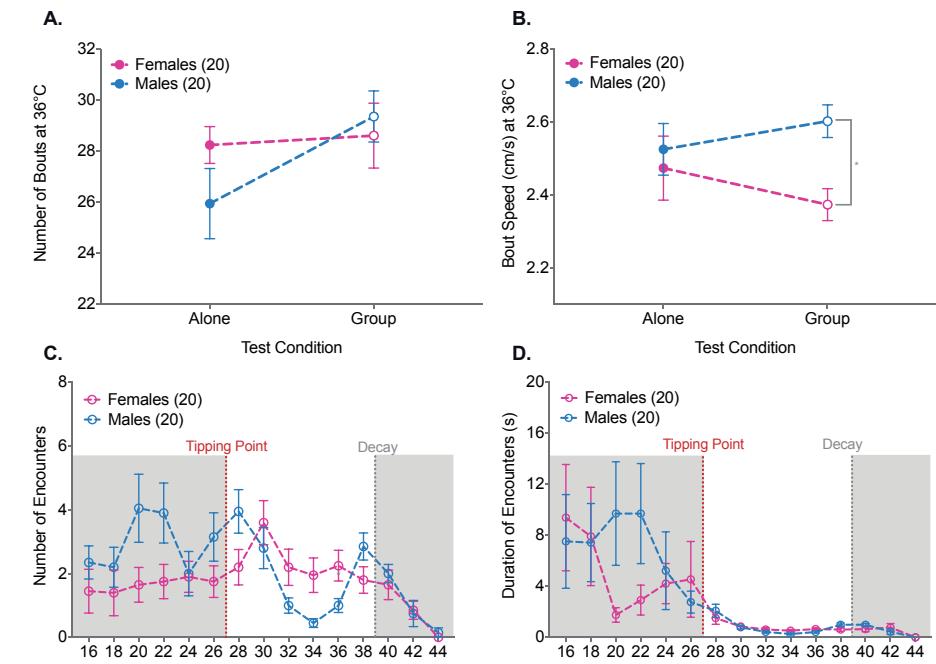


Figure 2 Bouts of Activity and Encounters of female and male flies. **A.** Interaction of sex and test conditions over number of bouts at 36°C (Two-way RM ANOVA: $F_{1,76}=1.849$, $p=0.1780$). **B.** Interaction of sex and test condition over bout speed at 36°C (Two-way RM ANOVA: $F_{1,76}=1.895$, $p=0.1726$) with a significant difference when between female and male flies tested in a group (Two-way RM ANOVA: $F_{1,76}=4.74$, $p=0.0280$). **C.** Number of encounters within same-sex groups (Two-way RM ANOVA: $F_{1,38}=1.895$, $p=0.1767$). Data is divided in three phases: response at comfortable temperatures (grey shaded area before tipping point); response at temperatures after the activation of temperature receptors (white area between tipping point and decay); and decay at painful temperatures (grey shaded area after decay). **D.** Duration of encounters of female and male flies (Two-way RM ANOVA: $F_{1,38}=0.6556$, $p=0.4232$). Data is divided in response at comfortable temperatures, response after activation of temperature receptors, and decay. Data are mean \pm s.e.m.

We considered that social interactions could be another fundamental factor determining the speed of flies. As flies stop moving to interact, more interactions could reduce the

overall speed of grouped individuals. We found that number of encounters between flies varies according to the temperature flies are exposed to (Fig. 2C): in a first phase in temperatures below 27°C, at which flies move at a low speed (Fig. 1A and 1B), males have more encounters than females; in a second phase at harmful temperatures beyond 27°C, at which flies move faster, males show a 'U' shape pattern in the number of encounters while females show a consistent shape after a short increase at the beginning of this harmful phase; and in a third phase of temperatures at which pain receptors are activated (beyond 38°C; Lee et al., 2005; Sokabe and Tominaga, 2009), both males and females show a gradual decay in the number of encounters. Interestingly, females and males have comparable average distances between flies at all temperatures (Fig. S1) and a similar length of interactions once they cross over the threshold of comfortable temperatures (Fig. 2D), despite differences below this threshold. This suggests that flies might not be capable of modulating the duration of their encounters or stop moving at high temperatures and proposes that not all locomotion factors can be controlled based on the presence of others. However, the consistency of the number of encounters between females in the first two phases, suggest that females continually modulate their locomotion to maintain a relative closeness by interacting with each other as stress increases, while males do not seem to prioritize interactions with the same regard. We therefore propose that the slower speed of female bouts of activity at 36°C when grouped compared to alone emerges from their slowing down to interact with each other.

The pheromone profile of the others affects the speed of females and males tested in a group

Social interactions are equally regulated by the cues sent by an individual and by the way its interacting partners perceive those cues. Sexual differences observed in the response to harmful temperatures in same-sex groups might in consequence depend on a non-sex specific response to sex-specific signals produced by the group members or on a sex-specific response to the presence of non-sex specific signals. To separate between these two possibilities, we took advantage of the sex-specific pheromone profile of female and male *Drosophila*. Sex pheromones act as recognition cues that tag individuals as being female or male to others, irrespective of their actual genetic sex. We therefore masculinized the pheromone pattern of female flies and feminized the pheromone pattern of male flies by changing the sex only of the pheromone-producing cells without affecting the rest of the animal. We hypothesised that if grouped masculinized females and feminized males behaved like wild-type flies of the same sex, then the social response to harmful temperatures depends on an individual's sex-specific response to non-sex-specific cues. However, if masculinized females behave similar to wild-type males and feminized males similar to wild-type females then the response to harmful temperatures depends on the non-sex-specific response to sexually dimorphic cues. Our results confirmed the latter hypothesis: masculinized females are faster in groups than alone (Fig. 3A; comparison to same sex controls Fig. S2A), resembling groups of wild-type males, while feminized males showed a similar speed in groups and alone, mimicking groups of wild-type females (Fig. 3A; comparison to same sex controls Fig. S2B). The sex-specific cues emitted by the other flies, but not the intrinsic sex of each individual, are thus sufficient to predict the speed of the group.

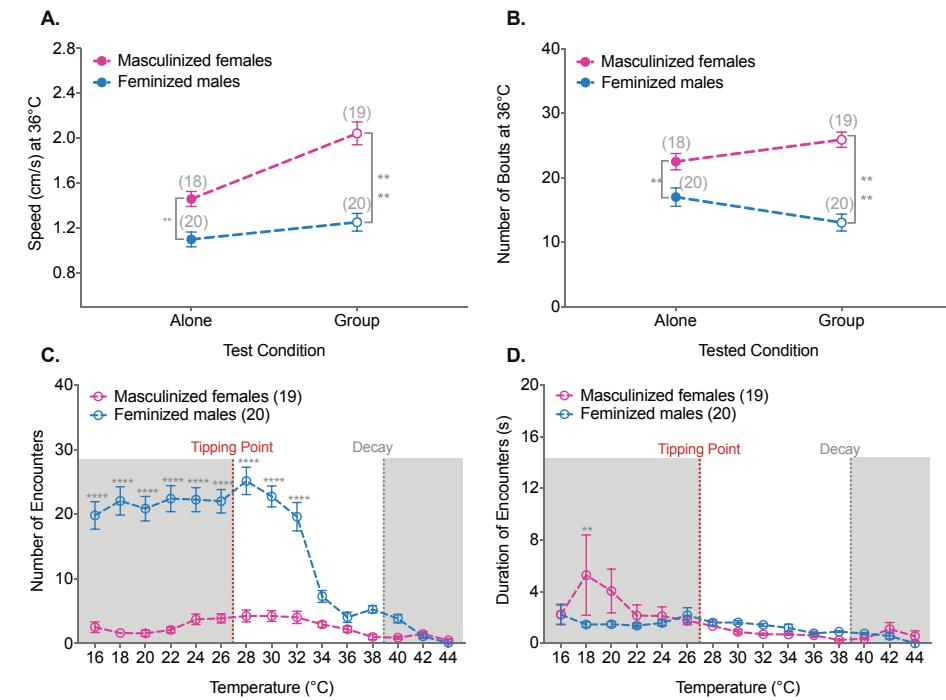


Figure 3 Locomotion and encounters of masculinized female and feminized male flies.
A. Speed of masculinized females (pink) and feminized males (blue) at 36°C. The interaction between genotype and test condition is significantly different (Two-way RM ANOVA: $F_{1,73} = 7.312$, $p=0.0085$). Flies are significantly different when tested alone (Two-way RM ANOVA: $F_{1,73} = 52.18$, $p=0.0044$) and in a group (Two-way RM ANOVA: $F_{1,73} = 52.18$, $p<0.0001$). **B.** Number of bouts of activities of masculinized females (pink) and feminized males (blue) at 36°C. The interaction between genotype and test condition is significantly different (Two-way RM ANOVA: $F_{1,73} = 7.901$, $p=0.0063$). Flies are significantly different when tested alone (Two-way RM ANOVA: $F_{1,73} = 49.29$, $p=0.0084$) and in a group (Two-way RM ANOVA: $F_{1,73} = 49.29$, $p<0.0001$). **C.** Number of encounters between masculinized female (pink) and feminized male (blue) flies are significantly different (Two-way RM ANOVA: $F_{1,37} = 185.6$, $p<0.0001$, *** $p<0.00001$). **D.** Duration of encounters between masculinized female (pink) and feminized male (blue) flies are not significantly different (Two-way RM ANOVA: $F_{1,37} = 1.062$, $p=0.3092$). Data are mean \pm s.e.m.

The pheromone profile of the others also seemed to influence the bouts of activity and the encounters between grouped flies. The number of bouts of activity at 36°C of masculinized and feminized flies followed a similar pattern to that of wild-type flies when flies were alone, but an inverted pattern was registered when flies were grouped (Fig. 3B). Masculinized females, in particular, showed an increase in their number of bouts when tested in a group that was larger than their controls (Fig. S2C) and resembled wild-type males. Grouped feminized males had a constantly high number of encounters between 16°C and 32°C (Fig. 3C), with a rapid loss of function at higher temperatures (Patton and Krebs, 2001), and both feminized and masculinized flies had shorter lasting encounters (Fig. 3D) than wild-types. Nevertheless, not all aspect of bouts and encounters were identical to the opposite sex once sex pheromones were reversed. Feminized males showed a decrease in their number of bouts at 36°C (Fig. 3B) when tested in a group, unlike their male controls with a wild-type pheromone profile (Fig. S2D) and unlike wild-type females. Grouped masculinized females maintained a constant number of encounters until the

decay phase (Fig. 3C), just as the wild-type females, suggesting that females' search for others is an intrinsic behaviour that does not depend on the sex of the surrounding flies. Moreover, the increased number of encounters between feminized males was most likely due to their continuous attempts at courting the other males, which is not what is seen in the encounters between females. This indicates that the motivation for each sex to seek others is driven by different factors. Taken together, the results of bouts and encounter suggest that the behavioural response to harmful temperature does not only depend on the pheromone profile of others, as indicated by measuring the speed of flies, but also on sex-specific intrinsic factors.

Mechanosensory interactions are necessary for the female response to stressful temperatures

To determine which sensory modalities regulate the maintenance of female interactions at stressful temperatures, we compared the response of wild-type females to the response of olfactory, gustatory, auditory, mechanosensory mutants, and wild-type flies in a dark environment. We used only female flies for these experiments to avoid potential mating attempts between males that could difficult interpreting results. Controls and mutants of the tested sensory modalities had all similar speeds when tested alone or in a groups, except *piezo*^{ko} females that increased their speed at harmful temperatures when grouped compared to when alone, which is typical of the response of wild-type males (Fig. 4A).

These mutants also had fewer bouts of activity at harmful temperatures than wild-type flies when individuals were tested alone (Fig. 4B). Given that *piezo* encodes for a mechanoreceptor, we concluded that sensing touch is necessary for grouped females to move at the same speed as single flies and that it is possible that mechanosensory perception of others suppresses the need to move more when flies are stressed and in a group. Indeed, mutants had a smaller number of encounters during comfortable temperatures than wild-type flies (Fig. 4C) and their encounters were short through all temperatures (Fig. 4D). However, the number of encounters between mutants increased almost to the level of wild type flies during stressful temperature between 30°C and 38°C. This suggests that females seek each other during stressful situations and that the duration of the interactions depends on tactile feedback. A second peak at 42°C in the encounters between mutants could represent a final attempt at seeking the effect of interacting with others despite lack of haptic feedback, supporting the conclusion that physical contact is fundamental in the effect of females flies over each other.

We expected to find a similar effect in olfactory or gustatory mutants as that seen in sex pheromone reversed flies (Fig. 3) because olfaction and taste detect close contact CH (reviews Ferveur, 2005; Sánchez-Alcañiz and Benton, 2017). However, it is possible that the response to pheromone detection depends on the interplay and crossover between gustatory and olfactory perception, as described for male social interactions (review Billeter and Levine, 2015; Laturney and Billeter, 2016; Wang and Anderson, 2010; Wang et al., 2011), leading to behavioural effects only when both components of the system are blocked. To test this, we created flies with a double mutation for olfactory and gustatory CH perception and found that they were faster when tested in a group than when tested alone unlike wild-type flies (Fig. 5). This supports the conclusion that olfaction and taste

are both involved in pheromone detection (Wang and Anderson, 2010) within groups of flies, and strengthens the role of the pheromone profile of the others in coordinating the response to stress when flies are grouped. Unfortunately, we were only capable of testing a small sample of these double mutants and further studies are necessary to confirm our findings.

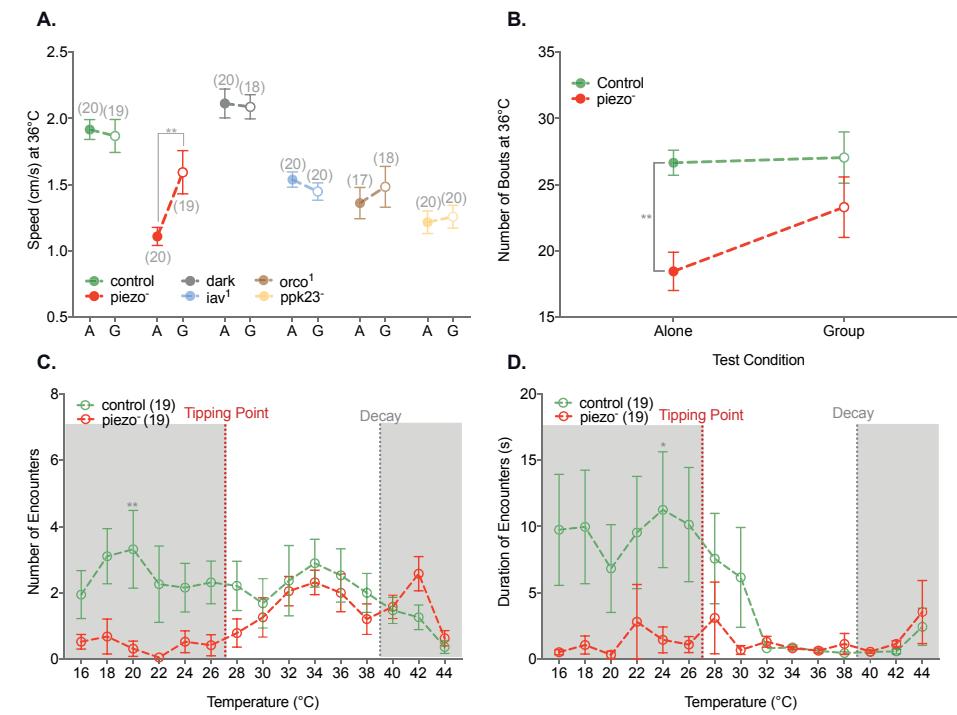


Figure 4 Speed at 36°C of sensory mutants and number of bouts and encounters of mechanosensory mutants. **A.** Speed at 36°C wild-type (green) flies, mechanosensory (red), auditory (blue), olfactory (brown), and gustatory (yellow) mutant flies, and wild-type flies exposed to a dark environment (grey) tested alone or in a group. Significant differences were only in mechanosensory mutants tested alone and in a group (Two-way RM ANOVA: $F_{1,25} = 3.189$, $p=0.0045$ for mechanosensory mutant, $p=0.9999$ for controls, $p=0.9937$ for auditory mutants, $p=0.9737$ for olfactory mutant, $p>0.9999$ for gustatory mutant and flies tested in a dark environment). **B.** Number of bouts of activity at 36°C of wild-type flies (green) and mechanosensory mutants (red). The interaction between genotype and test condition is not significantly different (Two-way RM ANOVA: $F_{1,73} = 1.72$, $p=0.1938$). Flies tested alone were significantly different (Two-way RM ANOVA: $F_{1,73} = 12.32$, $p=0.0017$) while flies tested in a group were not (Two-way RM ANOVA: $F_{1,73} = 12.32$, $p=0.2459$). **C.** Number of encounters between wild-type flies (green) and mechanosensory mutants (red) are significantly different (Two-way RM ANOVA: $F_{1,36} = 6.151(1, 36)$, $p=0.0179$, ** $p<0.01$). **D.** Duration of encounters between wild-type flies (green) and mechanosensory mutants (red) are significantly different (Two-way RM ANOVA: $F_{1,36} = 5.103$, $p=0.0300$, * $p<0.05$). Data are mean \pm s.e.m.

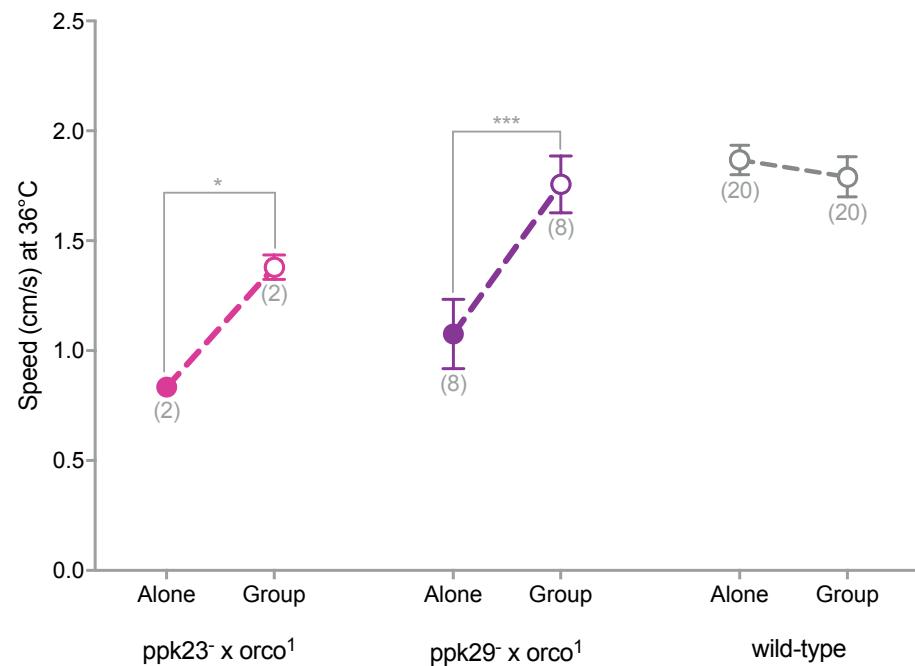


Figure 5 Speed at 36°C of olfactory and gustatory double mutants. Double mutants for olfactory and gustatory perception had significantly higher speeds when tested in a group than when tested alone (*Orco*-/*ppk23* in pink Two-way RM ANOVA: $F_{1,28} = 32.25$, $p=0.0364$; *Orco*-/*ppk29* in purple Two-way RM ANOVA: $F_{1,28} = 32.25$, $p=0.00015$). A=Alone, G=Group. Wild-type flies (grey) were not significantly different when alone or in a group (Two-way RM ANOVA: $F_{1,38} = 0.3941$, $p=0.5339$). Data are mean \pm s.e.m.

Discussion and Conclusion

We tested the hypothesis that sex and social context interact to determine the locomotor response of *Drosophila* to stressful temperatures by exposing male and female flies alone or in groups to gradually increasing temperatures. Traditional descriptions of flies' locomotor behaviour, based on observations of single flies walking at 24°C, describe males as moving with a more consistent pattern than females with less activity periods, even though both sexes walk comparable distances (Belgacem and Martin, 2002). We observe a similar performance in our single flies at 36°C, as females have more activity bouts than males (Fig. 2A and 3B) and both sexes move at similar speeds (Fig. 1D; although a significant difference was seen within pheromone mutants in Fig. 3A), which would directly correlate to total distance walked. We further demonstrated that this sexually dimorphic response changes when flies are placed in a same-sex group. While grouped females behave similarly to single flies, grouped males increased their locomotion through a boost in number and speed of activity bouts (Fig. 1B, 2A and 2B). Remarkably, this difference between single and grouped flies was only observed at harmful temperatures, as there was no effect of social condition at low temperatures (Fig. 1C). This suggests that

stress plays a significant role in modifying the locomotor response of grouped *Drosophila* in a sex-specific manner. Stressed grouped flies also respond differently according to the pheromone profile of the other flies, as shown by masculinized females and feminized males. Masculinized females increased their speed and number of activity bouts at 36°C when grouped compared to single flies, as would be expected from wild-type males (Fig. 3A and 3B), while feminized males move more consistently within test conditions (Fig. 3A and 3B), as would be expected from wild-type females. This suggests that the locomotor response to stress is, at least partially, dependent on the social context in which flies are immersed.

The locomotor behaviour of flies is controlled by a set of approximately 10 neurons in the *parc intercerebralis* (PI) of *Drosophila*'s brain that send projections to the *corpus allatum* (CA) in which they regulate the production of juvenile hormone (JH; Belgacem and Martin, 2002; Gatti et al., 2000). This cluster in the PI was shown to be sexually dimorphic, as expression of the sex-determination restriction factor, *transformer*, in male brains can feminize their locomotor activity through alteration of the metabolism of JH (Belgacem and Martin, 2002). The concentrations of JH are also affected by *Drosophila*'s insulin-like peptides acting on the insulin receptor (InR) of the CA (Belgacem and Martin, 2007; Rauschenbach et al., 2014). Blocking or knocking down the InR led to increased hydrolysis of JH, which feminized the locomotor pattern of males. Interestingly, insulin is a fundamental component of the stress response of flies that coordinates an increase in the concentration of biogenic amines, such as dopamine, octopamine and ecdysteroids, as well as JH, under different type of stressors (reviewed in Grunenko and Rauschenbach, 2018). Dopamine is a particularly interesting component of this network for our research, as manipulating dopamine has a sexually dimorphic effect over the social interactions of flies, measured by social spacing (Fernandez et al., 2017). Moreover, knockdown of InR in CA of flies exposed to heat stress reduced the concentrations of both, JH and dopamine of female flies while affecting only the concentrations of JH of male flies (Argue and Neckameyer, 2013; Rauschenbach et al., 2014). These results suggest that flies possess a sexually dimorphic locomotion brain centre, the PI, that projects to an area, the CA, in which stress dependent insulin signals could produce a sexually dimorphic physiological response that affects locomotion mainly through JH and social relations through dopamine. However, further studies are yet necessary to confirm or correct this suggestion.

A next step in this line of research would be to identify the role of dopamine in the observed sexual dimorphism. In particular, as dopamine has been correlated to social behaviour in multiple species (O'Connell and Hofmann, 2011) and as dopamine production is stimulated through touch (Maruyama et al., 2012), it would be of interest to explore its involvement in the sexually dimorphic pattern of encounters seen in our experiments. We observed that females keep a consistent number of interactions through comfortable and harmful temperatures irrespective of the pheromone profile of their peers (Fig. 2C and 3C) while males modify their number of interaction due to temperature (Fig. 2C) and court each other when they are feminized (Fig. 3C). The response of wild-type males could emerge from an attempt to avoid each other as temperature increases, until a point when active avoidance is too costly and flies simply continue running to try to escape and unintentionally run into each other, producing an apparent increase in the number

of contacts. The response of females, on the other hand, suggests an active interest in seeking each other. A previous study using females demonstrated that moving flies touch stationary ones to motivate them to escape the area of an aversive odour (Ramdy et al., 2015), while human studies shown that female patients react more favourable to touch than males (Dickson, 1999) and that across cultures females are more touch oriented than males (Dibiase and Gunnoe, 2004). It has been proposed that females favour group relations to form networks that facilitate defending offspring and that these associations actively reduce stress (Taylor et al., 2000). It is possible then that the consistency in females' encounters that we found and its dependency on touch (Fig. 4) correlates to a basic evolutionary mechanism of female response to stress across species that could be conditioned by dopamine and its sexually dimorphic effects.

Studying group behaviour would also entail considering the effect of varying social conditions during development. For this chapter, all flies were raised in isolation to eliminate possible biases caused by social encounters before testing. However, previous studies have shown that prior social experience can affect flies' behaviour (Goncharova et al., 2016; Simon et al., 2012) and we have seen that flies raised in a group behave differently than flies raised alone (Box 'Effect of Developmental and Experimental Social Conditions on the Response to Stress' after this chapter). In general, female flies have a consistent speed at stressful temperatures while male flies move faster when in a group independently of developmental social experience. However, both female and male flies raised in a group are slower than flies grown isolated when tested alone. This suggests an effect of sudden isolation that warrants further exploration to understand the importance of grouping for each sex. Females also show more and longer encounters when they have been raised in a group, while males show a higher increase in encounter number before the phase of decay if they have encountered others before the test. This strengthens the importance of social interactions for females, which is reinforced if previously exposed to others, and supports that males might become aggressive at highly stressful temperatures, especially if they have previously faced more males. This highlights the qualitative differences in the interactions between females and males and suggests that experience is a fundamental factor in determining how each fly interacts with others.

The main conclusion from this chapter and the Box 'Effect of Developmental and Experimental Social Conditions' is that studies in *Drosophila* facilitate making different combinations of social experience, access to others, and type of stressor, to identify and better understand sex differences in the stress response.

Authors and Contributors

A.S-P designed the study and performed the statistical analysis. A.S-P and S.L performed the experiments. A.S-P and J-C.B interpreted the study and wrote the manuscript.

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Supplementary Figures

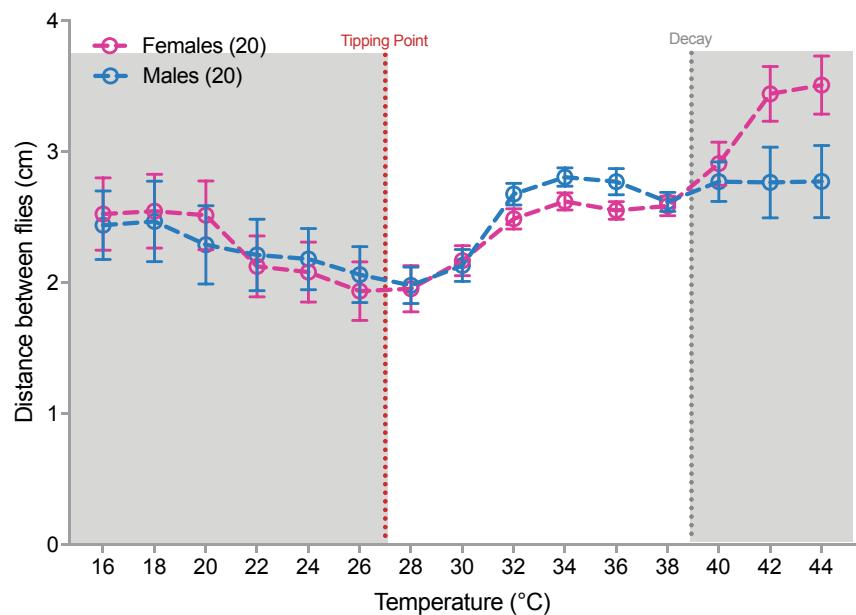


Figure S1 Mean distance between female and male flies. Mean distance between female groups and male groups are not significantly different (Two-way RM ANOVA: $F_{1,568} = 0.8397$, $p=0.3625$). Data is divided in response at comfortable temperatures, response after activation of temperature receptors, and decay. Data are mean \pm s.e.m.

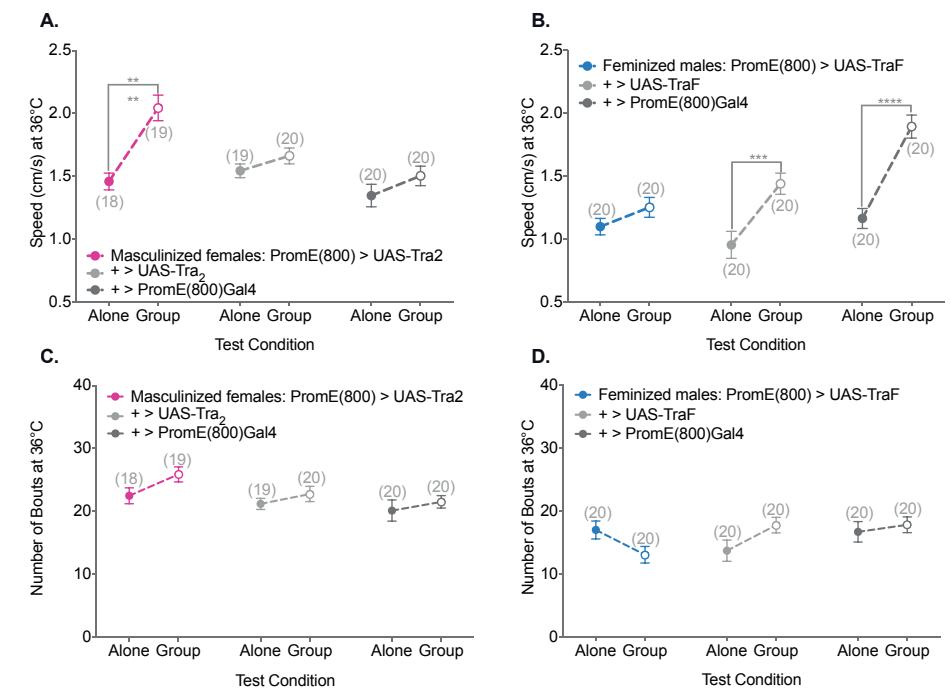


Figure S2 Speed and number of bouts of activity of masculinized females and feminized males at 36°C. **A.** Speed at 36°C of masculinized female flies (pink) tested alone is significantly different from masculinized females tested in a group (Two-way RM ANOVA: $F_{2,110} = 20.49$, $p<0.0001$) while controls (light grey Two-way RM ANOVA: $F_{2,110} = 20.49$, $p=0.6264$; and dark grey Two-way RM ANOVA: $F_{2,110} = 20.49$, $p=0.3850$) do not differ between test conditions. **B.** Speed at 36°C of feminized male flies (blue) tested alone is not different than feminized male flies tested in a group (Two-way RM ANOVA: $F_{1,114} = 42.94$, $p=0.5003$) while controls (light grey Two-way RM ANOVA: $F_{1,114} = 42.94$, $p=0.0003$; and dark grey Two-way RM ANOVA: $F_{1,114} = 42.94$, $p<0.0001$) are significantly different between test conditions. **C.** Number of bouts at 36°C of masculinized females (pink) tested alone or in a group are not different (Two-way RM ANOVA: $F_{1,224} = 2.668$, $p=0.1746$) nor are controls (light grey Two-way RM ANOVA $F_{1,224} = 2.668$, $p=0.4083$ and dark grey Two-way RM ANOVA: $F_{1,224} = 2.668$, $p=0.9920$). **D.** Number of bouts at 36°C of masculinized males (blue) tested alone or in groups are not significantly different (Two-way RM ANOVA: $F_{1,224} = 2.668$, $p=0.4083$) nor are controls (light grey Two-way RM ANOVA: $F_{1,224} = 2.668$, $p=0.9576$ and dark grey Two-way RM ANOVA: $F_{1,224} = 2.668$, $p=0.9758$).

Box: Effect of Developmental and Experimental Social Conditions on the Response to Stress

Social isolation can affect an organism's cognition, adult development and behavioural response. Rodents kept alone during juvenile stage become hyperactive and react with more aggression and less social recognition when exposed to others as adults (Lupien et al., 2009). Nematodes raised without peers display delayed development, an altered neural connectivity, and dampened mechanosensory responses (Rose et al., 2005). Flies reared in solitary vials show a lower social index and behave more aggressively than flies from rich environments (Goncharova et al., 2016; Simon et al., 2012). It is possible that the result presented in the Chapter "Qualitative differences between male and female social interactions modulate stress response in *Drosophila melanogaster*" emerged from an interaction with the solitary rearing conditions of our flies and not solely from innate sex differences. To test this, we raised flies in groups of three same-sex individuals and tested them in our temperature gradient protocol on their own or in the same group they grew up with. Females raised and tested in a group behave identical to flies raised isolated and tested single or in a group (Fig. 1A). Meanwhile, females raised in group and tested isolated had a higher speed during comfortable temperatures but reached the

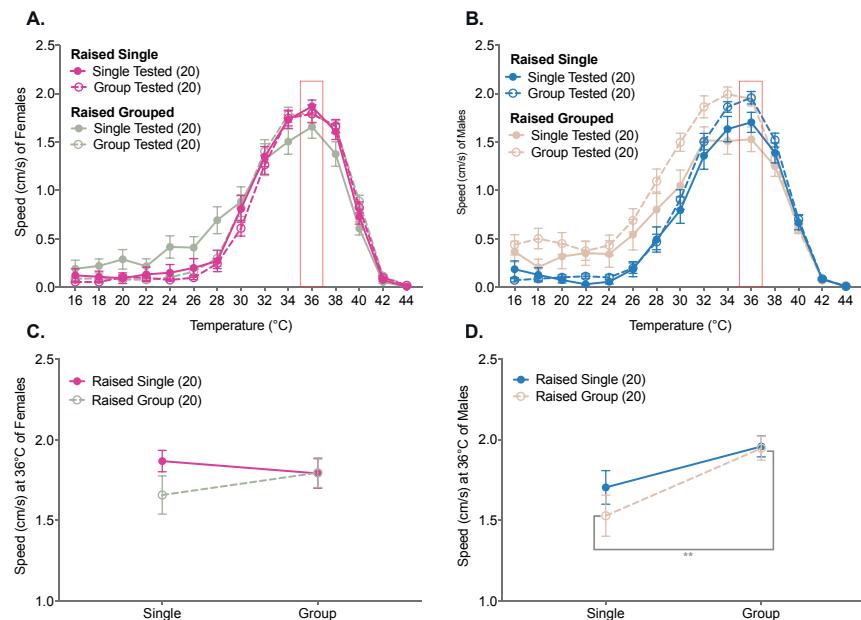


Figure 1 Speed of female and male flies with single and group rearing and testing conditions.
A. Speed of female flies raised in isolation (pink) or in groups of three flies (gold) and tested as single flies (full circles and solid line) or in groups of three flies (empty circles and dashed line). **B.** Speed of male flies raised in isolation (blue) or in groups of three flies (beige) and tested as single flies (full circles and solid line) or in groups of three flies (empty circles and dashed line). **C.** Speed at maximum speed temperature of female flies raised in isolation or in groups of three and tested as single flies or in groups of three (Two-way RM ANOVA: $F_{1,76} = 0.1383$, $p = 0.7110$). **D.** Speed at maximum speed temperature of male flies raised in isolation or in groups of three and tested as single flies or in groups of three (Two-way RM ANOVA: $F_{1,67} = 0.1158$, $p = 0.0011$, ** $p < 0.01$).

lowest maximum speed at 36°C (Fig. 1C).

Male flies raised in a group were faster at comfortable temperatures than flies raised in isolation (Fig. 1B) and reached a similar maximum speed to flies raised isolated (Fig. 1D). However, just as with females, males raised in group and tested in isolation reached the lowest maximum speed of all groups. These results show that the largest effect of varying social conditions on temperature performance is suddenly becoming isolated. Previous studies have shown that flies reared in isolation and then exposed to a group behave like flies socialized during rearing (Wang and Anderson, 2010). This suggests that the effect of developmental isolation on social behavior can be rapidly changed following initial social interactions. Our results support this observation by demonstrating the all flies tested in groups reached similar maximum speeds for their sex independent on how flies were raised. However, our results also suggest that growing in a group does affect social behaviour as both, female and male flies, react differently when suddenly finding themselves alone in comparison to when they have always been alone.

Flies who have grown in a group also interact differently with each other than flies that have developed in isolation. Females raised in a group have more frequent and longer encounters (Fig. 2A and 2C) than females grown alone. Encounters are more frequent

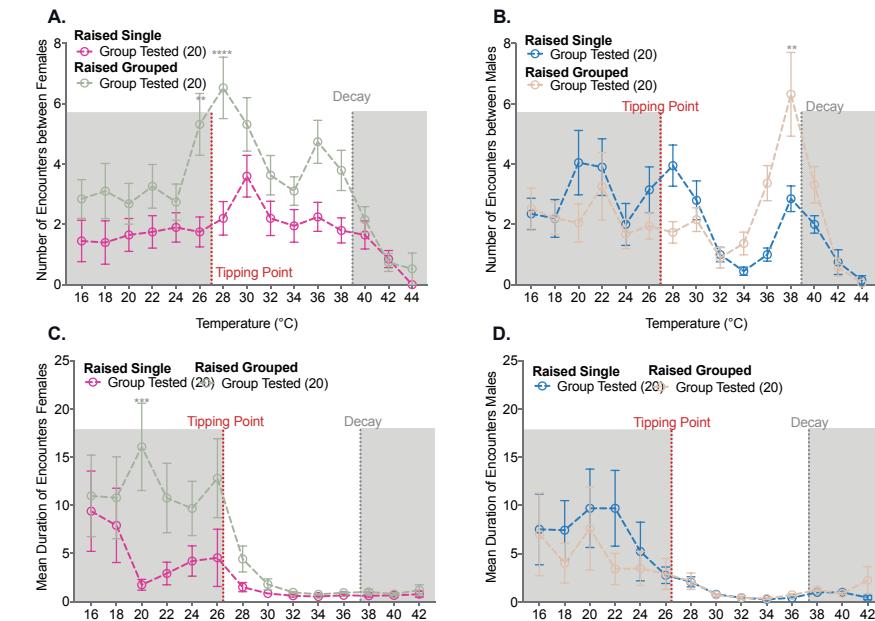


Figure 2 Number and duration of encounters between females and between males raised in isolation or in a group. **A.** The number of encounters between females raised in isolation (pink) or in groups of three flies (gold) are significantly different (Two-way RM ANOVA: $F_{1,38} = 24.35$, $p < 0.001$, ** $p < 0.01$, *** $p < 0.001$). **B.** The number of encounters between males raised in isolation (blue) or in groups of three flies (beige) are not significantly different, except for 38°C (Two-way RM ANOVA: $F_{1,37} = 0.0359$, $p = 0.8508$, ** $p < 0.01$). **C.** The durations of encounters between females raised in isolation (pink) or in groups of three flies (gold) are significantly different (Two-way RM ANOVA: $F_{1,38} = 4.505$, $p = 0.0404$, *** $p < 0.001$). **D.** The duration of encounters between males raised in isolation (blue) or in groups of three flies (beige) are not significantly different (Two-way RM ANOVA: $F_{1,38} = 0.3051$, $p = 0.5840$).

when temperatures change from comfortable to uncomfortable, and when the threshold for painful temperatures (38°C) is almost reached (Fig. 2A). This suggests that contact with others is important in the response of females to stressful situations independent on how they were raised.

Males have slightly more encounters when they have grown isolated than in a group during comfortable temperatures and the first few uncomfortable temperatures (Fig. 2B). When harmful temperatures are reached, males that developed in a group have a sudden peak in the number of encounters and then quickly decay to the level of males raised in isolation. The duration of the encounters, however, does not differ between the groups (Fig. 2D). These results could be explained by the larger concentrations of pheromones that males raised in groups are exposed to. Larger and denser groups of flies stimulate pheromone production, which increases male aggressive behaviour and avoidance (Goncharova et al., 2016; Miyamoto and Amrein, 2008; Wang and Anderson, 2010). This could lead the males developed in a group to avoid each other more actively during comfortable and the first uncomfortable temperatures, showing higher speeds (Fig. 1B) and a lower number of encounters (Fig. 2B) than males raised in isolation. As temperature cross the painful threshold, the stress response could stimulate more aggression between males, which would be higher in the already stimulated males from group rearing, causing a peak in encounter number before a quick functional decay.

Our results demonstrate that social conditions during rearing and testing affect behaviour and should always be carefully considered. Flies reared in isolation behaved similarly to flies reared in a group when tested in a group: females seek each other and males avoid each other. Noticeably, the response is more intense in flies reared with others. However, flies reared in a group and isolated for testing behave differently than flies reared in isolation. This suggests that sudden isolation acts as a stressor by itself and that it can be a confounding factor when comparing grouped and single flies. To avoid this, it is recommendable to keep flies isolated before testing when comparing flies tested alone or in a group, as done in Chapter “Qualitative differences between male and female social interactions modulate stress response in *Drosophila melanogaster*”. Once a setting is selected, combinations can increase in complexity by introducing mutants to wild-type groups, mixing flies that have not met before, or combining flies with different degrees of experience with regard to an environment or task. The changes in behaviour due to each of these variables will allow us to explore more of the network underlying social behaviour and will promote the fly as a model of complex social systems.

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Manuscript in preparation

Offspring developmental temperature is more relevant than maternal environment in determining adult temperature performance of *Drosophila melanogaster*

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Abstract

Besides inherited genes and developmental environment, an organism's phenotype can be modified by parental effects, a predictive change produced by parental influence on their offspring to better prepare them to face future environmental challenges, such as exposure to extreme temperatures. Assessing the adaptive significance of parental effects is highly interesting as consequences can be beneficial or harmful to the offspring; parental influence could produce an adaptive change if offspring conditions are similar to that of their parents. However, parental effects could also lead to non-adaptive offspring phenotypes when the environment differs or when changes are related to carry-over effects that reduce offspring phenotypical flexibility. The fruit fly *Drosophila melanogaster* has been a useful model to unravel the underlying mechanism of multiple behavioral and phenotypical characteristics. Flies live in habitats that span a wide range of temperatures and have shown apparent

parental effects, such as flies from parents raised in warm environments developing faster and having a higher heat tolerance than flies with parents from colder areas. Only one study has looked specifically at temperature-related parental effects in *Drosophila*, demonstrating a strong influence of maternal thermal environment on offspring survival from egg to adult. Here, we used a split-brood match and mismatch design to estimate maternal effects on offspring response to temperature. Mothers from an inbred population of flies were exposed to a cold (18°C) or hot (29°C) environment and their brood was split between matched and mismatched conditions. We found maternal effects on offspring climbing speed. The response to gradually increasing temperatures and to heat or cold-shocks depended mostly on the phenotypic plasticity of the offspring to the environment they were raised in, independent of maternal influence.

Keywords

Maternal effect, Matched and mismatched, temperature environment, *Drosophila*, fitness.

Introduction

Every environment produces challenges that organisms must face to survive. When these challenges are predictable, parents may be capable of influencing the development of their offspring to better face them (Engqvist and Reinhold, 2016; Mousseau and Fox, 1998) by, for example, changing egg composition, immune factors, or stimulating epigenetic changes (Groothuis et al., 2005; Ledón-Rettig et al., 2013). This parental influence, known as ‘anticipatory parental effects’ or ‘adaptive transgenerational plasticity’, functions as a cue that directs offspring plasticity into shaping a phenotype that takes advantage of that information (Agrawal, 1999; Crean and Bonduriansky, 2014; Galloway and Etterson, 2007; Mousseau and Fox, 1998; Mousseau et al., 2009; Uller, 2008). Parental effects (Marshall and Uller, 2007), together with inherited genes and the developmental environment, determine offspring phenotype (Adrian-Kalchhauser et al., 2018; Mousseau et al., 2009). Understanding parental effects is thus important to understanding how an individual phenotype develops.

Parental effects buffer offspring resistance against environmental stressors in plants and insects (Agrawal, 1999; Agrawal, 2002; Galloway, 1995; Mousseau and Dingle, 1991), increase disease resistance of crustaceans (Mitchell and Read, 2005) and beetles (Roth et al., 2010), and benefit development and stress tolerance of fish (Munday, 2014; Salinas and Munch, 2012). However, parental effects can also be non-adaptive or even maladaptive when parental and offspring environment differ and therefore parental influence decreases offspring performance (Crean and Marshall, 2009; Marshall and Uller, 2007), or when they are merely carry-over effects of the parental environment that do not have any adaptive value for the offspring (Engqvist and Reinhold, 2016; Nettle and Batteson, 2015; Uller et al., 2013). For example, prenatal stress in mothers can lead to susceptible smaller offspring in fish (Munday, 2014), and diminish offspring learning and social coping in rodents and non-human primates (Kofman, 2002). Maternal environment can also affect germination cycles in plants (Donohue, 2009), population size of soil mites (Plaistow and Benton, 2009), and size of earwig (Raveh et al., 2016) without any anticipatory value. In addition, the potential value of parental effects depends on the time lapse between environmental cue perception by the parents and selection on the offspring, the degree of variation of said cue, the adaptive value of modifying offspring phenotype, and the plastic capacity of the offspring (Auge et al., 2017; Engqvist and Reinhold, 2016). Thus, anticipatory and adaptive parental effects are expected to evolve only when parental and offspring environment sufficiently correlate, and when offspring plasticity is limited and parental input would confer an advantage (Adrian-Kalchhauser et al., 2018; Auge et al., 2017; Engqvist and Reinhold, 2016; Gibert et al., 2001). Although parental effects could be fundamental in understanding how species could adapt to rapidly changing environments, the prevalence and significant of parental effects still remains poorly understood (Sultan, 2007; Uller et al., 2013).

In this study we investigated the anticipatory nature of maternal effects in the context of temperature adaptation using the fruit fly *Drosophila melanogaster*. Temperature influences all levels of biological organization (Good, 1993) and small ectotherms, such as fruit flies, are particularly susceptible to this ambient variable (Hoffmann et al., 2003). *Drosophila* are

present in habitats that span multiple climates to which they have selectively adapted to (Jezovit et al., 2017), while still remaining plastic if developed at a different temperature. For example, populations from temperate areas are more resistant to desiccation than populations from tropical zones (Hoffmann et al., 2003; Kellermann et al., 2012) and flies from warmer areas perform better at warmer temperatures while flies from colder regions fare better in cold scenarios (Gibert et al., 2001). At the same time, flies from the same population raised in warmer temperatures are more resistant to heat-shock (Gibert et al., 2001; Gilchrist et al., 1997) and prefer higher temperatures (Good, 1993), while flies raised in cold temperatures are faster in cold environments (Gilchrist et al., 1997) and have higher survival rate when exposed to a cold-shock (Watson and Hoffmann, 1995). Apparent parental effects also affect flies. Offspring of flies raised in warm temperatures have a faster developmental speed (Crill et al., 1996; Gilchrist, 1996), a higher knockdown temperature, smaller body size (Crill et al., 1996), and reduced cold tolerance (Watson and Hoffmann, 1995) when compared to offspring of flies raised in colder temperatures. However, these studies have not specifically separated maternal effects from offspring plasticity or carry-over effects. One recent study addressed this distinction by using a match and mismatch offspring design (Mohan et al., 2018). This design allows differentiating offspring plasticity from adaptive maternal effects by exposing offspring from the same mother to different temperature environments, although it was not possible to completely eliminate the conceivable influence of temperature-dependent carry-over affecting the parents before egg laying (Engqvist and Reinhold, 2016). Results from these experiments demonstrated that only survival from egg to adult had a strong dependency on maternal influence. Developmental speed, body size, and fecundity were mostly affected by offspring environment, with a minimum influence of maternal condition. In the series of experiments presented here we used the same split-brood match and mismatch design to test maternal effect over offspring response to temperature, which was not previously tested. We exposed mothers to a cold (18°C) or hot (29°C) environment and then split their clutches among matched and mismatched scenarios. Once offspring developed to adulthood, we examined offspring response to temperature based on recovery from heat- and cold-shock, climbing speed, and speed at gradually increasing temperatures to look for potential temperature-related maternal effects.

Methods

Fly rearing and split-brood match-mismatch temperature treatment

Drosophila melanogaster Oregon-R stock flies were raised in LD 12:12 at 25°C on fly food medium containing agar (10 g/L), glucose (167 mM), sucrose (44 mM), yeast (35 g/L), cornmeal (15 g/L), wheat germ (10 g/L), soya (10 g/L), molasses (30 g/L), propionic acid and Tegosept (for food medium preparation see Gorter et al., 2016). For experiments, approximately 500 flies were transferred to an egg-laying cage with a removable egg-laying dish of 100mm x 15mm layered with 3 ml of a solution composed of 20g agar, 26g sucrose, 52g glucose, and 9% (v/v) red grape juice spotted with a fresh dab of dry yeast

mixed with water (Mohan et al. in progress) also kept at 25°C in LD 12:12 incubator. The egg-laying dish was removed after 24h and kept in the same incubator as the egg-laying cage. Larvae were collected 24h later and transferred in groups of 50 to vials of 25mm x 95mm containing 6ml of fly food medium. Flies developed to adulthood at 25°C in LD 12:12. Virgin males and females were collected under CO₂ anaesthesia. Virgin males were placed in vials in groups of 20 flies inside an incubator at 25°C with LD 12:12 cycles. Virgin females were individually placed in vials with 6ml of food and transferred within 1h of collection to a walk-in climate chamber at either 18°C ($\pm 1^\circ\text{C}$ range) or 29°C ($\pm 1^\circ\text{C}$ range) with LD 12:12 cycles. Female flies were acclimated for 24hrs, after which they were paired with one virgin male and allowed to mate for 24hrs within their respective climate chambers. After this period, males were removed and individual females were transferred to an inverted egg-laying vial fitted on top of a 1.5 cm food medium patch. Females were changed to a new patch every 1h during 12h. For the flies at 29°C, this process started immediately after male removal. For flies at 18°C, this process started 48h after male removal as flies require a longer time to start depositing sufficient eggs for our experiments at this temperature. Eggs were collected from each batch immediately after female removal and placed in groups of 2-15 in vials containing 6ml food. The total number of eggs per vial depended on the amount of eggs deposited by each mother, as each brood was split in two equal parts. This created two matched conditions: offspring

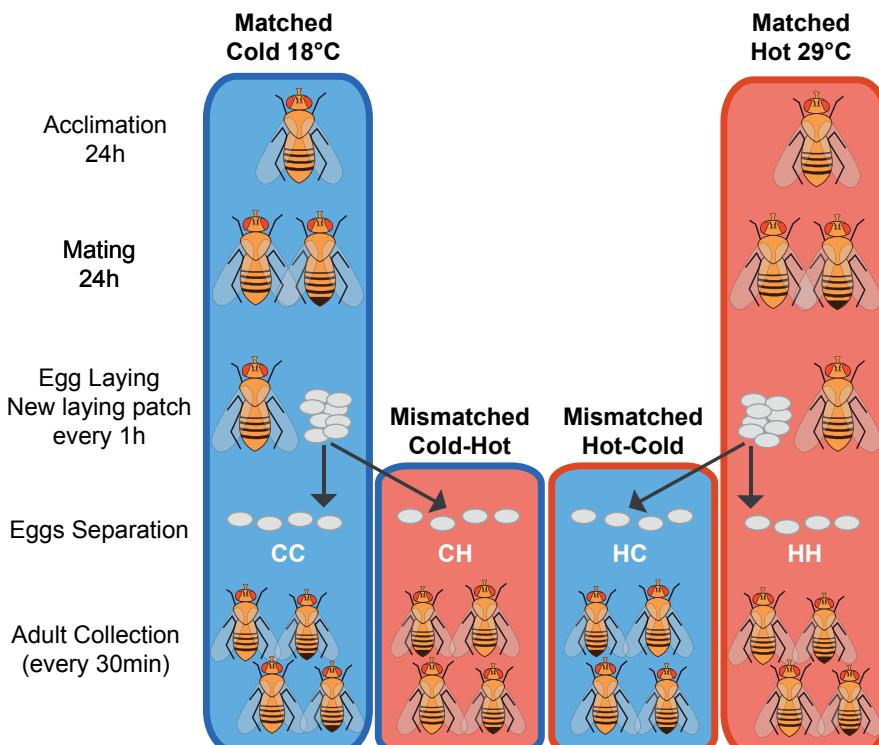


Figure 1 Match and Mismatch protocol. Mother flies were placed in a temperature chamber at 18°C or 29°C and allowed to acclimate for 24h. They were then paired with a male and allowed to mate for 24h. Eggs were separated in two groups per mother: one stayed in the same temperature chamber (matched: CC and HH), and one was taken to the opposite temperature chamber (mismatched: CH and HC). Adults were collected within 30 minutes after eclosion.

from 18°C mothers grown at 18°C (CC) and offspring from 29°C mothers grown at 29°C (HH); and two mismatched conditions: offspring from 18°C mothers grown at 29°C (CH) and offspring from 29°C mothers grown at 18°C (HC). The process is illustrated in Figure 1. Offspring were checked daily every 30min between 6:00 am and 6:00 pm from two days before expected eclosion time to two days after expected eclosion time. Vials at 29°C eclosed 7 days after egg collection while vials at 18°C eclosed 14 days after egg collection. This ensured that all offspring were collected within 30 minutes of eclosion and placed in individual vials kept in the same temperature in which they were raised before temperature response experiments.

Temperature performance measurements

Temperature performance was tested between 2h and 6h after eclosion. The delay was introduced to allow flies from both temperatures to extend their wings, as flies from 18°C matured slower than flies from 29°C, and to have a window of opportunity in which to test flies that eclosed at the same time. The first female and first male collected from each vial were used to test their response to gradually increasing temperatures in a temperature-controlled arena (as described in Soto-Padilla et al., 2018). Briefly, one fly was transferred to a 2.5 x 7.5 cm arena using a mouth aspirator and allowed to walk freely for 7 minutes at 16°C to get accustomed to the arena, after which the temperature was increased 2°C every 60 seconds until 44°C. Flies were continually video recorded with a high definition webcam (Logitech® c920, Logitech Europe S.A., Lausanne, Switzerland) and tracked using custom-made software (Python Software Foundation Version 2.7.6, <http://www.python.org>; Soto-Padilla et al., 2018). Fly centroid data was imported into RStudio and a custom script (RStudio Team: 2016, Version 1.0.143) was used to calculate the average speed per temperature used for analysis.

The second and third collected females of each vial were used for heat and cold-shock experiments. The similarity between the temperature reaction norms of males and females (Soto-Padilla et al., 2018; also confirmed in the similarity of sexes in the temperature curves in Supplementary Table 1) allowed us to only use female flies for these other temperature response tests. For the heat-shock recovery test, flies were placed individually in a 40x8x0.8-1 mm glass vial that was placed inside 25mm x 95mm empty vial and submerged in a hot bath set at 42°C for 7 minutes. Recovery time was measured between the moment flies were taken out of the bath and the moment they started walking again. For the cold-shock recovery test, flies were placed individually in a 40x8x0.8-1mm glass vial that was placed inside 25mm x 95mm empty vial, which was then placed in ice at 0°C for 5h 45 min. Ice temperature was measured every 3-4 h to ensure temperature consistency. Time to recovery was measured between the moment flies were taken out of the ice and the moment they started walking again.

The climbing speed test was done with females used for heat-shock and cold-shock recovery test prior to these experiments. Flies were taken from the climate chambers and taken to a room at 25°C to be transferred to an apparatus with six attached empty vials of 25mm x 95mm and a scale to measure distance. The apparatus was tapped once from a fixed height to force all flies to fall to the bottom of the vial. Photos were taken every

second for 5 seconds with a webcam (Logitech® c920, Logitech Europe S.A., Lausanne, Switzerland). The process was repeated 5 times for each group of six flies. Photos were imported to ImageJ (ImageJ bundled with 64-bit Java 1.8.0_112, <https://imagej.nih.gov/ij/>) to calculate average walking speed based on the distance moved in consecutive images. The three largest values of the five samples were used for final measurements to ensure capturing the maximum speed.

Data Analysis

Data was analyzed using Bayesian inference in R (*brms* version 2.9.0, Bürkner, 2018; *rstan* version 2.18.2, R Core Team 2018 Version 3.5.2). The (Bürkner, 2018) exposure to gradually increasing temperatures was analyzed through a hierarchical generalized additive model with a gamma hurdle distribution, with a log link function for the gamma part and a logit link for the hurdle part. The hurdle parameter (σ) and the shape parameter of the gamma distribution (k) were fitted with thin plate spline smoothers with respect to temperature as single predictor. The scale parameter of the gamma distribution (θ) was fitted with separate global smoothers with respect to temperature for each of the four treatments and an intercept that varied by sex and mother ID. For each individual fly a separate “random smoother” was fitted (factor-smoother interaction basis type; adapted from model GS in Pedersen et al., 2019). The model was run with 10 parallel chains with 2,000 iterations each, where the first 1,000 were used as warm up and discarded. Priors for population-level effects were normal distributions with a mean of 0 and a standard deviation of 10, while priors for standard deviations of group-level effects were Student’s t distributions with a mean of 0, a standard deviation of 10 and 3 degrees of freedom (default *brms* prior). Trace plots, effective sample sizes (range of effective sample sizes: 485 – 6887) and R-hat (Gelman and Rubin, 1992) values ($1 < \text{R-hat} < 1.02$) confirmed proper convergence.

Results from heat-shock, cold-shock, and climbing speed experiments were analyzed using a multivariate Gaussian response model. For each of the three experiments, the response variable was fitted separately for the matched and mismatched conditions, yielding a multivariate model with a total of six potentially correlated response variables. Each response variable was fitted with an intercept that varied by condition, mother ID and individual ID. This setup allowed us to estimate within-mother correlations between (1) within-experiment measurements on siblings from matched and mismatched conditions, (2) between-experiment measurements on siblings, and (3) between-experiment measurements on the same individual. The multivariate model was run with 4 parallel chains, with 5,000 iterations each, where the first 1,000 were used as warm up and discarded. Priors for population-level effects and group-level standard deviations were the same as above, and for correlation coefficients the *brms* default prior of an LKJ ($\eta = 1$) distribution was used. As before, trace plots, effective sample sizes (range of effective sample size: 196 – 9326) and R-hat values ($1 < \text{R-hat} < 1.04$) confirmed convergence.

Results

Maternal temperature affects climbing speed but not cold- or heat-shock recovery

The cold-shock (Fig. 2A) and heat-shock (Fig. 2B) recovery tests showed that offspring’s environment is the main determinant of recovery speed (Table 1). Flies raised at 18°C recovered faster from the cold-shock, while flies raised at 29°C recovered faster from the heat-shock, with those from mothers kept at 18°C recovering slightly faster in both tests (Table 2). The climbing speed test showed an effect of both offspring and maternal environments (Fig. 2C; Table 1: 89% directional posterior probability of maternal condition effect and 91% directional posterior probability of offspring condition effect). Offspring raised at 29°C were faster than offspring raised at 18°C; however, offspring from mothers kept at 29°C were faster than their counterparts from mothers kept at 18°C (Table 2). The pattern of differences observed in the multiple comparison tests (Table 2) suggests there is an apparent additive effect of having a mother kept in 29°C and developing at 29°C to produce an increased climbing speed.

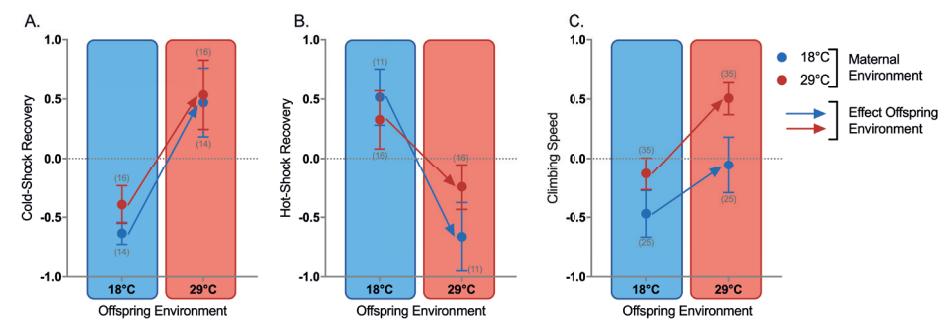


Figure 2 Response to temperature tests. **A.** Cold-shock recovery. Flies kept at 18°C recovered faster. **B.** Heat-shock recovery. Flies kept at 29°C recovered faster. **C.** Climbing speed. Test performed at 25°C. Flies kept at 29°C walked faster, with those from mothers also kept at 29°C walking the fastest. Data are mean and s.e.m.

Maternal temperature has a minor influence in offspring’s response to gradually increasing temperature

The gradually increasing temperature curve suggests that offspring raised at 29°C from mothers kept also at 29°C were the fastest flies (Fig. 3A), consistent with the hypothesis that mothers influence the overall motility of their offspring, although this was not quite statistically significant (Fig 3B-D). Indeed, the analysis of the gradually increasing temperature curve showed that the offspring environment and the changing temperatures were the main determinants of the speed of the flies, with those raised at 29°C moving faster at relatively high temperatures than those raised at 18°C. Flies raised at 29°C also decayed later than flies raised at 18°C (Fig. 3A).

Estimate	Trait	Mean	SD	Lower 90% CI	Upper 90% CI	Directional post. prob.
Mother condition	Cold-shock Recovery	-0.25	0.35	-0.82	0.33	0.77
Offspring condition		-1.12	0.37	-1.72	-0.53	1.00
M x O interaction		-0.14	0.50	-0.97	0.67	0.61
Mother condition	Heat-shock Recovery	0.19	0.37	-0.41	0.81	0.69
Offspring condition		1.17	0.42	0.48	1.87	1.00
M x O interaction		0.60	0.53	-0.28	1.47	0.88
Mother condition	Climbing Speed	-0.34	0.27	-0.78	0.09	0.89
Offspring condition		-0.40	0.29	-0.88	0.06	0.91
M x O interaction		0.23	0.38	-0.39	0.83	0.73

Table 1 Summary of posterior distribution for multivariate model for cold-, heat-shock recovery and climbing speed

Note: Directional posterior probability represents the posterior probability that the tested effect has the same sign as the mean. Mother and offspring interaction (MxO).

Comparison	Parameter	Estimate	Estimate Error	Lower 90% CI	Upper 90% CI	Directional post. prob.
CC - CH	Cold-shock Recovery	-1.12	0.37	-1.72	-0.53	1.00
CC - HC		-0.25	0.35	-0.82	0.33	0.77
CC - HH		-1.23	0.36	-1.81	-0.65	1.00
CH - HC		0.87	0.34	0.32	1.43	0.99
CH - HH		-0.11	0.35	-0.68	0.46	0.62
HC - HH		-0.98	0.34	-1.53	-0.43	1.00
CC - CH	Heat-shock Recovery	1.17	0.42	0.48	1.87	1.00
CC - HC		0.19	0.37	-0.41	0.81	0.69
CC - HH		0.76	0.33	0.22	1.31	0.99
CH - HC		-0.98	0.44	-1.70	-0.28	0.99
CH - HH		-0.41	0.40	-1.09	0.23	0.85
HC - HH		0.57	0.32	0.04	1.10	0.96
CC - CH	Climbing Speed	-0.40	0.29	-0.88	0.06	0.91
CC - HC		-0.34	0.27	-0.78	0.09	0.89
CC - HH		-0.97	0.26	-1.39	-0.57	1.00
CH - HC		0.07	0.28	-0.40	0.53	0.59
CH - HH		-0.57	0.27	-1.00	-0.13	0.98
HC - HH		-0.63	0.24	-1.04	-0.22	0.99

Table 2 Pairwise comparisons between all four conditions for multivariate model on cold-shock recovery, heat-shock recovery and climbing speed

Note: Directional posterior probability represents the posterior probability that the tested comparison has the same sign as the mean. First letter maternal temperature and second letter offspring temperature (C=18°C; H=29°C).

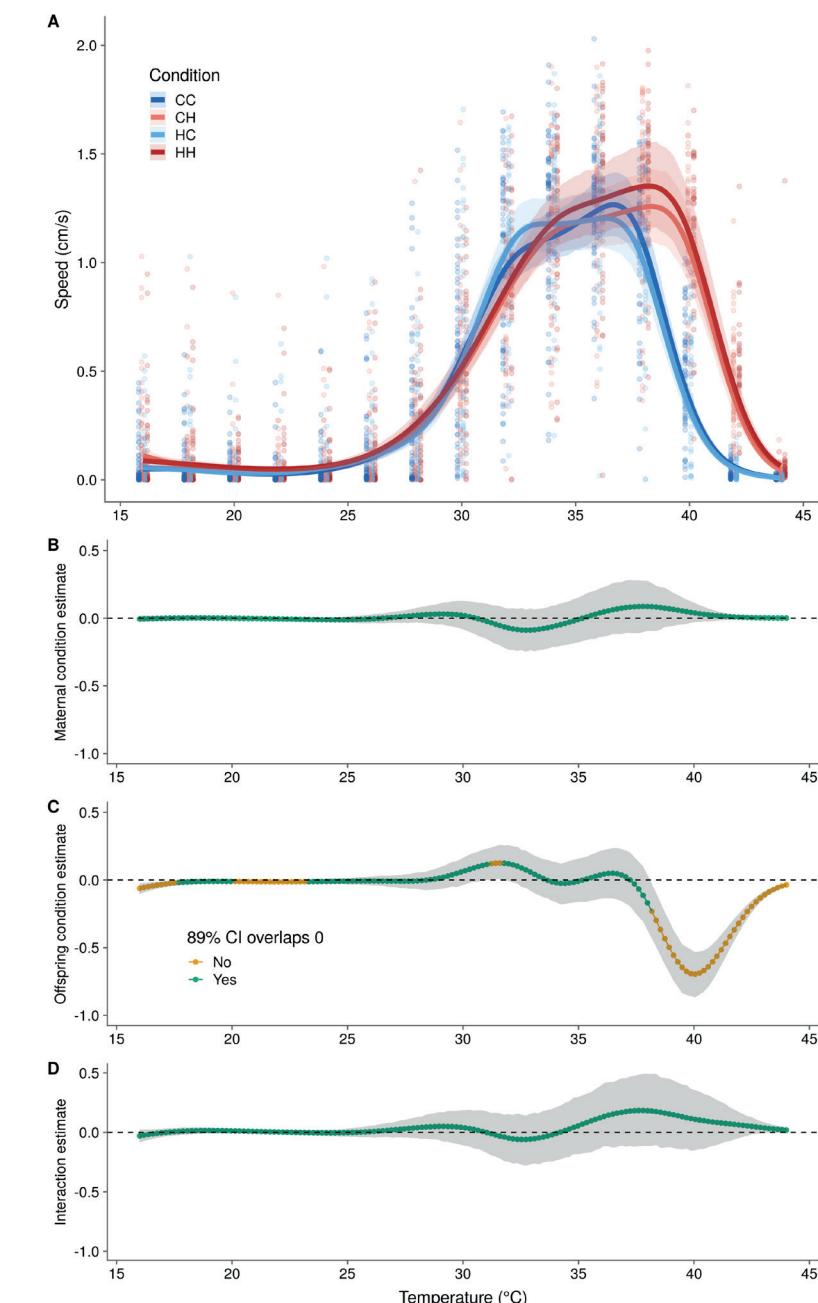


Figure 3 Temperature response curve and main effects of fitted curve. A. Fit curve of speed response to gradually increasing temperature. Flies raised at 29°C move faster at lower temperatures and decay later than flies raised at 18°C. B-D. Main effects of fitted temperature response curve for maternal condition B., offspring condition C., and their interaction D.. Ribbons represent 89% highest density interval of posterior distribution. Green points represent values not statistically different from zero and yellow points represent values statistically different from zero.

Discussion and Conclusion

We used a split-brood match and mismatch design to explore maternal effects on offspring response to temperature. We found that offspring response was mainly determined by the environment in which the offspring developed, with those raised at 18°C recovering faster from cold-shock, those raised at 29°C recovering faster from heat-shock, and those raised at 29°C moving faster in the climbing speed test and when exposed to gradually increasing temperatures (Fig. 2 and 3). Maternal effects, however, were hinted at by subtle differences between offspring raised at the same temperature but coming from 18°C mothers or 29°C mothers: offspring from 18°C mothers recovered faster in both, cold- and heat-shock test, and were slower in the climbing speed test and when exposed to gradually higher temperatures after being raised at 29°C. This could have emerged as consequence of carry-over effects of the cold temperature over mothers and not from an anticipatory maternal effect. Flies reared in cold environments have larger bodies with greater fat content and slower metabolism when compared to flies from warm environments (Adrian et al., 2016; Czarnoleski et al., 2013; Klepsat et al., 2013; Li and Gong, 2015). It is possible that mothers exposed to a cold environment transferred these characteristics to their offspring, conferring a higher resistance to extreme temperatures due to the extra fat layer protecting the core of the fly, which could have reduced the intensity of the effect of extreme temperatures in our shock tests, accelerating recovery. A greater fat content has been linked to a slower metabolism (Brookheart and Duncan, 2016; Palu et al., 2017), which could explain the slower walking rate of offspring from 18°C mothers in the climbing speed test. As offspring effects would have emerged as a consequence of the phenotypic change due to temperature in the mothers, they could be considered carry-over effects, and not anticipatory maternal influence.

Future studies should focus on exploring differences in metabolic processes, genetic changes, and individual factors that could affect the complex dynamics between development and maternal effects. A split-brood match and mismatch experimental protocol is still advisable, as it allows comparing offspring from the same mother instead of distinct lineages. However, the work presented here suffered from an important limitation that should be considered in future endeavors: the egg collection scheme, based on the maximum egg laying times of mothers in cold or warm environments, implied that eggs were collected from 3 day old mothers at 29°C and from 5 day old mothers at 18°C. We chose this scenario because we sought to maximize offspring production to have comparable sample sizes from each temperature. Subsequent replications of this experimental method should account for possible effects of maternal age and attempt to prevent such consequences. Moreover, designers of future experiments should consider analyzing younger flies in the larval stage instead of adults. Developmental experience could modify flies' phenotype and produce a loss of maternal influence, which is less likely to occur in younger stages. Only through the full understanding of this factor would it be possible to fully use *Drosophila* as a model of parent effects.

Authors and Contributors

A.S-P and M.S.M. designed the study, performed the experiments, interpreted the results, and wrote the manuscript. M.S.M performed the statistical analysis. I.P. gave advice regarding the statistical analysis and the writing of this chapter. J-C.B guided the development and advice on the writing of this chapter.

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Supplementary Tables

Estimate	Parameter	Mean	SD	Lower 95% CI	Upper 95% CI
Group-Level effects: Mother (70 levels)					
Intercept	θ	0.05	0.03	0.00	0.12
Population-Level effects:					
Intercept	θ	-1.86	0.06	-1.99	-1.74
Condition CH		0.43	0.08	0.26	0.59
Condition HC		0.00	0.08	-0.16	0.17
Condition HH		0.52	0.09	0.36	0.70
Sex (Male)		0.03	0.05	-0.07	0.13
Temp x Condition CC		-3.67	0.61	-4.85	-2.46
Temp x Condition CH		-6.16	0.60	-7.34	-4.98
Temp x Condition HC		-3.96	0.62	-5.19	-2.74
Temp x Condition HH		-4.73	0.67	-6.06	-3.44
Intercept	k	0.63	0.03	0.58	0.68
Temp		-0.56	0.44	-1.42	0.30
Intercept	σ	-4.37	0.29	-4.99	-3.88
Temp		4.38	1.55	1.58	7.70

Supplementary Table 1 Full summary of the results obtained from a hierarchical generalized additive model for speed

Note: Environment of mother (M) and offspring (O). Group-Level effects are the standard deviation of the maternal random effects; Population-Level effects are the fixed effects of the model. The probability of zero (σ) and the shape parameter of the gamma distribution (k) were fitted with a smooth curve where temperature was the only variable. The scale parameter of the gamma distribution (θ) was fit with a separate smooth curve for each condition, sex as a fixed effect, mother ID as a random effect, and individual ID as a random smooth.

Note: This table is divided into three sections: Group-Level effects are the standard deviation of the maternal random effects (σ) and maternal level correlations between parameters (cor); Population-Level effects are the fixed effects of the model; Family specific parameters in this model are the standard deviations (σ) of each parameter. Environment temperature of mother (M) and offspring (O). Subscript M indicates matched conditions between mother and offspring and MM mismatched conditions between mother and offspring. Subscript CR indicates Cold-shock recovery time, HR indicates heat-shock recovery time and CS indicates climb speed.

Estimate	Trait	Mean	SD	Lower 95% CI	Upper 95% CI
Group-Level Effects: Mother (33 levels)					
σ_{M-CR}	Cold-shock Recovery	0.61	0.28	0.05	1.08
σ_{MM-CR}		0.58	0.25	0.06	1.01
σ_{M-HR}	Heat-shock Recovery	0.22	0.16	0.01	0.58
σ_{MM-HR}		0.41	0.27	0.02	0.96
σ_{M-CS}	Climbing speed	0.52	0.34	0.02	1.20
σ_{MM-CS}		0.24	0.17	0.01	0.63
$cor_{M-CR,M-HR}$		-0.43	0.32	-0.86	0.38
$cor_{M-CR,M-CS}$		-0.09	0.36	-0.74	0.64
$cor_{M-HR,M-CS}$		0.07	0.36	-0.63	0.72
$cor_{M-CR,MM-CR}$		-0.09	0.34	-0.70	0.59
$cor_{M-HR,MM-CR}$		0.04	0.34	-0.62	0.67
$cor_{M-CS,MM-CR}$		-0.05	0.37	-0.72	0.67
$cor_{M-CR,MM-HR}$		-0.06	0.34	-0.69	0.62
$cor_{M-HR,MM-HR}$		0.11	0.34	-0.60	0.72
$cor_{M-CS,MM-HR}$		0.04	0.37	-0.67	0.71
$cor_{MM-CR,MM-HR}$		-0.13	0.37	-0.76	0.62
$cor_{M-CR,MM-CS}$		0.06	0.36	-0.63	0.70
$cor_{M-HR,MM-CS}$		-0.14	0.36	-0.76	0.60
$cor_{M-CS,MM-CS}$		-0.01	0.38	-0.71	0.69
$cor_{MM-CR,MM-CS}$		-0.03	0.37	-0.71	0.67
$cor_{MM-HR,MM-CS}$		0.01	0.37	-0.69	0.69

Supplementary Table 2 Full summary of the results obtained from a multivariate model for matched and mismatched cold-shock recovery time, heat-shock recovery time, and climbing speed

Estimate	Trait	Mean	SD	Lower 95% CI	Upper 95% CI
Matched Offspring (60 levels)					
σ_{M-CR}	Cold-shock Recovery	0.41	0.26	0.02	0.96
σ_{M-HR}	Heat-shock Recovery	0.33	0.23	0.01	0.85
σ_{M-CS}	Climbing speed	0.49	0.28	0.03	0.97
$cor_{M-CR,M-HR}$		0.00	0.50	-0.89	0.88
$cor_{M-CR,M-CS}$		-0.22	0.45	-0.90	0.77
$cor_{M-HR,M-CS}$		0.03	0.45	-0.84	0.85
Mismatched Offspring (60 levels)					
σ_{MM-CR}	Cold-shock Recovery	0.54	0.27	0.03	1.04
σ_{MM-HR}	Heat-shock Recovery	0.53	0.34	0.03	1.18
σ_{MM-CS}	Climbing speed	0.60	0.30	0.04	1.07
$cor_{MM-CR,MM-HR}$		0.02	0.50	-0.86	0.88
$cor_{MM-CR,MM-CS}$		-0.38	0.41	-0.94	0.65
$cor_{MM-HR,MM-CS}$		-0.01	0.44	-0.84	0.83

Supplementary Table 2 (continuation) Full summary of the results obtained from a multivariate model for matched and mismatched cold-shock recovery time, heat-shock recovery time, and climbing speed

Estimate	Trait	Mean	SD	Lower 95% CI	Upper 95% CI
Population-Level effects:					
Condition CC		-0.64	0.26	-1.15	-0.13
Condition HH		0.58	0.24	0.11	1.07
Condition CH		0.51	0.27	-0.02	1.04
Condition HC		-0.24	0.20	-0.64	0.16
Condition CC		-0.47	0.19	-0.85	-0.09
Condition HH		0.50	0.17	0.17	0.83
Condition CH		0.47	0.25	-0.01	0.97
Condition HC		-0.39	0.24	-0.87	0.07
Condition CC		-0.66	0.35	-1.36	0.02
Condition HH		0.32	0.26	-0.19	0.83
Condition CH		-0.06	0.22	-0.50	0.37
Condition HC		-0.13	0.18	-0.48	0.23
Family specific parameters:					
σ_{M-CR}	Cold-shock Recovery	0.43	0.27	0.03	0.98
σ_{MM-CR}		0.40	0.25	0.03	0.90
σ_{M-HR}	Heat-shock Recovery	0.66	0.25	0.12	1.01
σ_{MM-HR}		0.49	0.27	0.05	1.01
σ_{M-CS}	Climbing speed	0.68	0.33	0.08	1.26
σ_{MM-CS}		0.68	0.27	0.13	1.09

Supplementary Table 2 (continuation) Full summary of the results obtained from a multivariate model for matched and mismatched cold-shock recovery time, heat-shock recovery time, and climbing speed



Manuscript in preparation

Assessment of *Drosophila* interval timing ability through temperature-based conditioning

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Abstract

Time perception in the seconds to minutes range, known as interval timing, is necessary for a wide range of cognitive functions, including learning and decision-making. However, the underlying basis of interval timing remains elusive. The fruit fly *Drosophila melanogaster* is a well-known model organism that has helped to unravel the underlying components of other time-related processes, such as circadian timing. These flies also possess brain areas homologous to those linked to interval timing in mammals, suggesting that the fly is also capable of interval timing. Here we attempted to test *Drosophila*'s interval timing skills using a temperature-controlled arena and auditory and visual cues of specific

durations. Flies were to associate each cue with a specific area of the arena being safe (22°C), while the rest of the arena was heated (40°C). We found that flies did not associate the temporal cues with particular arena areas, as there were no consistent behavioural differences between flies exposed to informative short and long cues, and flies exposed to non-informative cues or no cue at all. However, flies in all conditions moved faster and remained longer in the safe location in later trials compared to earlier trials. This suggests that flies were following the temperature characteristics of the test to guide their behaviour.

Keywords

Drosophila, interval timing, temperature response, temperature-controlled arena

Introduction

The perception of time is a crucial component of multiple cognitive functions, such as learning and memory, and a force that guides our behaviour (Buhusi and Meck, 2005; Matell and Meck, 2000; Merchant et al., 2013). In the seconds to minutes range, time perception is known as interval timing, a process involved in associative learning and decision-making that permits predicting the near future and coordinating appropriate behavioural responses (Machado et al., 2009; Sohn and Carlson, 2003; Van Rijn et al., 2014; Wittmann, 2013). The underlying cellular and molecular substrates that regulate interval timing are not well understood (Tucci, 2012), although neurophysiological and psychological studies have proposed models of these components (Kotz et al., 2016; Lake and Meck, 2013; Merchant et al., 2013). Validating these models would require performing experimental studies at the single neuron level in behaving animals, which poses a challenge for vertebrate studies. This limitation could be surpassed by studies in insects, whose stereotypically organized brains with fewer neurons permit single neuron exploration.

Experimental techniques that allow unravelling the cellular and molecular basis of behaviour are highly advanced for the fruit fly *Drosophila melanogaster*. This fly has been used to understand complex processes such as learning and memory (Ofstad et al., 2011; Waddell, 2010), social behaviour (Ramdyia et al., 2015; Schneider et al., 2012), circadian rhythm (Sehgal, 2017; Yao and Shafer, 2014), and sleep cycles (Donlea et al., 2014). *Drosophila* react to disruption of what was expected at a certain time (van Swinderen, 2007), and computational models suggest that flies are capable of sequence learning (Arena et al., 2015). The brain of this fly also possesses structures that are functional homologues to the vertebrate brain areas related to interval timing, such as the insect central complex that is comparable to the mammalian basal ganglia, a core structure in interval timing models (12,20). Thus, it seems plausible that flies are capable of processing temporal information in the interval timing range and therefore might constitute a potential model to unravel the neural basis essential to this process.

To use flies to explore interval timing, it is necessary to first demonstrate that flies are capable of perceiving and using temporal regularities in the interval timing range. To do so, we placed *Drosophila melanogaster* in a temperature-controlled arena in which they had to find one comfortable (safe) location while the rest of the arena was heated up. An auditory or visual cue of specific duration indicated which section of the arena was safe. Flies were to associate the temporal cue with the precise safe location to demonstrate the use of interval timing. To differentiate the response to the temporal cues from the response to the temperature settings of our experiments, we compared a group of flies exposed to timed signals to a group of flies exposed to non-informative auditory or visual stimuli, and to a group exposed to no stimuli. We found that flies from all groups reached the safe location faster and spent more time in it as trials progressed. However, we failed to find significant differences between the groups that would have indicated that flies were using the timed cues to guide their behaviour. These findings suggest that the temperature component of the experiments influenced flies' behaviour, even though our approach might not have been suitable to identify interval timing in *Drosophila*.

Methods

Drosophila rearing and stocks

Drosophila melanogaster Canton-S (CS) and Oregon-R (OR) wild-type flies were raised in LD 12:12 at 25°C on fly food medium (Gorter et al., 2016). Female flies were collected using CO₂ anaesthesia on the day of eclosion, placed in groups of 20 flies in 25x95mm rearing vials with 6.5 ml of food, and tested at 5-7 days old.

Temperature controlled arena and interval timing protocols

Flies were tested individually in an automated temperature-controlled arena which consisted of three adjacent copper tiles of 2.5 x 2.5 cm mounted on a thermal mechanism (see 23,24). The temperature of each tile could be independently set to any temperature between 15°C and 50°C ($\pm 0.2\text{--}0.5^\circ\text{C}$), allowing tiles of different temperatures at the same time. Each fly was placed in the arena with the three tiles set at 22°C and was allowed to explore it for 60 seconds prior to the start of the protocol it was assigned to. Flies could be assigned to only one of the following three protocols:

Behavioural experiments

Protocol 1: Left and Right

The goal of *Protocol 1* was to condition flies to associate left and right tiles with a short (300ms) or long (900ms) stimulus (Fig 1A). Flies could be assigned to an auditory group exposed to an auditory stimulus of 450Hz at 80db, or to a visual group exposed to a visual green light stimulus (520nm) produced by a 16x8 LED matrix (MAX7219). Frequencies and wavelengths were based on those perceived by *Drosophila* (Belusic, 2011; Göpfert and Robert, 2002). Within each group, flies were divided into three conditions: experimental condition with short and long stimulus (20 flies per group); a control condition of flies exposed to an equal medium length non-informative stimulus (600ms; 20 flies per group); or a control condition in which flies did not receive any stimulus (20 flies per group). For both, auditory and visual groups, the left tile was associated with the short stimulus and the right tile with the long stimulus for half the subjects, while this set-up was inverted for the other half. The presentation of the short or long stimulus was semi-randomized, as stimulus of one length (either short or long) could be presented maximum three times in a row.

Each fly within this protocol was exposed to 30 consecutive trials. A trial consisted of a start phase and a test phase, each lasting 60 seconds. The start phase entailed motivating the flies to move to the middle tile ('Fig. S1'Start Position') set at 22°C by increasing the temperature of the left and right tiles to 34°C. This produced a consistent start

position between trials and between flies. The test phase began with the presentation of the stimulus followed by the heating up of the start position tile and the side tile not associated with the stimulus (Fig. S1'Unsafe Tile') to 40°C, while the tile linked to the stimulus (Fig. S1'Safe Tile') was cooled to 22°C. A custom script (MATLAB and Statistic Toolbox release 2014a, The Mathworks Inc., Natick, Massachusetts, US) controlled the transition between phases, the stimulus presentation, and the temperature changes. This setup eliminated the need to physically manipulate the fly for each new trial. Successful use of interval timing by flies from the test group would have been demonstrated if they had moved to the safe tile more efficiently than the control groups as the trials advanced.

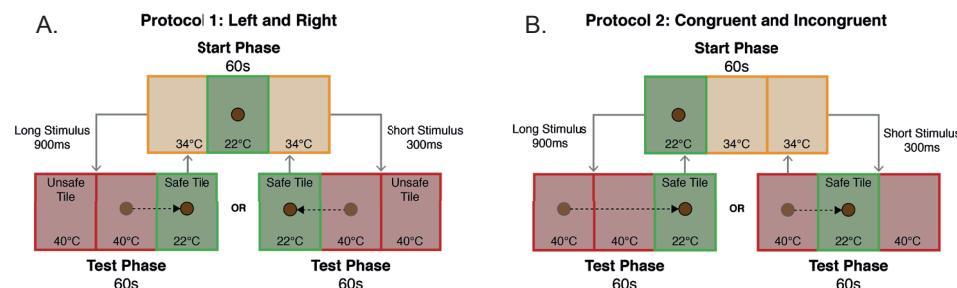


Figure 1 Protocols 1 and 2. **A.** Diagram of *Protocol 1* showing start and test phase durations, tile temperatures, and expected fly response. The dash line indicates the idealized path a fly should follow to successfully perform during the test phase. **B.** Diagram of *Protocol 2* showing start and test phase durations, tile temperatures, and expected fly response. The dash line indicates the idealized path a fly should follow to successfully perform during the test phase

Protocol 2: Congruent and Incongruent

This protocol was designed to facilitate congruency between the duration of the stimulus and the length of the distance that a fly had to walk to reach the safe tile (Fig. 1B). Congruency between stimulus and response has been shown to reduce response time and to be less cognitively demanding than incongruent situations (Barsalou, 1999; Egner, 2007) and hence could facilitate associating the duration of a tone with the location of the safe tile. Flies were exposed to the same auditory stimuli, temperature settings between start and trial phases, and number of trials as used in *Protocol 1*. However, the start position was moved from the middle tile to one of the side tiles (left for 10 flies and right for 10 flies) during the start phase. This allowed associating the closer middle tile with the short tone and the further opposite side tile with the long tone for the test phase, creating congruency between the duration of the stimulus and the distance from the start position to the safe tile (congruent condition). To control for the effect of congruency, a second group (20 flies) was tested in an incongruent condition: with the middle tile linked to the long stimulus and the opposite side tile indicated by the short stimulus. To control for the effect of timed cues, a third group (20 flies) was exposed to the same protocol without any auditory stimulus provided (non-informative condition). Flies exposed to the congruent condition were expected to reach the far tile when it was safe sooner than flies exposed to the incongruent or to non-informative conditions if congruency aided in coordinating flies' behaviour. If flies were using only the temporal information but not the congruency between stimulus and distance walked, flies exposed to the congruent or incongruent

conditions would have outperformed flies in the non-informative condition. Groups were compared only based on the far tile because flies had to cross over the heating middle tile to reach it, which would be expected to happen more efficiently if flies had associated the far location with the cues provided to them. Reaching the middle tile only required flies to walk outside of the heating start position, which they would have done independently of the auditory information presented during the experiment.

Protocol 3: Alternative Intervals

This protocol is a replication with *Protocol 1*, but with a wider range of short and long auditory stimuli durations to test for flies' sensitivity for duration. Each fly was exposed to one of the following short and long stimuli combinations (20 flies per combination):

Short Stimulus	-	Long Stimulus
100ms	-	1100ms
200ms	-	1000ms
300ms	-	900ms
400ms	-	800ms
500ms	-	700ms
600ms	-	600ms

Flies exposed to each of the combinations were compared to flies exposed to 300ms and 900ms stimuli within this protocol to observe if other stimuli lengths improved performance. Groups were also compared to flies exposed to a single medium length stimulus (600ms) for both, left and right tiles as control for the effect of the time cue.

Data processing and statistical analysis

Flies were video recorded (Logitech® c920, Logitech Europe S.A., Lausanne, Switzerland) and then tracked using custom-made software (Python Software Foundation Version 2.7.6, <http://www.python.org>) as described in Chapter 2 (Soto-Padilla et al., 2018b). Fly location data was imported into a custom script (RStudio Team: 2016, Version 1.0.143) to calculate motility measurements (Fig. S1 'Motility Measurements') and perform statistical analyses. Flies that died (no movement in two consecutive phases) were excluded (<2% of sample) and test phases in which flies did not start in the start position tile were eliminated. Normality was assessed using a D'Agostino-Pearson normality test and when necessary data was rescaled using an aligned rank transformation (Villacorta, 2015; Wobbrock et al., 2011). Learning indexes for the selection of the safe, close, or previously safe tiles were calculated by subtracting wrong choices from the correct choices and dividing by the total number

of test phases. Differences between groups in these learning indexes were analysed using a One-way Analysis of Variance (ANOVA) with a post hoc Tukey multiple comparisons test.

A linear mixed-effects model (*nlme* version 3.1-139, R Core Team 2018 Version 3.5.2) was used to analyse if conditions differ from each other through the 30 test phases in each of the motility measurements and in the learning index for safe tile. The model incorporated

the condition in which a fly was tested, test phases, and one of the motility measurements or the learning index for the safe tile as fixed effects. Independent models were run for each of the motility measurements and for the learning index for the safe tile. For every model, condition was considered a categorical variable and test phase was considered a continuous linear variable. The particular identity of each fly was incorporated as random effects. A two-way mixed ANOVA, with phases as the within-subjects factor and condition as the between-subjects factor, was used to obtain *F* test values. P-values for the difference between conditions were determined using a pairwise comparison (Tukey multiple comparisons test). Data was imported into GraphPad Prism (Version 7.0a) for graphing.

Results

Drosophila selects the closest tile more often than the safe tile

To investigate interval timing in *Drosophila* we placed flies in a temperature-controlled arena and exposed them to an auditory or visual stimulus of which the duration was associated to either the left-most or right-most tile that remained at non-aversive temperature (Fig. 1A; Safe Tile). Flies exposed to short and long stimuli were compared with flies exposed to stimuli of equal duration or to no stimulus at all in their learning index to safe tile. As we did not find evidence of flies exposed to short and long stimuli selecting the safe tile more often than flies exposed to stimuli of equal duration or to no stimulus (Fig. 2A and 2D), our results do not support the hypothesis that flies can use this type of temporal information to guide their navigation. Interestingly, flies from all conditions moved to the safe location more often than chance, independent of the stimuli they were exposed to (Fig. 2A, 2D and S2). We considered two possibilities to explain this observation: First, flies could have remembered which tile was safe in the previous test phase and walked to that location; as the same safe location could be randomly repeated up to three times, flies memory would have increased the likelihood of selecting the safe tile in this repeated sequences, and in consequence artificially increase the learning index to safe tile. However, flies selected the previously safe tile as often as chance (Fig. 2B and 2E), which suggest that previous experience was not contributing to their behaviour. A second possibility was that flies moved to the closest tile relative to their position at the beginning of the test phase and that the close tile was coincidentally the safe tile. Flies indeed move to the closer tile more often than chance (Fig. 2C and 2F), perhaps searching for the closer edge as flies prefer arena boundaries (Soibam et al., 2012). Interestingly, the close tile was selected more often if it was also the safe tile (77.91% of times) than if it was not (61.13% of times). These suggest that a combination of closeness, probably due to distance from edge, and safeness, perhaps indicated by colder air temperature above the safe tile, were determinants of fly movements.

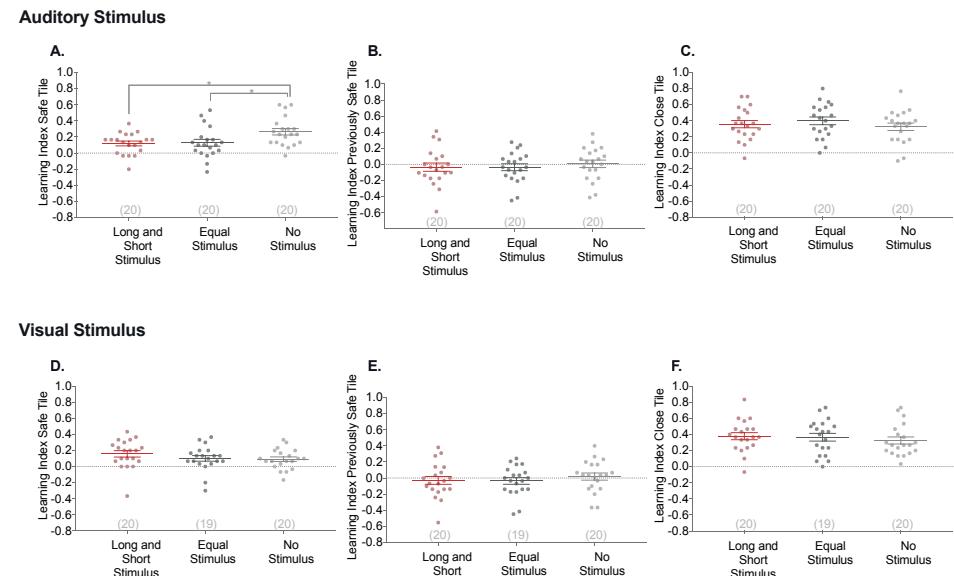


Figure 2 Learning index of flies going to the safe tile first, the closest tile first, or the previously safe tile first. **A.** Learning index of CS flies that went to the safe tile first after being exposed to long and short, equal or no auditory stimulus within *Protocol 1* (One-way ANOVA: $F_{2,57} = 4.626$, $p=0.014$, $*p<0.05$). **B.** Learning index of CS flies that went to the closest tile first after being exposed to long and short, equal or no auditory stimulus within *Protocol 1* (One-way ANOVA: $F_{2,57} = 0.634$, $p=0.534$). **C.** Learning index of CS flies that went to the previously safe tile first after being exposed to long and short, equal or no auditory stimulus within *Protocol 1* (One-way ANOVA: $F_{2,57} = 0.263$, $p=0.769$). **D.** Learning index of CS flies that went to the safe tile first after being exposed to long and short, equal or no visual stimulus within *Protocol 1* (One-way ANOVA: $F_{2,57} = 1.073$, $p=0.349$). **E.** Learning index of CS flies that went to the closest tile first after being exposed to long and short, equal or no visual stimulus within *Protocol 1* (One-way ANOVA: $F_{2,57} = 0.374$, $p=0.689$). **F.** Learning index of CS flies that went to the previously safe tile first after being exposed to long and short, equal or no visual stimulus within *Protocol 1* (One-way ANOVA: $F_{2,57} = 0.444$, $p=0.644$). Data are mean per fly through 30 trials \pm s.e.m.

Drosophila behaviour is affected by experimental conditions

Above, we discussed whether the duration of the stimuli affected the selection of the safe tile. It is, however, possible that flies exposed to short and long stimuli performed better than flies exposed to stimuli of equal duration or no stimuli in other motility measurements. For example, flies exposed to short and long stimuli could reach the safe location faster or spend more time on the safe tile. To test this, a linear-mixed effect model was used to compare the three stimuli conditions in multiple motility measurements (Supplementary Table 1, Table 2, and Table 3). We found that conditions differ only in selected motility measurements and not in overall performance. For example, flies exposed to short and long auditory stimuli differed from the other two conditions in their distance walked before reaching safe tile (Fig. 3D) while their time in safe tile was not different (Fig. 3A), and flies

exposed to short and long visual stimuli differed from flies exposed to a single equal cue in their time inside safe tile in their distance to safe tile (Fig. 3B and 3E) while not differing in their time or speed to safe tile (Fig. S3B and S3H). These differences specific to particular motility measurements suggest that the type of stimuli affected specific features of fly behaviour, even though overall performance was similar between conditions.

Conditions could have been similar in *Protocol 1* because the experimental design was too complex for flies to process. To reduce complexity, we tested flies in a new protocol in which congruency between the duration of the stimulus and the time to walk to the safe tile was expected to facilitate the task (Fig. 1B *Protocol 2*). Flies exposed to the congruent and incongruent conditions differed in their time in safe tile (Fig. 3C), probably due to differences in the time to reach safe tile (Fig. S3C) based on a close to significant difference in the distance to safe tile ($p=0.067$; Fig. 3F). Overall speed of flies did not differ between groups ($p=0.91$; Fig. S3I). Although this might suggest that congruency allowed flies to reach the safe tile faster and spend more time in it, the similarity of means between the groups in time in safe (50.6 ± 2.5 s congruent; 50.1 ± 2.6 s incongruent; 50.4 ± 2.9 s control) and time to reach (7.3 ± 1.0 s congruent; 7.1 ± 1.4 s incongruent; 7.0 ± 1.5 s control) suggest that no group actually performed better than the other. The significant differences we found are probably due to the shape of the curve of each condition. This suggests that the condition could have affected how flies responded, despite not causing an overall impact over the final behavioural output.

The linear mixed-effects model also showed that some motility measurements had were affected by the phase the fly was in. This was more common for auditory stimuli within *Protocol 1* than for visual stimuli within the same protocol or the motility measurements within *Protocol 2* (Supplementary Table 1 and Table 2). The graphical results demonstrate that flies quickly increase their performance after the first few trials and then reach a plateau (Fig. 3 and S3); however, variability between phases is more notable at visual inspection for flies exposed to visual stimuli within *Protocol 1* or to *Protocol 2*, than for flies within *Protocol 1* (e.g. Fig. 3 A-C and Fig. S3 A-C). This further supports that the type of stimuli can affect flies' response, and additionally suggests that the experimental configuration of the temperature-controlled arena can influence how flies perform.

Drosophila reacts similarly to diverse time intervals

It could be argue that our results from *Protocol 1* and *Protocol 2* were linked to the inability of flies to distinguish between the short (300ms) and long (900ms) stimuli. To explore this possibility, we tested the response of flies to other combinations of stimuli durations in *Protocol 3*. As depicted in Figure 4, none of the combinations of short and long durations produced learning indices significantly different from stimulus of equal duration (600ms) or the short and long stimuli used in *Protocol 1* and *Protocol 2*.

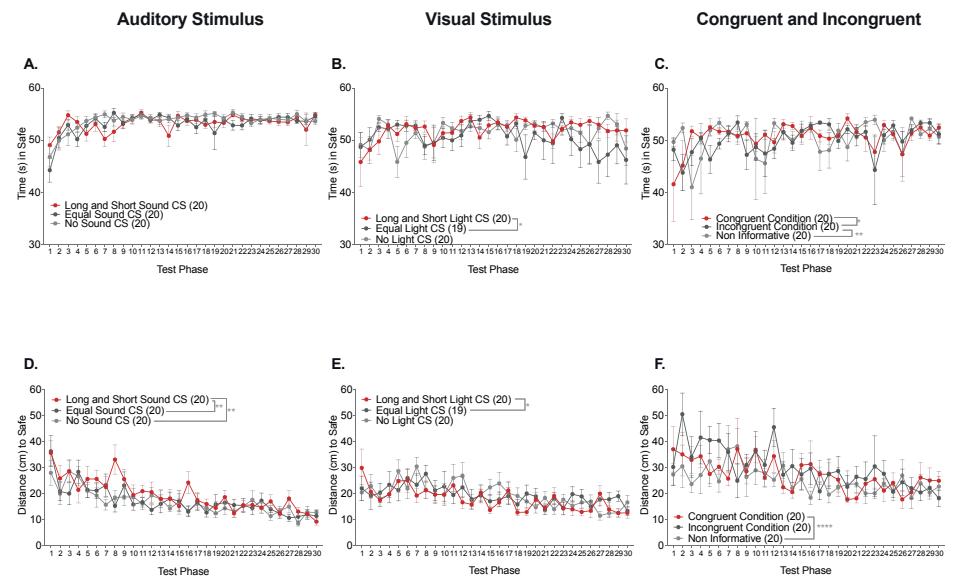


Figure 3: Time in safe tile and distance walked to safe tile during each test phase. **A.** Time on safe tile of CS flies exposed to long and short, equal or no auditory stimulus within *Protocol 1*. **B.** Time on safe tile of CS flies exposed to long and short, equal or no visual stimulus within *Protocol 1*. **C.** Time on safe tile of CS flies exposed to congruent, incongruent, or non-informative conditions within *Protocol 2*. **D.** Distance to safe tile of CS flies exposed to long and short, equal or no auditory stimulus within *Protocol 1*. **E.** Distance to safe tile of CS flies exposed to long and short, equal or no visual stimulus within *Protocol 1*. **F.** Distance to safe tile of CS flies exposed to congruent, incongruent, or non-informative conditions within *Protocol 2*. Data are mean \pm s.e.m. Asterisks indicate significant differences between groups (* $p>0.05$, ** $p>0.01$, *** $p>0.001$, **** $p>0.0001$; Tukey multiple comparisons test).

Discussion and Conclusion

To investigate interval timing in *Drosophila* we attempted to condition flies to move to a safe tile of a temperature-controlled arena according to the duration of an auditory or visual stimulus. Our experiments failed to find direct support for the use of interval timing in *Drosophila* because flies exposed to stimuli of short and long durations did not perform significantly better than flies exposed to stimuli of equal duration or no stimuli at all. To ensure this failure was not specific to the inbred wild-type strain we used (CS), we repeated the *Left and Right* experiments with another wild-type strain (OR; Fig. S4, S5 and S6; Supplementary Table 4 and Table 5). Since those flies behaved similarly to CS we conclude that fly genotype was unlikely to drive these findings.

We observed that flies from all conditions selected the safe tile more often than chance (Fig. 2A and 2D). A combination of flies tendency to move to the tile closest to them (Fig. 2C and 2F) and another non-identified feature of the safe tile probably explained part of this result.

Protocol 3: Alternative Intervals

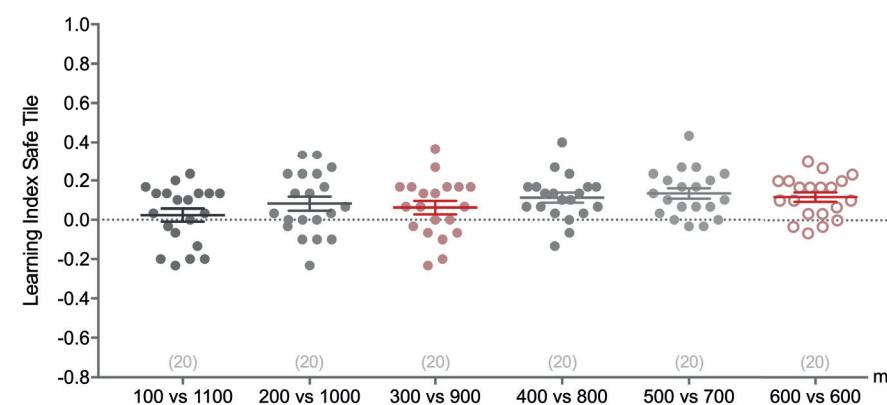


Figure 4: Safe tile learning index between groups exposed to stimuli of different durations.

A. Comparison of learning index of selecting the safe tile between CS groups exposed to long and short stimuli of different durations (One-way ANOVA: $F_{5,114} = 1.859$, $p=0.1070$). Data are mean \pm s.e.m

Future work to fully identify the features of the safe tile that might aid in fly selection are important to understand fly behaviour better and plan for more appropriate approaches to determine *Drosophila* interval timing capacity. Understanding the behavioural effects of the different conditions in *Protocol 1* and *Protocol 2* would work for this same ending. For example, flies selected the safe tile more often without auditory stimuli than with auditory stimuli (Fig. 2A), while flies exposed to long and short visual stimuli differed from flies exposed to an equal visual stimuli in their time in and distance to safe tile (Fig. 3B and 3E). These suggest that different type or combination of stimuli produced different responses from the flies, even though these differences did not reflect use of interval timing. Future studies could take advantage of our set-up to further explore flies' behavioural determinants, such as whether different sounds or lighting produce more precise conditioning and use this information to develop more accurate test to demonstrate interval timing.

We also observed that flies within *Protocol 1* and *Protocol 2* improved their response within the first few trials and they quickly increase the amount of time inside safe tile (Fig. 3A-C). This was probably due to the reduction in the time to reach safe tile and the time to start moving (Fig. S3A-F), both of which were expected to improve as consequence of practice (Newell, 1980). The time to reach safe tile probably decreased as a consequence of the gradual reduction in the distance walked to safe tile (Fig. 3D-F). These shorter distances could have emerged from flies exploring the arena less in late trials compared to early phases, as flies explore more within the first minutes of exposure to a new environment (Soto-Padilla et al., 2018a). However, if this were the only explanation, flies would not have reduced the distance walked throughout the whole experiment. As the distance walked became continuously smaller, our results indicate that flies became better at moving to the safe tile as phases progressed. It is possible that flies were using the temperature component

of the experiment to determine these more efficient paths. If this were the case, our results would suggest that the temperature information is a highly salient stimulus for flies, which could have prevented them from using the time component of the cues presented to them. Future endeavours should consider this possibility to design more suitable experiments and fully determine *Drosophila*'s interval timing.

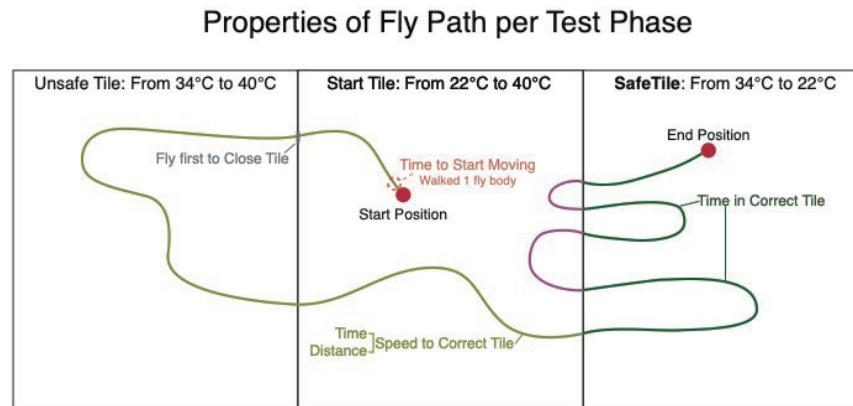
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Supplementary Figures

Additional File 1

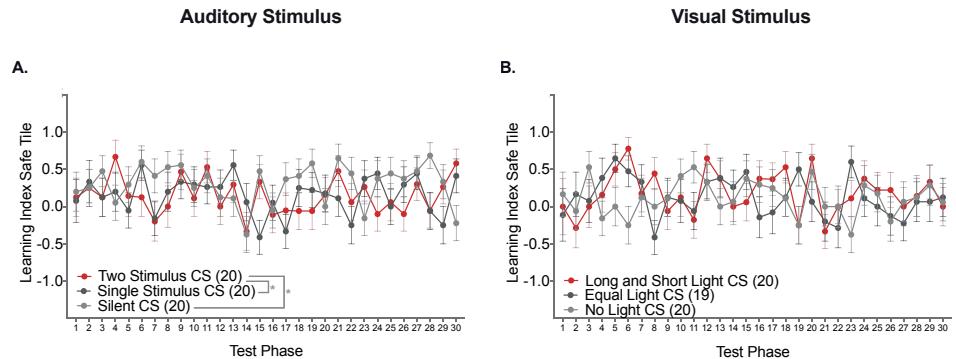
Measurements of fly motility



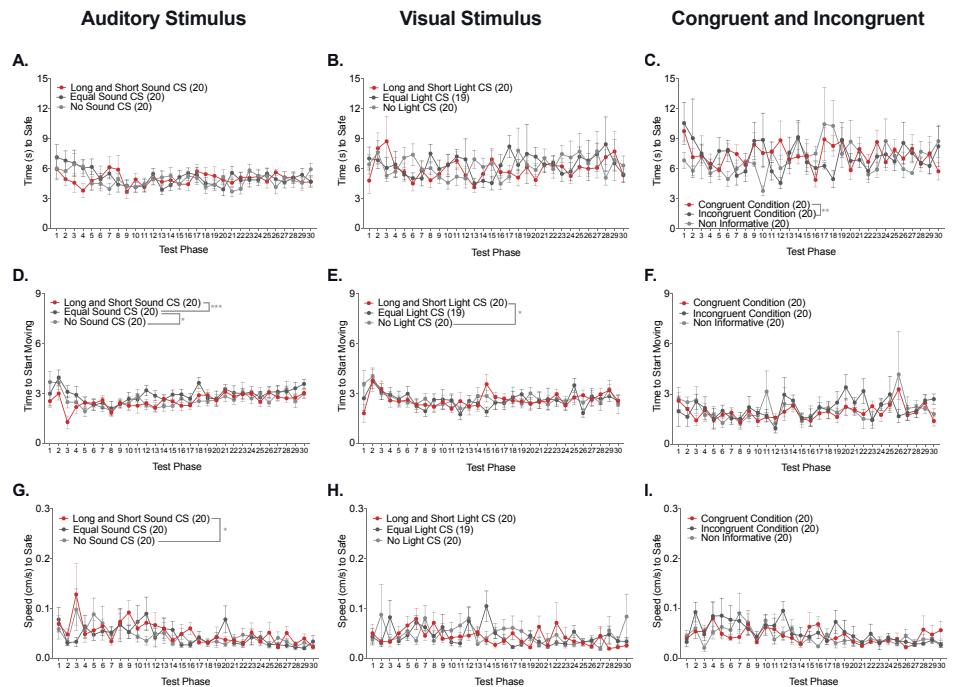
Measurement	Description
Start position	Register of the tile in which a fly is in at the beginning of the test phase to eliminate those that did not begin in the tile at 22°C of the start phase.
Time to start moving	Time to walk since the beginning of the test phase. Walking is defined as a fly that moves at least one fly distance (25 pixels) within three frames.
First to safe tile (at 22°C)	Register of whether flies move first to the tile at 22°C during the test phase. Indicates if flies select the tile associated with the stimulus in the experimental groups.
Time to safe tile (at 22°C)	Time between the beginning of the test phase and the first moment a fly crosses the border of the safe tile for that test phase.
Speed to safe tile (at 22°C)	Mean speed at which a fly walks towards the safe tile during the test phase. Measured only during the time to safe tile.
Distance to safe tile (at 22°C)	Distance walked before reaching the safe tile during the test phase.
Time in safe tile (at 22°C)	Time inside the safe tile during the test phase. If a fly left this tile and came back both moments are added for the total time.
First to close tile	Register of whether flies move to the closest tile relative to their position in the start tile once the test phase has begun.
First to previously safe tile	Register of whether flies move to the tile that was safe in the previous test phase during the current test phase. This measurement does not exist for the first test phase.

Supplementary Figure 1 Motility measurements. Flies start each test phase in the middle tile, most commonly standing still (no displacement for 5 frames). Their Start Position can be closer to the left side tile or the right-side tile depending on which half of the middle tile they are standing in. Flies are considered to be moving once they displace one fly body distance (25px in 3 frames). Once the centroid on top of the fly crosses the border of a side tile the fly is considered to be in the Safe or Unsafe tile as first choice. The Time to Safe tile is counted from the moment the fly starts moving until the centroid crosses the border of the Safe

tile. The Time in Safe tile adds all the moments the fly step inside the Safe tile after having step outside of it. Table with all measurements used for data analysis.

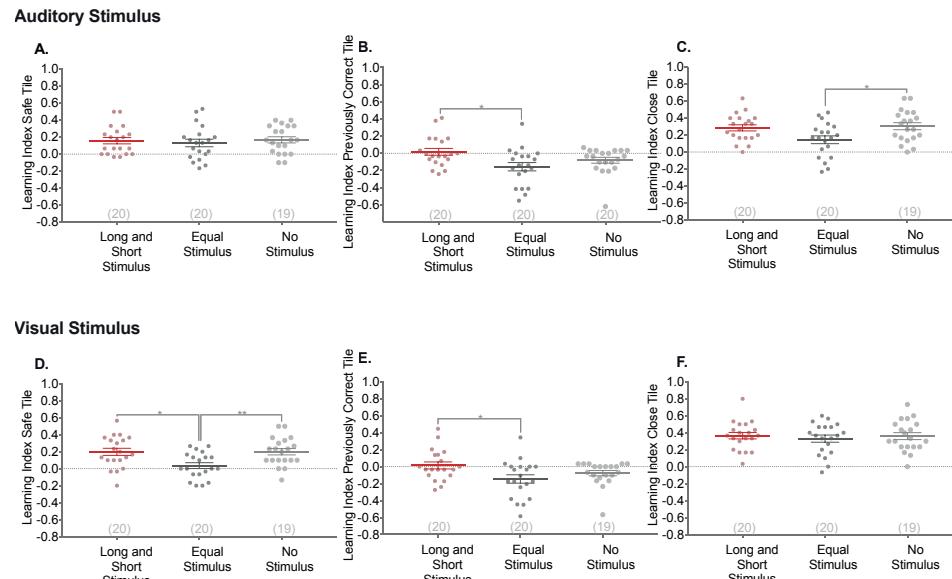


Supplementary Figure 2 Learning index of flies going first to the safe tile through the 30 test phases. A. Learning index of CS flies exposed to long and short, equal or no auditory stimulus within *Protocol 1* going to the safe tile first. B. Learning index of CS flies exposed to long and short, equal or no visual stimulus within *Protocol 1* going to the safe tile first. Data are mean \pm s.e.m. Asterisks indicate significant differences between groups ($*p>0.05$, $**p>0.01$, $***p>0.001$, $****p>0.0001$; Tukey multiple comparisons test).

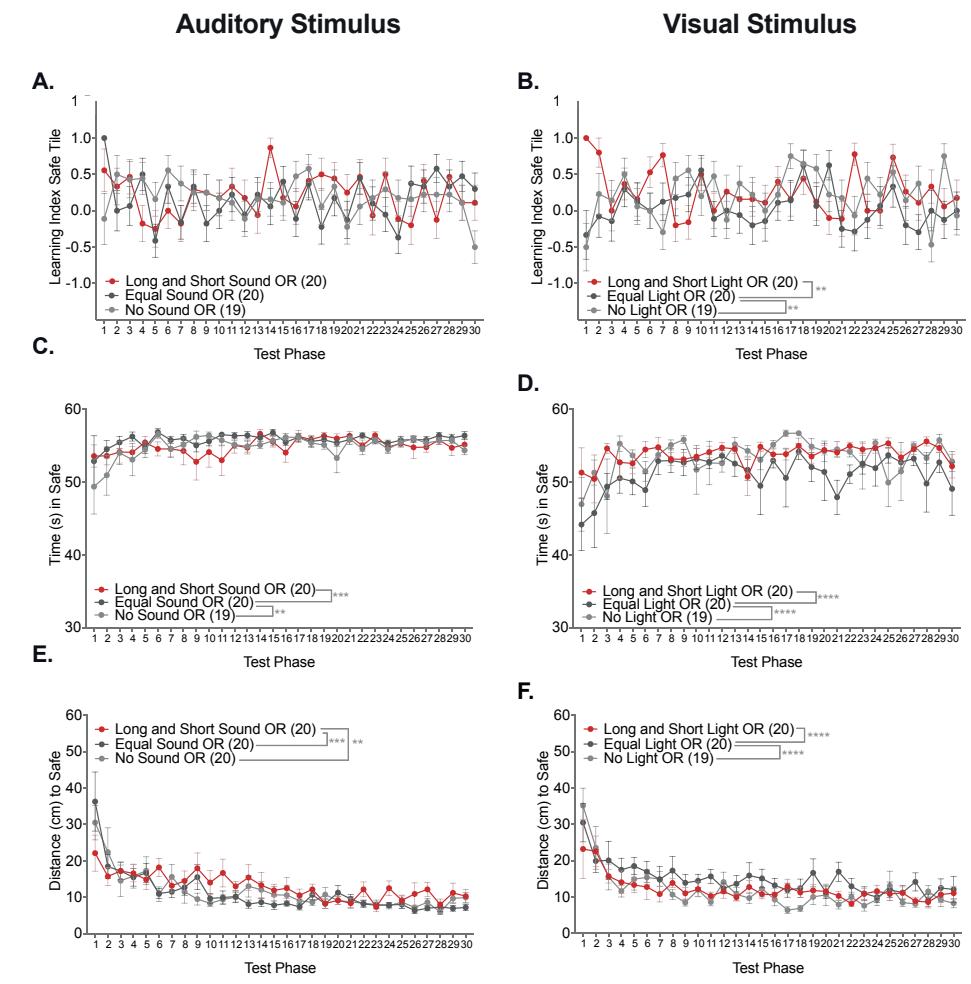


Supplementary Figure 3 Time to safe tile, time to start moving, and speed to safe tile of CS flies. A. Time to reach safe tile of CS flies exposed to long and short, equal or no auditory stimulus within *Protocol 1*. B. Time to reach safe tile of CS flies exposed to long and short, equal or no visual stimulus within *Protocol 1*. C. Time to reach safe tile of CS flies exposed to congruent, incongruent, or non-informative

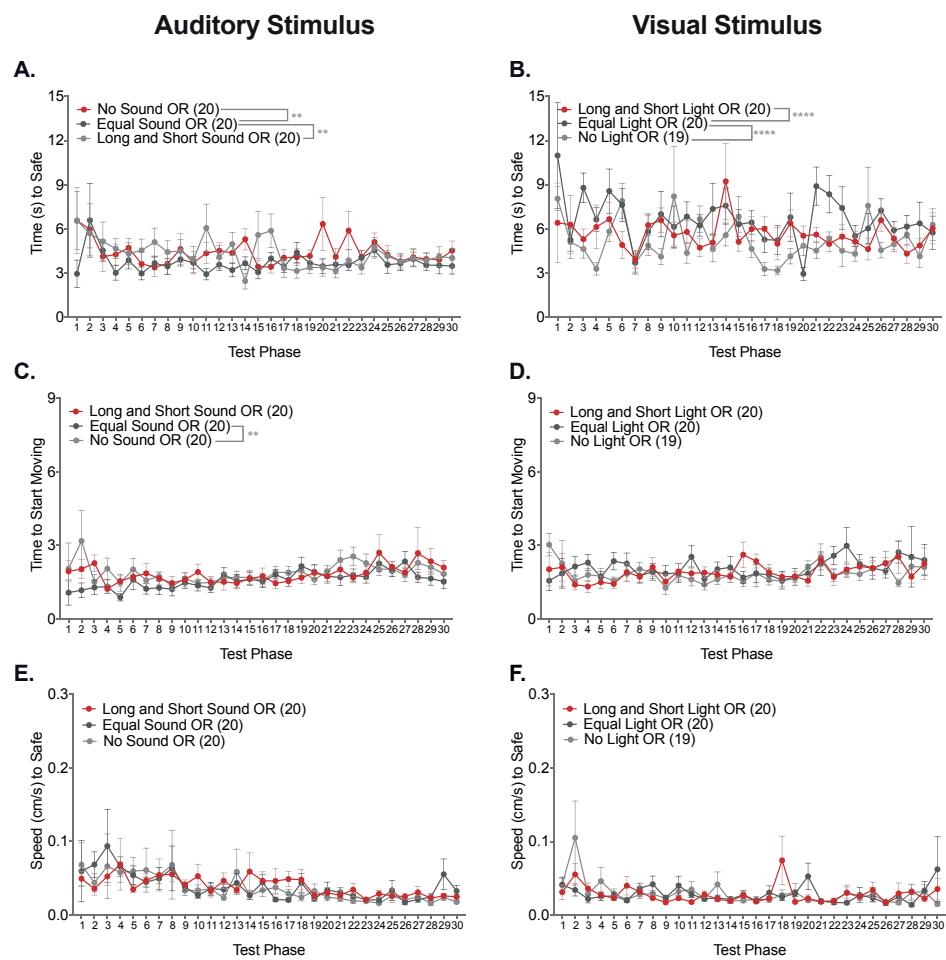
conditions within *Protocol 2*. **D**. Time to start moving of CS flies exposed to long and short, equal or no auditory stimulus within *Protocol 1*. **E**. Time to start moving of CS flies exposed to long and short, equal or no visual stimulus within *Protocol 1*. **F**. Time to start moving of CS flies exposed to congruent, incongruent, or non-informative conditions within *Protocol 2*. **G**. Speed to safe tile of CS flies exposed to long and short, equal or no auditory stimulus within *Protocol 1*. **H**. Speed to safe tile of CS flies exposed to long and short, equal or no visual stimulus within *Protocol 1*. **I**. Speed to safe tile of CS flies exposed to congruent, incongruent, or non-informative conditions within *Protocol 2*. Data are mean \pm s.e.m. Asterisks indicate significant differences between groups (* $p>0.05$, ** $p>0.01$, *** $p>0.001$, **** $p>0.0001$; Tukey multiple comparisons test).



Supplementary Figure 4 Learning index of flies going to the safe tile first, the closest tile first, or the previously safe tile first of OR flies. **A.** Learning index of OR flies that went to the safe tile first after being exposed to long and short, equal or no auditory stimulus within *Protocol 1* (One-way ANOVA: $F_{2,57} = 0.230$, $p=0.796$). **B.** Learning index of OR flies that went to the closest tile first after being exposed to long and short, equal or no auditory stimulus within *Protocol 1* (One-way ANOVA: $F_{2,57} = 4.466$, $p=0.016$, * $p<0.05$). **C.** Learning index of OR flies that went to the previously safe tile first after being exposed to long and short, equal or no auditory stimulus within *Protocol 1* (One-way ANOVA: $F_{2,57} = 4.374$, $p=0.017$, * $p<0.05$). **D.** Learning index of OR flies that went to the safe tile first after being exposed to long and short, equal or no visual stimulus within *Protocol 1* (One-way ANOVA: $F_{2,57} = 6.141$, $p=0.004$, * $p<0.05$, ** $p<0.01$). **E.** Learning index of OR flies that went to the closes tile first after being exposed to long and short, equal or no visual stimulus within *Protocol 1* (One-way ANOVA: $F_{2,57} = 0.220$, $p=0.803$). **F.** Learning index of OR flies that went to the previously safe tile first after being exposed to long and short, equal or no visual stimulus within *Protocol 1* (One-way ANOVA: $F_{2,57} = 3.697$, $p=0.031$, * $p<0.05$). Data are mean \pm s.e.m.



Supplementary Figure 5 Learning index to safe tile first during 30 trials, time in safe tile, and distance walked to safe tile of OR flies. **A.** Learning index of OR flies exposed to long and short, equal or no auditory stimulus within *Protocol 1* going to the safe tile first. **B.** Learning index of OR flies exposed to long and short, equal or no visual stimulus within *Protocol 1* going to the safe tile first. **C.** Time inside safe tile of OR flies exposed to long and short, equal or no auditory stimulus within *Protocol 1*. **D.** Time inside safe tile of OR flies exposed to long and short, equal or no visual stimulus within *Protocol 1*. **E.** Distance to safe tile of OR flies exposed to long and short, equal or no auditory stimulus within *Protocol 1*. **F.** Distance to safe tile of OR flies exposed to long and short, equal or no visual stimulus within *Protocol 1*. Data are mean \pm s.e.m. Asterisks indicate significant differences between groups (* $p>0.05$, ** $p>0.01$, *** $p>0.001$, **** $p>0.0001$; Tukey multiple comparisons test).



Supplementary Figure 6 Time to safe tile, time to start moving, and speed to safe tile of OR flies. **A.** Time to reach safe tile of OR flies exposed to long and short, equal or no auditory stimulus within *Protocol 1*. **B.** Time to reach safe tile of OR flies exposed to long and short, equal or no visual stimulus within *Protocol 1*. **C.** Time to start moving of OR flies exposed to long and short, equal or no auditory stimulus within *Protocol 1*. **D.** Time to start moving of OR flies exposed to long and short, equal or no visual stimulus within *Protocol 1*. **E.** Speed to safe tile of OR flies exposed to long and short, equal or no auditory stimulus within *Protocol 1*. **F.** Speed to safe tile of OR flies exposed to long and short, equal or no visual stimulus within *Protocol 1*. Data are mean \pm s.e.m. Asterisks indicate significant differences between groups (* $p>0.05$, ** $p>0.01$, *** $p>0.001$, **** $p>0.0001$; Tukey multiple comparisons test).

	Predictor	<i>Protocol 1: Left and Right</i>		<i>Protocol 2</i>	
		Auditory Stimulus	Visual Stimulus	Congruent and Incongruent	
Times in Safe	Condition	2.03 (2, 1527)	0.131	4.097 (2, 1418)	0.017
	Phase	41.11 (1, 1527)	<.0001	0.187 (1, 1418)	0.665
	Condition x Phase	0.27 (2, 1527)	0.762	8.501 (2, 1418)	<.0001
Distance to Safe	Condition	5.893 (2, 1525)	0.003	4.121 (2, 1397)	0.016
	Phase	187.398 (1, 1525)	<.0001	75.628 (1, 1397)	<.0001
	Condition x Phase	2.043 (2, 1525)	0.130	0.616 (2, 1397)	0.540
Times to Safe	Condition	0.946 (2, 1525)	0.389	1.619 (2, 1401)	0.198
	Phase	10.408 (1, 1525)	0.001	1.318 (1, 1401)	0.251
	Condition x Phase	0.124 (2, 1525)	0.884	0.107 (2, 1401)	0.899
Time to Start Moving	Condition	7.508 (2, 1525)	0.001	2.774 (2, 1411)	0.063
	Phase	21.889 (1, 1525)	<.0001	3.569 (1, 1411)	0.059
	Condition x Phase	0.048 (2, 1525)	0.953	0.233 (2, 1411)	0.792
Speed to Safe	Condition	3.081 (2, 1525)	0.046	0.173 (2, 1397)	0.841
	Phase	40.102 (1, 1525)	<.0001	3.860 (1, 1397)	0.050
	Condition x Phase	2.547 (2, 1525)	0.079	0.287 (2, 1397)	0.750
First to Safe	Condition	4.527 (2, 1527)	0.011	0.8 (2, 1418)	0.450
	Phase	0.065 (1, 1527)	0.799	1.737 (1, 1418)	0.188
	Condition x Phase	0.011 (2, 1527)	0.989	0.587 (2, 1418)	0.556

Supplementary Table 1 Main linear-mixed effects model to predict effect of Condition, Phase and their interaction over motility measurements of CS flies: Omnibus test results.

		Protocol 1: Left and Right							
		Auditory Stimulus				Visual Stimulus			
		Predictor	b	SE	t test	p	b	SE	t test
Time in Safe	Condition: Equal Tone	-0.528	0.579	-0.913	0.362	2.238	1.081	2.070	0.039
	Condition: Long and Short Tones	-0.199	0.581	-0.343	0.732	0.054	1.065	0.050	0.960
	Phase	0.090	0.022	4.019	0.000	0.067	0.042	1.581	0.114
	Condition Equal Tone x Phase	0.002	0.032	0.066	0.948	-0.204	0.061	-3.356	0.001
	Condition Long and Short Tones x Phase	-0.019	0.032	-0.609	0.543	0.027	0.060	0.450	0.653
Distance to Safe	Condition Equal Tone	-2.055	0.920	-2.234	0.026	1.084	0.928	1.169	0.243
	Condition: Long and Short Tones	-1.271	0.934	-1.360	0.174	-0.062	0.961	-0.065	0.949
	Phase	-0.499	0.036	-13.691	0.000	-0.325	0.038	-8.638	0.000
	Condition Equal Tone x Phase	0.068	0.051	1.335	0.182	-0.047	0.053	-0.901	0.368
	Condition Long and Short Tones x Phase	0.033	0.052	0.642	0.521	0.055	0.054	1.016	0.310
Time to Safe	Condition: Equal Tone	-0.111	0.210	-0.527	0.598	0.173	0.348	0.497	0.619
	Condition: Long and Short Tones	0.005	0.213	0.024	0.981	0.002	0.360	0.006	0.995
	Phase	-0.027	0.008	-3.214	0.001	0.016	0.014	1.164	0.245
	Condition Equal Tone x Phase	-0.002	0.012	-0.149	0.882	0.000	0.020	0.023	0.982
	Condition Long and Short Tones x Phase	0.006	0.012	0.485	0.627	0.008	0.020	0.385	0.700
Time to Start Moving	Condition: Equal Tone	-0.043	0.127	-0.339	0.735	0.282	0.291	0.967	0.334
	Condition: Long and Short Tones	0.251	0.128	1.959	0.050	-0.102	0.300	-0.341	0.733
	Phase	0.023	0.005	4.675	0.000	0.022	0.012	1.912	0.056
	Condition Equal Tone x Phase	-0.001	0.007	-0.164	0.869	-0.002	0.017	-0.118	0.906
	Condition Long and Short Tones x Phase	-0.001	0.007	-0.143	0.886	0.011	0.017	0.639	0.523
Speed to Safe	Condition: Equal Tone	-0.014	0.008	-1.846	0.065	0.001	0.012	0.111	0.912
	Condition: Long and Short Tones	-0.011	0.008	-1.424	0.155	0.006	0.013	0.460	0.645
	Phase	-0.002	0.000	-6.326	0.000	-0.001	0.000	-1.993	0.047
	Condition Equal Tone x Phase	0.001	0.000	1.178	0.239	0.000	0.001	0.169	0.866
	Condition Long and Short Tones x Phase	0.000	0.000	1.075	0.283	-0.001	0.001	-0.723	0.470
First to Safe	Condition: Equal Tone	-0.068	0.064	-1.067	0.286	0.068	0.067	1.014	0.311
	Condition: Long and Short Tones	-0.080	0.064	-1.246	0.213	0.043	0.066	0.648	0.517
	Phase	0.000	0.002	-0.068	0.946	-0.001	0.003	-0.288	0.773
	Condition Equal Tone x Phase	-0.001	0.004	-0.144	0.885	-0.004	0.004	-0.977	0.329
	Condition Long and Short Tones x Phase	0.000	0.004	-0.031	0.975	0.000	0.004	-0.070	0.945

Supplementary Table 2 Main linear-mixed effects model to predict effect of Condition, Phase and their interaction over motility measurements of CS flies within *Protocol 1*.

		Protocol 2			
		Congruent and Incongruent			
Predictor	b	SE	t test	p	
Time in Safe	Condition: Congruent	-2.907	1.363	-2.133	0.033
	Condition: Incongruent	0.679	1.316	0.516	0.606
	Phase	-0.001	0.055	-0.023	0.981
	Condition Congruent x Phase	0.141	0.077	1.837	0.067
	Condition Incongruent x Phase	-0.196	0.076	-2.583	0.010
Distance to Safe	Condition: Congruent	-4.473	1.507	-2.968	0.003
	Condition: Incongruent	6.552	1.541	4.253	0.000
	Phase	-0.376	0.061	-6.150	0.000
	Condition Congruent x Phase	0.153	0.087	1.763	0.078
	Condition Incongruent x Phase	-0.265	0.086	-3.066	0.002
Time to Safe	Condition: Congruent	-0.066	0.235	-0.282	0.778
	Condition: Incongruent	-0.304	0.240	-1.267	0.206
	Phase	0.010	0.010	1.080	0.281
	Condition Congruent x Phase	0.006	0.014	0.416	0.678
	Condition Incongruent x Phase	0.000	0.013	-0.016	0.987
Time to Start Moving	Condition: Congruent	0.149	0.324	0.459	0.646
	Condition: Incongruent	-0.330	0.331	-0.998	0.318
	Phase	0.025	0.013	1.901	0.058
	Condition Congruent x Phase	-0.011	0.019	-0.576	0.565
	Condition Incongruent x Phase	0.002	0.018	0.131	0.896
Speed to Safe	Condition: Congruent	0.006	0.022	0.272	0.786
	Condition: Incongruent	0.026	0.023	1.139	0.255
	Phase	-0.001	0.001	-0.977	0.329
	Condition Congruent x Phase	-0.001	0.001	-0.917	0.359
	Condition Incongruent x Phase	-0.001	0.001	-0.827	0.409

Supplementary Table 3 Main linear-mixed effects model to predict effect of Condition, Phase and their interaction over motility measurements of CS flies within *Protocol 2*.

		Protocol 1				
		Auditory Stimulus		Visual Stimulus		
Predictor		F test	p	F test	p	
Times in Safe	Stimulus	9.09 (2, 1498)	0.000	24.892 (2, 1454)	<.0001	
	Phase	16.35 (1, 1498)	0.000	4.135 (1, 1454)	0.042	
	Stimulus x Phase	1.6 (2, 1498)	0.203	0.211 (2, 1454)	0.810	
Distance to Safe	Stimulus	10.596 (2, 1497)	<.0001	19.171 (2, 1436)	<.0001	
	Phase	166.661 (1, 1497)	<.0001	81.055 (1, 1436)	<.0001	
	Stimulus x Phase	0.896 (2, 1497)	0.408	1.794 (2, 1436)	0.167	
Times to Safe	Stimulus	8.232 (2, 1498)	0.000	16.947 (2, 1440)	<.0001	
	Phase	1.037 (1, 1498)	0.309	10.034 (1, 1440)	0.002	
	Stimulus x Phase	2.134 (2, 1498)	0.119	0.856 (2, 1440)	0.425	
Time to Start Moving	Stimulus	5.083 (2, 1492)	0.006	0.870 (2, 1439)	0.419	
	Phase	4.170 (1, 1492)	0.041	0.039 (1, 1439)	0.843	
	Stimulus x Phase	0.971 (2, 1492)	0.379	0.699 (2, 1439)	0.497	
Speed to Safe	Stimulus	1.835 (2, 1497)	0.160	0.005 (2, 1436)	0.995	
	Phase	65.12 (1, 1497)	<.0001	7.659 (1, 1436)	0.006	
	Stimulus x Phase	3.645 (2, 1497)	0.026	2.576 (2, 1436)	0.077	
First to Safe	Stimulus	0.367 (2, 1498)	0.693	5.991 (2, 1454)	0.003	
	Phase	0.051 (1, 1498)	0.821	0.322 (1, 1454)	0.571	
	Stimulus x Phase	2.64 (2, 1498)	0.072	1.006 (2, 1454)	0.366	

Supplementary Table 4 Main linear-mixed effects model to predict effect of Condition, Phase and their interaction over motility measurements of OR flies: Omnibus test results.

		Protocol 1: Left and Right							
		Auditory Stimulus			Visual Stimulus				
Predictor		b	SE	t test	p	b	SE	t test	p
Time in Safe	Condition: Equal Tone	1.728	0.539	3.206	.001	-2.113	1.047	-2.017	.044
	Condition: Long and Short Tones	0.259	0.542	0.477	0.633	0.752	1.041	0.722	0.470
	Phase	0.074	0.021	3.474	.001	0.066	0.042	1.580	0.114
	Condition Equal Tone x Phase	-0.052	0.030	-1.765	0.078	-0.038	0.059	-0.643	0.520
	Condition Long and Short Tones x Phase	-0.019	0.030	-0.641	0.522	-0.014	0.058	-0.242	0.809
Distance to Safe	Condition: Equal Tone	-0.459	0.676	-0.679	0.497	-0.409	0.728	-0.562	0.574
	Condition: Long and Short Tones	-0.240	0.672	-0.356	0.722	2.471	0.732	3.376	.001
	Phase	-0.340	0.026	-12.880	.000	-0.262	0.029	-9.057	.000
	Condition Equal Tone x Phase	0.002	0.037	0.063	0.950	-0.050	0.041	-1.213	0.225
Time to Safe	Condition: Long and Short Tones x Phase	-0.044	0.037	-1.195	0.232	-0.025	0.042	-0.611	0.541
	Condition: Equal Tone	0.197	0.242	0.812	0.417	-0.596	0.372	-1.603	0.109
	Condition: Long and Short Tones	-0.837	0.241	-3.475	.001	1.362	0.373	3.647	.000
	Phase	-0.010	0.009	-1.060	0.289	-0.048	0.015	-3.218	.001
	Condition Equal Tone x Phase	0.000	0.013	-0.025	0.980	-0.002	0.021	-0.108	0.914
Time to Start Moving	Condition: Long and Short Tones x Phase	0.024	0.013	1.811	0.070	-0.022	0.021	-1.058	0.290
	Condition: Equal Tone	0.767	0.333	2.306	.021	0.048	0.383	0.126	0.900
	Condition: Long and Short Tones	-0.780	0.331	-2.356	.019	-0.163	0.382	-0.426	0.670
	Phase	0.026	0.013	2.020	.044	-0.003	0.015	-0.180	0.857
	Condition Equal Tone x Phase	-0.020	0.018	-1.063	0.288	-0.013	0.021	-0.603	0.547
Speed to Safe	Condition: Long and Short Tones x Phase	0.024	0.018	1.308	0.191	0.025	0.021	1.183	0.237
	Condition: Equal Tone	-0.007	0.006	-1.287	0.198	0.006	0.003	1.735	0.083
	Condition: Long and Short Tones	0.016	0.006	2.911	.004	-0.006	0.004	-1.802	0.072
	Phase	-0.002	0.000	-8.013	.000	0.000	0.000	-2.717	.007
	Condition Equal Tone x Phase	0.000	0.000	0.457	0.647	0.000	0.000	-1.987	.047
First to Safe	Condition: Long and Short Tones x Phase	-0.001	0.000	-2.542	.011	0.000	0.000	1.970	0.049
	Condition: Equal Tone	-0.155	0.066	-2.358	.019	-0.028	0.067	-0.415	0.678
	Condition: Long and Short Tones	-0.088	0.066	-1.325	0.185	0.066	0.067	0.992	0.322
	Phase	-0.005	0.003	-1.900	0.058	0.002	0.003	0.836	0.403
	Condition Equal Tone x Phase	0.008	0.004	2.265	.024	-0.005	0.004	-1.202	0.230
	Condition: Long and Short Tones x Phase	0.005	0.004	1.473	0.141	-0.005	0.004	-1.257	0.209

Supplementary Table 5 Main linear-mixed effects model to predict effect of Condition, Phase and their interaction over motility measurements of OR flies within *Protocol 1*.



General Synthesis

Andrea Soto Padilla

Environmental factors, such as light, humidity and temperature, apply selective pressures that determine what types of organisms can thrive in different ecosystems. When environments change, as when mean temperatures increase, organisms are forced to adapt to the new conditions to survive. For example, the higher temperatures brought by the recent acceleration in climate change led species of birds, squirrels, and flowering plants to breed and bloom earlier in the year (Dietzl et al., 2007; Nussey et al., 2005; Réale et al., 2003), whilst species of mosquitos have began to remain active for longer before going dormant for the winter (Bradshaw and Holzapfel, 2001). Meanwhile, extreme weather events (temperatures above a given threshold for an area; in Coumou and Rahmstorf, 2012), caused by the shifting climate, have produced the death of desert bird species, coral reefs, and trees (Allen et al., 2010; Hughes et al., 2003; McKechnie and Wolf, 2010).

Temperature changes have a dramatic influence on organisms' survival because temperature directly affects the rate at which enzymatic reactions occur. Enzymes are the main catalyst of all biochemical reactions required for life; lower temperatures produce slower enzymatic activity, while higher temperature lead to faster enzymatic action (Santhosh, 2018). However, enzymes are incapable of working at all temperatures; below a minimum temperature enzymes will lack sufficient energy to produce a biochemical reaction, while beyond a maximum temperature, enzymes denaturalize and decouple from their substrates, preventing them from working (Huey and Kingsolver, 1989; Martinez del Rio and Karasov, 2010). This implies that organisms are restricted to spending their lives within temperature ranges at which their enzymes can function to ensure their survival (Martin and Huey, 2008). As temperature in the wild fluctuates through multiple time scales, from seasonal changes to daily thermal variations (Colinet et al., 2015), organisms have evolved two main mechanisms to maintain their body temperature within the permissive limits: endotherms, on the one hand, use physiological processes to actively regulate their body temperature; ectotherms, on the other hand, adapt to thermal variations using behavioural responses through which they seek areas that are in their temperature tolerance range (Abram et al., 2017).

Ectotherms are divided in large and small organisms. Large ectotherms, such as lizards and turtles, have a large body mass that delays the effects of heating and cooling and allows them to tolerate abrupt temperature changes without immediate metabolic consequence (Stevenson, 1985). Meanwhile, small ectotherms such as insects acquire the temperature of their environment almost instantly (Angilletta, 2006; Stevenson, 1985), which quickly translates into metabolic and behavioural changes. For example, temperature predicts ants' walking speed with such accuracy that ant locomotion can be used to determine how warm it is (Martinez del Rio and Karasov, 2010). The close relationship between temperature, biochemical reactions, and insect behaviour, permits using principles of enzyme kinetics to represent insect's thermal performance curves (Logan et al., 1976). This could create the impression that insect behaviour is no more than the reflection of the direct effect of temperature on the rate of their biochemical reactions. However, a closer inspection of the insect world has revealed examples that question this conclusion: triatomid bugs and migratory locusts change their temperature preference according to their nutritional status (Coggan et al., 2011), probably to increase the assimilation of certain nutrients at a more efficient thermal range (Clissold et al., 2013); some species of cockroaches prefer colder environments when environmental humidity is low (Deal, 1941; Gunn, 1933), indicating a combined effect of moisture and thermal information; and worker ants deprived of certain nutrients seek lower temperatures (Porter and Tschinkel, 1993), most likely to increase their longevity when resources are limited. These observations suggest that insects combine external and internal stimuli to control their behavioural response to temperature (Gallio et al., 2011), and that they are not simple victims of changes in enzymatic processes. Most likely, the final behavioural output is a combination of the direct effect of temperature over insect's biochemical reactions and their capacity to integrate this effect with other physiological inputs to coordinate their response.

The work presented here tested the capacity of *Drosophila melanogaster* to adapt to diverse temperature challenges. The fly has been a fundamental research tool for over a hundred years (Bellen et al., 2010), helping us elucidate the mechanisms behind heredity, development of the nervous system, behavioural regulation, and even social interactions (Bellen et al., 2010; Ramdya et al., 2017). Temperature studies have shown that flies possess a complex temperature sensing system, with central and peripheral receptors dedicated to particular temperature ranges or rates of temperature change (Frank et al., 2015; Gallio et al., 2011; Hamada et al., 2008; Luo et al., 2017; Ni et al., 2013; Tang et al., 2013). Flies also possess a neural substrate where temperature information appears to be integrated with other stimuli to regulate their behavioural response (Frank et al., 2017; Gallio et al., 2011). These findings mean that *Drosophila* can be used as model to understand how small ectotherms process temperature information to guide their behaviour, and to predict how climate change might affect the manner in which these organisms react. To test *Drosophila*'s response to thermal challenges, a device capable of fast and precise temperature changes controlled in time and space, where flies do not require frequent manipulation, was necessary. Development and implementation of such a device is presented in **Chapter 2** as a new temperature-controlled arena. In **Chapter 3**, flies were exposed to gradually increasing temperatures within this arena to explore the importance of their thermosensors in their locomotor response to temperature. Wild-type flies moved faster as temperature increased beyond their maximum threshold of comfortable temperatures ($>27^{\circ}\text{C}$), until a

maximum point of performance ($\sim 36^{\circ}\text{C}$) was reached, after which their speed decreased. Meanwhile, flies lacking central thermosensors did not increase speed at any temperature, while flies lacking a peripheral thermosensor increased speed but at a lower rate and maximum than wild-type flies. These data suggest that a functioning thermosensory system is required for flies to respond to changing temperatures, and confirms that insect's behavioural response to temperature is not based on a passive biochemical effects but on neural regulation. Nonetheless, future studies should consider exploring the biochemical effects of the thermosensory receptors related to temperature changes. Even though the final behavioural output of flies is linked to the cognitive handling of the temperature information, it is reasonable to expect that the function of the thermosensors directly relates to their biochemical response to changing temperatures. This would open the door to exploring how biochemical information is translated to cognitive processing and help understand the development and evolution of thermal adaptation.

Cognitive control over the temperature response allows *Drosophila* to regulate their thermal reaction according other relevant environmental factors. **Chapter 4** shows that male and female flies tested alone have similar temperature response curves. However, when surrounded by same-sex peers, individual male flies increased their speed at high temperature ($34\text{--}38^{\circ}\text{C}$), while female flies remain at a similar speed as females tested alone. Surprisingly, this sexual dimorphism depends on the perceived sex of the other group members: when male flies are modified to express female pheromones (identifying them as females to others), they stop increasing their speed in the presence of others, while females made to express male pheromones move faster when tested in a group. As the feminization and masculinization processes affects only the pheromones produced by the flies but not their internal state, it is safe to conclude that it is the perceived sex of the others that conditions this sexually dimorphic difference. One of the reasons behind this divergence between the sexes is the amount of interactions sought by male and female flies at high temperatures: females contact each other more often than males. Mutant females lacking mechanosensory receptors move faster at high temperatures than their wild-type counterparts, which suggests that touching each other reduces the intensity of the stress response of normal female flies. Mechanosensory mutant females also increase social interactions when exposed to high temperatures, which further supports that social contact is important for females to deal with stress, at least the one brought by increasing temperature. Interestingly, this resembles the response to stress of males and females of mammalian species: rodent, non-human primates, and human females have a strong inclination for seeking others when stressed (tend-and-befriend), while males often follow a fight-or-flight pattern (Genovesio et al., 2015; Taylor et al., 2000). Analyses of the functional and neurochemical patterns between males and females of mammalian species have shown that the larger concentrations of oxytocin and oxytocin receptors of females could produce a stronger natural drive to seek others, which could be exacerbated during stress as higher concentrations of oxytocin reduce the impact of the stress response (Taylor et al., 2000). *Drosophila* do not produce oxytocin; nonetheless, flies hold a sexually differentiated stress response system (Neckameyer and Nieto, 2015) that produces sexually specific changes in dopamine concentrations (Argue and Neckameyer, 2013). As the dopaminergic and oxytocin brain circuits are intimately integrated in mammals (Baskerville and Douglas, 2010; Love, 2014), it is possible that a common dopaminergic pathway could explain the sexually dimorphic response to stress of *Drosophila* and

mammalian grouped females and males. *Drosophila* possess a known dopaminergic system with multiple markers for specific dopaminergic clusters (Mao and Davis, 2009; Xie et al., 2018), which implies that the system presented here can be used to investigate the mechanisms linking the sexually dimorphic response to stress and its interaction with social context.

Temperature affects not only how *Drosophila* respond to their immediate environment, but also how fly species distribute around the world (Jezovit et al., 2017; Kellermann et al., 2012). Different fly species have adjusted their thermal tolerance to the temperature range of the environment in which they exist, which has led to species-specific reaction to the same thermal challenge, as illustrated in *Figure 3* of **Chapter 2**. Flies' adaptation to a particular temperature range suggest that some species might face an impossible challenge when exposed to the increasing temperatures worldwide due to climate change. In fact, climate change is considered one of the main contributors of insects' population loss in the past few decades and a predicted factor of future decline (Sánchez-Bayo and Wyckhuys, 2019). Nevertheless, *Drosophila* might be able to quickly adapt to the changes around the world by transgenerational effects in which parents equip their offspring to better face future challenges. For example, flies from parents raised at 25°C or 29°C showed higher fitness than offspring from parents kept at 18°C (Gibert et al., 2001), while offspring from parents kept at either 18°C or 29°C survived more often when kept at the same temperature of their parents than when placed in the opposite environment (Mohan et al., 2018). These data suggest that *Drosophila* is able to use temperature information of their current environment as a cue to affect the development of their offspring and better prepare them for the environment they will face. At the same time, flies possess physiological mechanisms to respond to thermal fluctuations of daily life that do not require parental influence (Colinet et al., 2015). For example, flies exposed to brief periods of high temperature overproduced heat-shock proteins, which allows them to tolerate future periods of thermal stress (Roberts et al., 2003), while flies exposed to short moments of coldness have a metabolic transition from forming glycogen storages to increasing triglyceride stores, which augments their cold resistance (Marshall and Uller, 2007). These data suggest that individual phenotypic plasticity is sufficient to allow flies to adjust to new temperature environments, irrespective of the experience of their parents. As shown in the match and mismatch design of **Chapter 5**, in which mothers were exposed to 29°C (hot) or 18°C (cool) and their offspring developed at the same temperature or at the other temperature, the main contributor to a fly's response to thermal challenges is the environment in which that fly developed. Flies that grew in a hot environment recovered faster from heat-shock and had an overall faster movement, probably linked to a faster metabolism, than flies from a cool environment. Meanwhile, flies from a cool environment recovered faster from cold-shock than flies from a hot environment, demonstrating a greater cold resistance. However, offspring from 29°C mothers move faster in the climbing test and when exposed to gradually increasing temperatures, while offspring from 18°C mothers recovered faster after cold- or heat-shock, regardless of offspring environment. This suggests that maternal influence played a role in determining the fly's capacity to respond to temperature challenges. Maternal influence could have emerged from an anticipatory mechanism from the mothers after their sudden exposure to 29°C or 18°C; conversely, it could have arisen as a carry-over effect of physiological changes occurring in the mothers that allowed them to produce better or worse quality

eggs. Future studies should focus in segmenting these two possibilities by analyzing genetic changes in mothers and offspring and metabolic differences between flies from either environment. It would also be advisable to observe offspring at different development stages, as maternal effects could be stronger during early larvae stages (24–48 hours after egg-laying), where individuals may not yet have a formed thermosensory system and may still rely on accurate information from their parents. Another interesting angle would be to analyze fly species adapted to diverse climate niches, as cosmopolitan species, such as the ones used in **Chapter 5**, might depend more on environmental influence to determine their temperature tolerance while highly specialized lineages might have a stronger transgenerational component. Describing the details behind transgenerational and individual effects in diverse fly species could help predict how insects will adapt to climate change and how the current biodiversity might change in the coming decades.

The fast adaptation of *Drosophila melanogaster* to changing temperatures suggests that temperature changes could be used as particularly salient stimuli to explore other behaviours. In fact, flies' response to temperature has been used to explore the genetic basis of memory and learning (Ofstad et al., 2011; Putz and Heisenberg, 2002; Wustmann and Heisenberg, 1997; Wustmann et al., 1996; Zars and Zars, 2006; Zars et al., 2000), which suggests that other cognitive skills could be explored based on thermal response. In **Chapter 6**, flies were exposed to long and short visual or auditory stimuli paired with temperature changes to condition them to move to a particular area based on the presented stimulus. The intention was to demonstrate that flies could differentiate between short and long durations and hence that they could be used as a model of short-range time perception, known as interval timing. Interval timing is a fundamental component of learning, decision-making, and numerous other cognitive functions (Buhusi and Meck, 2005; Matell and Meck, 2000), neural bases of which still remain unknown. *Drosophila* could have served as a model of the wiring diagram at the single neuronal level (Kohl and Jefferis, 2011) of interval timing had they demonstrated to possess this skill. However, although flies demonstrated an ability to follow the temperature gradients they were exposed to, they did not show interval timing. This does not imply that *Drosophila* absolutely lack interval timing capacity, as it could reflect that this paradigm was not ideal to demonstrate this process in the fly. Further attempts could consider using other conditioning stimuli, such as a food reward, or other experimental set-ups, such as observing flying bouts, to fully explore interval timing in *Drosophila* and take advantage of the fly to understand this cognitive process.

Final Remarks

Drosophila melanogaster is a fascinating research organism. Flies permit answering questions from the neuron-by-neuron level to the population perspective, allowing us to explore genetic, physiological, behavioural and evolutionary components of a multitude of processes. Not surprisingly, flies have been fundamental in the understanding of male and female interactions (Billeter et al., 2009; Gorter et al., 2016; Laturney and Billeter, 2014; Yamamoto and Koganezawa, 2013), egg laying (Duménil et al., 2016), circadian rhythms (Yao and Shafer, 2014), sleep cycles (Donlea et al., 2014), learning and memory (Galili et al., 2011), temperature processing (Barbagallo and Garrity, 2015), and even social behaviour (Ramdyia et al., 2015; Schneider et al., 2012). Temperature is a particularly relevant component of a fly's life as it affects all aspects of *Drosophila*'s existence, from their development (Kjærsgaard et al., 2012) and distribution (Jezovit et al., 2017), to their survival (**Chapter 5**) and social communication (**Chapter 4**). Many of these aspects, such as the response to gradually increasing temperature (**Chapter 3**) or to sudden temperature changes (**Chapter 3 and Chapter 6**), can be explored in the new temperature-controlled arena here developed (**Chapter 2**). However, some aspects still require new inventions and innovative approaches to be examined. For example, flying might allow flies to tolerate warmer environments by reducing their exposure to a hot surface and instead exposing them to wind, which increases heat loss. It would be interesting to explore how diverse interventions, such as developmental temperature or maternal environment, affect the rate at which flies fly and land, and what does a change in this rate imply for mating, egg deposition, and overall survival. One can imagine that if flies spend more time flying due to an increase in surface temperatures, their energy expenditure might increase and their opportunities to mate and lay eggs might reduce, leading to shorter lifespans and less successful reproductive attempts, which could translate into the extinction of a species. On the other extreme, flies in colder areas suffering from longer and more intense cold weather could develop new strategies to cope with this challenge, such as grouping more often and for longer periods to keep warm. This could lead to the development of new communications strategies, such as shorter-range pheromones substituting longer-range cues, and to new behaviors between males and females to control the rate of courtship and mating while grouped. It would be fascinating to develop a method in which the mechanisms behind this adaptation could be studied and unraveled.

The work presented here could also be further complemented by an exploration of the genetic and metabolic components of *Drosophila*'s thermal response. Fly larvae that develop in cold or hot environments (13°C - 29°C) have differential gene expression (Chen et al., 2015), which suggests that genetic plasticity plays a fundamental role in conferring flies cold and hot resistance. It is worth exploring if adult flies are capable of the same genetic plasticity, and if these modifications could be inherited by the next generation, which could accelerate offspring adaptation to the impeding climate change. It would also be interesting to investigate whether genetic changes in larvae correlate to changes in adulthood and whether these changes could restrict further adaptability later in life. Differences between species in these aspects could explain why some *Drosophila* inhabit multiple climates while others are restricted to specific temperature ranges. Knowing that some species might not be able to cope with the impeding changes of climate change

could help predict how ecosystems might vary around the world and what consequences this might imply for human development and survival.

This thesis has covered diverse aspects of *Drosophila*'s response to temperature. It has shown that flies must perceive temperature to properly react to it, that temperature affects how flies interact with each other, that the environment of mothers and offspring affects how flies respond to thermal challenges, and that flies can use temperature information to predict where they should move. It has also presented a new temperature-controlled arena that could be exploited to explore similar temperature-related aspects of other insects, or even of larger species if the technical components are properly adapted. Overall, this thesis demonstrates that temperature matters, and that understanding how the fly deals with it is fundamental for a deeper comprehension of this vital environmental factor, but also of principles that might affect other species, such as the impact of climate change, parental effects, and how brains integrate sensory information.

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Summaries

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Nederlandse Samenvatting

Omgevingstemperatuur heeft een direct effect op hoe welvarend organismes zijn. Wanneer het milieu veranderd, zoals bijvoorbeeld de momentele de klimaatopwarming, worden organismes geforceerd zich aan te passen aan de nieuwe condities om te kunnen overleven. In sommige gevallen kunnen organismen hun gedrag aanpassen om te overleven in het nieuwe milieu. Andere organismes zijn echter niet in staat om zich op tijd aan te passen. Dit heeft als resultaat dat gehele soorten zijn uitgestorven, zoals sommige woestijnvogels, koraalriffen en bomen die niet bestendig waren tegen extreme weercondities. Temperatuur is een zeer belangrijke component van adaptatie en overleven, omdat het een direct effect heeft op het tempo waarop enzymatische reacties plaatsvinden. Enzymen en de reacties waar ze deel van uit maken spelen een hoofdrol in alle biochemische reacties. Enzymen kunnen echter alleen hun functie uitvoeren binnen beperkte temperaturen; onder een minimale temperatuur zullen enzymen geen biochemische reactie produceren, terwijl boven een maximale temperatuur enzymen ontbinden, wat ervoor zorgt dat ze niet meer werkzaam zijn. Dit suggereert dat organismes ze gebonden zijn te leven binnen temperatuur limieten om ervoor te zorgen dat enzymen kunnen functioneren.

De meeste organismen houden hun lichaamstemperatuur op peil door middel van twee verschillende mechanismen; aan de ene kant staan de endothermen welke fysieke mechanismen gebruiken om op te warmen of af te koelen; aan de andere kant staan de ectothermen welke afhankelijk zijn van de omgevingstemperatuur om hun eigen lichaamstemperatuur te bepalen. Voor de ectothermen betekent dit dat zij de temperatuur van de omgeving overnemen als hun lichaamstemperatuur en daardoor op zoek moeten gaan naar een plek met de gewenste temperatuur. Grote ectothermen, zoals hagedissen, ondervinden minder problemen in acute temperatuur verschillen, omdat hun lichaamsgewicht het opwarmen of afkoelen vertraagt. Daarentegen veroorzaken acute temperatuurverschillen bij kleine ectothermen, zoals insecten, acute veranderingen in lichaamstemperatuur, wat al snel tot veranderingen in het gedrag en metabolisme leidt door

het effect van temperatuur op de enzymen. In insecten wekt dit de indruk dat de reactie op temperatuur wordt veroorzaakt door het directe effect van temperatuur op enzymatische reacties. Dat zou echter alleen zo zijn als insecten geen complex zintuigssysteem zouden hebben om temperatuur waar te nemen, waarvan al is aangetoond dat ze dit systeem wel hebben. De fruitvlieg, *Drosophila melanogaster*, heeft bijvoorbeeld verscheidene centrale (hersenen) en perifere (antennes) temperatuur receptoren, die allemaal specifiek zijn voor een bepaald temperatuurbereik. Dit samen met de grote diversiteit van biologische vragen die over de laatste honderd jaar zijn beantwoord door het gebruik van *Drosophila*, zoals de mechanismes achter erfelijkheid, ontwikkeling van het zenuwstelsel en zelfs sociale interacties, leidde tot de eerste taak van mijn proefschrift; het ontrafelen of de reactie van fruitvliegen op temperatuur inderdaad alleen werd veroorzaakt door enzymatische processen of dat hun neurale zintuigssysteem voor temperatuur hier ook een rol in speelde.

Om dit te testen, zoals in **Hoofdstuk 2** beschreven, zijn de fruitvliegen blootgesteld aan een temperatuur-gecontroleerde arena, waarin de omgevingstemperatuur geleidelijk maar ook abrupt kon worden veranderd. The arena bestaat uit drie koperen tegels waarvan de temperatuur automatisch van wordt gecontroleerd, een metalen barrière rondom de tegels en een glazen plafon wat voorkomt dat de vliegen kunnen vliegen en ze dus forceert om te lopen. Tijdens de eerste experimenten, beschreven in **Hoofdstuk 3**, werden de fruitvliegen blootgesteld aan geleidelijk toenemende temperaturen om het belang van temperatuur sensoren vast te stellen op de loopsnelheid van de fruitvliegen op verschillende temperaturen. Naarmate de temperatuur steeg gingen normale vliegen steeds sneller lopen tot op een maximumsnelheid (op 36°C) waarna snelheid snel afnam. Vliegen die geen temperatuursensoren hebben in de hersenen lieten geen enkele toename in loopsnelheid zien in reactie op temperatuur wijzigingen, terwijl vliegen zonder temperatuurreceptoren in de antennes wel een toename in loopsnelheid lieten zien, maar niet in dezelfde mate als normale vliegen. Deze resultaten suggereren dat een functionerend temperatuur zintuigssysteem nodig is voor een reactie op toenemende temperaturen, en daarmee ook vaststelt dat de gedragsresponse op temperatuur niet alleen afkomstig is van enzymatische activiteit maar afhankelijk in van neuronale controle.

De cognitieve controle van *Drosophila*'s over hun reactie op temperatuur geeft de mogelijkheid aan dat vliegen niet-thermische en thermische sensorische signalen integreren om hun reactie op temperatuur te moduleren. Een van die signalen zou de aanwezigheid van anderen kunnen zijn. Hiervan is al bekend dat dit invloed heeft op de thermale reactie in verschillende organismes, zoals samenklonteren om warmte te behouden in koude klimaten. In **Hoofdstuk 4** is dit idee onderzocht door de reactie van alleen geteste vliegen te vergelijken met de reactie van vliegen getest in een groep. Vrouwtjes en mannetjes vliegen die alleen zijn getest gedragen zich vergelijkbaar. Mannetjes in een groep met andere mannetjes lieten echter een hogere loopsnelheid zien bij hoge temperaturen, terwijl vrouwtjes getest met andere vrouwtjes bewegen op dezelfde snelheid als de alleen geteste vliegen. Daarnaast lieten mannetjes, die waren gemanipuleerd om vrouwelijke feromonen uit te scheiden, geen hogere loopsnelheid zien in een groep net als normale vrouwtjes. Daarbovenop komt nog dat vrouwtjes, die waren gemanipuleerd om mannelijke feromonen uit te scheiden, wel een hogere loopsnelheid lieten zien op hoge temperaturen net als normale mannetjes. Dit suggereert dat de reactie van vliegen in een groep afhankelijk is van de vliegen waar ze van denken omgeven te zijn.

Dit resultaat kan worden verklaard door de manier waarop vliegen op elkaar reageren. Vrouwtjes die zich omgeven door andere vrouwtjes zoeken constant contact met elkaar, wat niet wordt gezien als vrouwtjes worden gegroepeerd met mannetjes of als alleen mannetjes gegroepeerd zijn. Dit interactieve gedrag tussen vrouwtjes vermindert de intensiteit van hun reactie op toenemende temperaturen, wat mogelijk een weerspiegeling is van een ontspannend effect van aanraking tijdens temperatuur geïnduceerde stress. Dit werd bevestigd doordat vrouwtjes die genen misten die gevoelsreceptoren coderen – waardoor deze vrouwtjes ongevoelig waren voor aanrakingen- een hogere loopsnelheid hebben bij toenemende temperaturen, terwijl ze ook opzoek bleven naar contact. Samen wijst dit er op dat stress veroorzaakt door warmte vrouwtjes motiveert om elkaar op te zoeken en dat de aanrakingen die daaruit volgen een kalmerend effect heeft op hun reactie. Zoogdieren zoals knaagdieren, niet-menselijke primaten en mensen laten interessant genoeg eenzelfde soort fenomeen zien: vrouwen gaan opzoek naar anderen als ze gestrest zijn, terwijl mannen het eenzame vecht of vlucht principe volgen in stressvolle situaties. Meerdere verschillen tussen de hersenen van mannen en vrouwen zijn al gevonden die deze resultaten zouden kunnen verklaren; interessant is dat de processen gerelateerd aan de stressreactie in *Drosophila* anders zijn in vrouwtjes en mannetjes, wat mogelijkheden biedt om de vlieg te gebruiken om meer te weten te komen over de mechanismes achter deze verschillen.

Vliegen kunnen zich ook aanpassen aan veranderende temperatuuromstandigheden door hun nakomelingen beter voor te bereiden op extreme temperaturen. Eerdere studies hebben bijvoorbeeld aangetoond dat de nakomelingen van vliegen levend in een 25°C of 29°C omgeving een hogere fitness hadden dan de nakomelingen van ouders levend in een 18°C omgeving. Dit suggereert dat *Drosophila* hun huidige omgevingstemperatuur kunnen gebruiken als signaal om de ontwikkeling van hun nakomelingen te beïnvloeden en hen beter voor te bereiden op de omgeving waar zij aan blootgesteld zullen zijn (bekend als anticiperende effecten). Dit idee hebben we getest in **Hoofdstuk 5** door de moeders bloot te stellen aan of 29°C (warm) of 18°C (koud) om daarna de nakomelingen zich te laten ontwikkelen in dezelfde temperatuur als de moeder of op de tegenovergestelde temperatuur. We vonden dat de reactie van de nakomelingen op temperatuur voornamelijk werd bepaald door de omgevingstemperatuur waarin de nakomelingen zich hebben ontwikkeld. Dit gezien de vliegen die zijn opgegroeid in de warme conditie sneller herstelden na blootstelling aan een hitte-shock en over het algemeen bewogen zij sneller, terwijl vliegen die zijn opgegroeid in de koude conditie, sneller herstelden na blootstelling aan een koelte-shock, wat een grotere resistentie voor kou aanduidt. De nakomelingen van moeders die in 18°C leefden herstelden zich echter beter dan nakomelingen van moeders die in 29°C leefden, wat laat zien dat er een gering maternaal effect is op de temperatuur response van hun nakomelingen. Dit betekent echter niet dat dat moeders anticiperen op de omgeving van hun nakomelingen en daardoor een doelgerichte verandering veroorzaakten. Dit is waarschijnlijk het gevolg van een overdracht van fysiologische veranderingen van de moeder die was blootgesteld aan 18°C, zoals een langzamer metabolisme wat een hogere hoeveelheid lichaamsvet veroorzaakt wat op zijn beurt weer een verhoogde resistentie voor warme en koude temperaturen met zich meebrengt. Verdere studies zijn nodig om vast te stellen of de maternale effecten daadwerkelijk worden veroorzaakt door een overdracht van fysiologische veranderingen of dat het toch gerelateerd is aan het anticiperen van de moeders. Inzicht hierin kan helpen

voorspellen hoe insecten zich zullen aanpassen aan de klimaatverandering.

De complexe interactie die *Drosophila* laten zien in relatie tot temperatuur suggereert dat temperatuur een opvallende stimulus is die kan helpen ander gedrag te onderzoeken. Met dit in gedachten werden de vliegen in **Hoofdstuk 6** blootgesteld aan korte of lange geluiden en beelden om te onderzoeken of zij dit konden correleren met de locatie van een comfortabele temperatuur. Dit werd gedaan met de intentie te laten zien dat vliegen een verschil konden maken tussen korte en lange tijdsduur en dat zij dus gebruikten konden worden als een diermodel in onderzoek naar een soort tijdsperceptie die bekend staat als intervaltiming. Intervaltiming is een fundamentele component van leren, besluitvorming, en vele andere cognitieve functies in mensen, waarvan de neurale basis tot op heden onbekend is. *Drosophila* hadden als model kunnen fungeren om de neurale circuits die intervaltiming ondersteunen te onderzoeken als zij hadden laten zien deze vaardigheid te bezitten. De vliegen hebben echter niet kunnen laten zien dat ze beschikken over intervaltiming, ondanks het feit dat ze wel in staat zijn om een temperatuurgradiënt te volgen. Dit betekent niet per se dat *Drosophila* niet over intervaltiming beschikken, maar dit toont aan dat de hier gebruikte proefopzet niet de beschikking had om dit te laten zien. Vervolgstudies om intervaltiming in *Drosophila* volledig te exploreren kunnen mogelijk andere geconditioneerde stimuli gebruiken, zoals een voedselbeloning, of andere experimentele proefopstellingen, zoals het observeren van hoe vaak de vliegen beginnen met vliegen.

Afsluitende Opmerkingen

Drosophila melanogaster is een fascinerend organisme om onderzoek mee te doen. Deze vliegen geven de mogelijkheid om vragen te beantwoorden variërend van het neurale niveau tot op populatieperspectief. Hierdoor kunnen genetische, fysiologische, gedrags- en evolutionaire componenten van een groot aantal processen worden onderzocht. Het is dan ook niet verrassend dat tot op heden vliegen fundamenteel zijn geweest in het begrijpen van mannelijke en vrouwelijk interacties, ei leg gedrag, circadiane ritmes, slaap cycli, leren en geheugen en zelf sociaal gedrag. In dit proefschrift zijn diverse aspecten van de reactie van een *Drosophila* op temperatuur beschreven. Zo is er beschreven dat vliegen temperatuur moeten kunnen voelen om er correct op te reageren, dat de manier waarop interacties plaatsvinden tussen vliegen door temperatuur beïnvloed wordt, dat de omgeving van moeders en nakomelingen effect hebben op de reactie van vliegen op thermische moeilijkheden en dat vliegen temperatuur informatie kunnen gebruiken om te bepalen waar ze naartoe moeten bewegen. Daarnaast is een nieuwe temperatuurgecontroleerde arena beschreven, die gebruikt zou kunnen worden om vergelijkbare temperatuur-gerelateerde aspecten in andere insecten te onderzoeken, of zelf van grotere soorten indien de technische aspecten adequaat worden aangepast. Alles samengenomen toont dit proefschrift dat temperatuur in alle aspecten van het gedrag van een vlieg een rol speelt, en dat het van fundamenteel belang is om te begrijpen hoe een vlieg met deze omgevingsfactor omgaat. Dit principe is van invloed op alle soorten en onderstreept de brede impact die klimaatverandering heeft en zal hebben op het leven.

Resumen en Español

La temperatura del medio ambiente afecta de forma directa la supervivencia de todos los seres vivos. Cuando el medio ambiente cambia, tal como está ocurriendo con el calentamiento global, los seres vivos se ven forzados a adaptarse a las nuevas condiciones para sobrevivir. En algunos casos, animales y plantas lograrán cambiar de acuerdo a lo que les demanda el medio ambiente; en otros, será demasiado tarde y las especies se extinguirán. Este impacto tan significativo de la temperatura sobre la supervivencia de las especies se debe a su efecto directo sobre las reacciones enzimáticas. Las enzimas guían todas las reacciones bioquímicas fundamentales para la vida; sin ellas, ninguno de los organismos conocidos existiría. Las enzimas requieren un rango de temperaturas específico para poder funcionar. Debajo de este rango, las enzimas son incapaces de producir una reacción, mientras que sobre el rango las enzimas se desnaturizan y pierden funcionalidad. Esto implica que cada ser vivo tiene que mantenerse dentro del rango de temperatura al cual sus enzimas son funcionales, de lo contrario su supervivencia se pone en riesgo.

Los animales utilizan dos mecanismos básicos para mantenerse dentro de su rango de temperaturas: los endotérmicos, o animales de sangre caliente, utilizan mecanismos fisiológicos para enfriar o calentar su cuerpo; los ectotérmicos, o animales de sangre fría, dependen de la temperatura en el exterior para regular su temperatura corporal. Esto significa que para los ectotérmicos estar en un área a una temperatura dentro del rango que necesitan es muy importante. Los ectotérmicos grandes, como los lagartos, pueden tolerar estar en zonas más frías o calientes ya que el tamaño de su cuerpo dilata la transferencia de temperatura del exterior a su interior; sin embargo, los ectotérmicos pequeños como los insectos adquieren la temperatura del medio ambiente casi de inmediato, lo que se traduce en efectos directos sobre el funcionamiento de sus enzimas y crea la impresión de que la reacción de estos pequeños seres a los cambios de temperatura a su alrededor, no sea más que un reflejo del efecto de la temperatura sobre sus reacciones enzimáticas. Sin embargo, los insectos han demostrado no ser tan simples. Por ejemplo, las moscas de la fruta *Drosophila melanogaster*, tienen una serie de sensores térmicos centrales (en el cerebro) y periféricos (en su antenas) dedicados a percibir rangos o cambios de temperatura específicos. Esto sugiere que *Drosophila* responde de forma más compleja a los cambios ambientales que un simple reflejo del efecto enzimático. Considerando que las moscas de la fruta han sido un animal de laboratorio por más de cien años y que han sido fundamentales para contestar todo tipo de preguntas biológicas - como mecanismos hereditarios, el desarrollo del sistema nervioso, o los procesos detrás de las interacciones sociales - la primera tarea de esta tesis fue utilizarlas para entender si su respuesta a los cambios de temperatura es un mero reflejo del efecto enzimático o en contraste dependen de un proceso cognitivo más complejo.

Para resolver este misterio, moscas *Drosophila* fueron colocadas en una arena cuya temperatura puede controlarse y cambiarse automáticamente, descrita en el **Capítulo 2**. La arena está rodeada por una barrera de metal que evita que las moscas escapen y cubierta por una tapa de vidrio, que evita que las moscas vuelen. En los experimentos presentados en el **Capítulo 3**, la temperatura de la arena se programó para incrementar

gradualmente para explorar el efecto que cada tipo de sensor de temperatura tiene sobre la velocidad a la que las moscas caminan conforme el ambiente se calienta. Una mosca normal camina más rápido conforme la temperatura incrementa hasta un punto de velocidad máxima ($\sim 36^{\circ}\text{C}$), después del cual la velocidad cae rápidamente y la mosca fallece. Una moca sin sensores térmicos centrales no incrementa su velocidad a ninguna temperatura, mientras que una mosca sin sensores térmicos periféricos camina más rápido en ambiente más calientes, pero nunca tan rápido como una mosca normal. Esto sugiere, en primer lugar, que receptores térmicos centrales y periféricos son necesarios para que las moscas presenten una respuesta normal a los cambios de temperatura y, en segundo lugar, que los insectos no responden al ambiente sólo por el efecto en sus enzimas, sino por el control cognitivo de sus reacciones.

El control cognitivo de *Drosophila* sobre su respuesta a la temperatura abre la posibilidad de que las moscas integren información térmica y no térmica para modular su comportamiento. Por ejemplo, las moscas podrían actuar diferente si otros están presentes, ya que en muchas especies la presencia de otros afecta la reacción a cambios de temperatura, como cuando una manada se junta para conservar calor. El **Capítulo 4** presenta la reacción de moscas estudiadas solas o en grupo para explorar esta idea. Moscas solas, ya sean hembras o macho, se comportan igual. En grupo, la moscas macho con otros machos muestran un incremento en su velocidad a altas temperaturas ($34\text{-}38^{\circ}\text{C}$), mientras que las hembras con otras hembras no muestran cambios. De forma fascinante, cuando las feromonas de los machos son manipuladas para simular feromonas de hembra, los machos dejan de acelerar a altas temperaturas; en contraste, cuando las hembras expresan feromonas de macho comienzan a moverse más rápido conforme la temperatura incrementa. Esto sugiere que la reacción de una mosca en grupo depende del sexo de las moscas que la rodean, probablemente por la forma en que hembras y machos interactúan entre sí. En particular, cuando moscas hembra se encuentran con otras hembras cada una busca interactuar con otras frecuentemente, a diferencia de lo que ocurren entre machos o cuando hembras y machos se mezclan. Las hembras continúan buscándose entre sí conforme la temperatura incrementa, incluso cuando su sensación del tacto ha sido mutada y no son capaces de percibir el contacto con otras. Es posible que las hembras utilicen estos contactos como mecanismo de relajación ante una situación estresante - el aumento de temperatura -, tal como se ha visto en otras especies más complejas (roedores, primates no humanos y humanos). En estos otros ejemplos, las hembras (o mujeres) tienden a agruparse con otros cuando se encuentran bajo estrés, mientras que los machos (u hombres) tienden a actuar de forma solitaria. Estas tendencias han sido explicadas por diferencias cerebrales entre los sexos. Curiosamente, *Drosophila* también posee áreas cerebrales relacionadas con estrés que son diferentes entre los sexos, lo que sugieren que estos insectos nos pueden ayudar a comprender mejor los mecanismos detrás de estas diferencias.

Las moscas de la fruta podrían ser capaces de adaptarse a los cambios ambientales a través de la preparación de sus descendientes para enfrentar climas extremos. Por ejemplo, en estudios previos se ha visto que descendientes de moscas mantenidas a 25°C o 29°C tienen una supervivencia más alta que los descendientes de moscas mantenidas a 18°C . Esto sugiere que *Drosophila* puede utilizar información sobre la temperatura de su ambiente como guía para influir en el desarrollo de sus descendientes y prepararlos mejor para el ambiente que podrían enfrentar en el futuro. Esto se conoce como efectos

anticipatorios de los padres y no ha sido satisfactoriamente demostrado en moscas. Por ello, se realizaron experimentos con esta idea en el **Capítulo 5** al exponer moscas madre a ambientes calientes (29°C) o ambientes fríos (18°C) y después permitir a sus descendientes desarrollarse en el mismo ambiente que las madres o en el ambiente opuesto. Se encontró que el mayor determinante de la respuesta de los descendientes a diferentes retos de temperatura es el ambiente en el que los descendientes se desarrollaron. Las moscas que crecieron en ambientes calientes se recuperaron más rápido de choques térmicos con altas temperaturas, mientras que las moscas que crecieron en ambientes fríos se recuperaron más rápido de choques térmicos con temperaturas frías. Sin embargo, las moscas que nacieron de madres expuestas a 18°C se recuperaron mejor que las que nacieron de madres expuestas a 29°C , lo que sugiere un efecto materno dependiente del ambiente de la madre sobre el desarrollo de su descendencia. Esto no implica necesariamente que las madres hayan tenido efectos anticipatorios sobre sus descendientes; más probablemente refleja un efecto fisiológico dado el cambio metabólico en las madres expuestas a 18°C , como el aumento en grasa corporal que pudiera haber causado una mayor resistencia a temperaturas extremas. Más estudios son necesarios para responder satisfactoriamente a la pregunta sobre la existencia de efectos anticipatorios en *Drosophila*. La resolución final de este misterio podría ayudar a predecir cómo se adaptarán los insectos al cambio climático y cómo se verá la biodiversidad de nuestro futuro cercano.

La forma compleja en que *Drosophila* interactúa con la temperatura sugiere que éste es un estímulo importante para las moscas que nos podría ayudar a investigar otros comportamientos. Con esto en mente, en el **Capítulo 6** las moscas fueron expuestas a luces o sonidos largos y cortos para explorar su capacidad de relacionar la duración del sonido con la localización de temperaturas cómodas en nuestra arena. De poder diferencias entre tonos cortos y largos, las moscas hubieran mostrado la capacidad de percibir intervalos de tiempo, un componente fundamental para el aprendizaje, la toma de decisiones y un sin fin de otras habilidades cognitivas en humanos cuyas bases neurales se desconocen. Las moscas hubieran servido entonces como modelo para investigar los circuitos neuronales detrás de la percepción de intervalos temporales; sin embargo, los resultados no demostraron que las moscas tuvieran la capacidad temporal deseada aunque sí mostraron mejorar en encontrar el lugar seguro gracias a la información térmica. Esto no prueba necesariamente que las moscas no perciben intervalos de tiempo, sino que señala que esta aproximación experimental careció del poder para mostrar esta capacidad. Experimentos en el futuro podrían utilizar otros estímulos, como la presencia de comida, o desarrollar otros paradigmas, como observar tiempo de vuelo y descanso, para desenmascarar las capacidades temporales de *Drosophila* y crear un nuevo modelo con el que explorar cómo percibimos el tiempo.

Notas Finales

La mosca de la fruta *Drosophila melanogaster* es un organismo experimental tremadamente interesante. Las moscas han permitido responder preguntas en todos los niveles, desde los componentes neurales hasta los efectos poblacionales, permitiéndonos explorar los componentes genéticos, fisiológicos, de comportamiento y evolutivos de una multitud de procesos. Las moscas han sido esenciales para entender muchas de las interacciones entre machos y hembras, la deposición de huevos, los ritmos circadianos, los ciclos de sueños,

aprendizaje y memoria e incluso las complejidades del comportamiento social. Esta tesis ha cubierto diversos aspectos relacionados con la respuesta de *Drosophila* a la temperatura. Ha mostrado que las moscas deben percibir y procesar la información térmica para poder reaccionar a ella, que la temperatura afecta cómo se relacionan las moscas unas con otras, que el ambiente de las madres y su descendencia afecta la capacidad de las moscas para responder a diversos retos térmicos y que las moscas pueden utilizar la información sobre la temperatura para predecir a dónde moverse. Esta tesis presenta también una nueva arena que podría utilizarse para explorar la relación con la temperatura de otros insectos o incluso de especies más grandes si los componentes técnicos son adaptados. En general, esta tesis demuestra que la temperatura afecta todos los aspectos del comportamiento de una mosca y que entender cómo lidiá *Drosophila* con estos efectos es fundamental para lograr una comprensión profunda de las consecuencias que este estímulo podría tener en la naturaleza dado el inevitable calentamiento global.



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