



## Review

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## ABSTRACT

Environmental temperature strongly affects physiology of ectotherms. Small ectotherms, like *Drosophila*, cannot endogenously regulate body temperature so must rely on behavior to maintain body temperature within a physiologically permissive range. Here we review what is known about *Drosophila* thermal preference. Work on thermal behavior in this group is particularly exciting because it provides the opportunity to connect genes to neuromolecular mechanisms to behavior to fitness in the wild.

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## 1. Introduction

Temperature has profound effects on the physiology, ecology, and fitness of ectotherms. Extreme temperatures are injurious and potentially lethal, but even temperatures within those lethal limits have major effects on performance and ultimately Darwinian fitness.

Ectotherms are not entirely at the mercy of the thermal environment. Although most have only limited abilities to

thermoregulate via physiological adjustments (Stevenson, 1985, but see Heinrich, 1993), many use behavioral adjustments (e.g. shuttling between sun and shade) to regulate body temperatures at remarkably narrow, species-specific levels (Cowles and Bogert, 1944).

Behavioral thermoregulation can be adaptive in two complementary ways. (i) It can help an animal avoid extreme heat or cold temperatures that could be damaging or lethal (Norris, 1967; Grant and Dunham, 1988). (ii) It can increase the time an animal spends at physiologically optimal temperatures (Huey et al., 2003). Indeed, thermal preferences (or “set point” temperatures in a lab thermal gradient) of species often correspond closely with temperatures that maximize diverse performance traits (e.g. sprint speed, digestion, enzyme kinetics, etc.; Dawson, 1975; Bennett, 1980; Huey, 1982; Huey and Bennett, 1987; Angilleta

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et al., 2002). Moreover, field studies show that thermoregulation generally enhances physiological performance in nature (Huey, 1983; Hertz et al., 1993).

Physiological and behavioral ecologists have long been fascinated by ectotherm thermoregulation, and have made this one of the best-studied disciplines in ecological physiology (Bennett, 1987). Most researchers have focused on reptiles, and primarily lizards, which are relatively easy to track and whose body temperatures are easily measured in the field. These studies have shown that the thermoregulatory behavior of lizards can drive habitat choice and behavioral activity patterns in the field (Heath, 1965; Porter et al., 1973; Huey, 1991; Bartholomew, 1964; Bennett, 1987), as well as influence physiological performance and survival (Huey and Stevenson, 1979; Christian and Tracy, 1981; Kingsolver and Watt, 1983).

Lizards and other reptiles have contributed greatly to our current understanding of ectotherm thermoregulation, but this group of organisms nonetheless poses some limitations. Although these taxa have proven useful for establishing links between thermoregulation and physiological performance (Bennett, 1980; Huey, 1982; Angilleta et al., 2002), they are impractical for linking thermoregulation with Darwinian fitness (but see Christian and Tracy, 1981). In this regard, insects are potentially better subjects because many can be reared in the lab, have short generation times, and are well understood genetically and developmentally. Of course, insects are also fascinating in their own right and are the dominant multi-cellular terrestrial organisms both in terms of biomass and species numbers.

Some aspects of thermal biology are well understood for ectothermic insects. For example, heat and cold tolerance have been well studied (Hoffmann et al., 2003; Lee and Denlinger, 1991), but other aspects have received limited attention. In particular, we know very little about how most ectothermic insects thermoregulate in the field or whether thermoregulation affects their fitness.

Here we review what is known about the ability of *Drosophila* to sense and regulate body temperature and about how this ability affects their fitness. We focus primarily on *Drosophila* because it is a suitable model for many types of studies (Powell, 1997). The genus is diverse phylogenetically, geographically, and ecologically. Many species are easily reared in the laboratory. Finally, *Drosophila melanogaster* has an abundance of molecular and genetic tools and is a leading model system for investigating metazoan biology. However, the thermal and physiological ecology of *Drosophila* are largely unknown. We hope that this review will encourage more studies along these lines.

We first review methods of measuring thermal preferences of insects. Then we address the neurobiological underpinnings of thermal sensation. Finally, we review a series of physiological, ecological, and evolutionary issues: ontogenetic patterns of thermoregulation, developmental and acclimation effects, correlations between thermal preferences and temperatures optimizing performance or fitness, and evolutionary diversification of thermal preferences.

## 2. Measurement of thermal preference

Temperature (or thermal) preference ( $T_p$ ) is the body temperature ( $T_b$ ) an organism chooses when provided with a range of potential temperatures. How does one measure  $T_p$ ? In the field, a complex and constantly changing thermal and biotic environment make field  $T_b$  unsuitable as estimates of  $T_p$  (Licht et al., 1966; Huey, 1982). Laboratory thermal gradients are straightforward to construct and use and provide consistent estimates of  $T_p$  (Licht et al., 1966). In fact, workers have used laboratory gradients to

estimate  $T_p$  of numerous insects since at least as early as 1922 (Barbour and Racine, 1967, and references therein; Deal, 1941, and references therein; Uvarov, 1931).

A gradient is typically created by heating and cooling opposite ends of a thermally conductive material (Barbour and Racine, 1967; Campbell, 1937; Chapman, 1965; Flinn and Hagstrum, 1998; Deal, 1941; Fogleman, 1978, 1979; Gunn, 1935; Prince and Parsons, 1977; Sayeed and Benzer, 1996; Thomson, 1938; Waddington et al., 1954; Yamada and Ohshima, 2003; Yamamoto and Ohba, 1982, 1984; Yamamoto, 1994a,b; Hedgecock and Russell, 1975; McDaniel et al., 1995; Uvarov, 1931), but other gradient designs have also been used. For example, heating the center of a sheet of aluminum creates a circular gradient of decreasing temperature away from the center. Also, authors have avoided potential discrepancies between gradient temperature and air temperature (particularly problematic for larger organisms) by embedding hollow glass tubes which provide a tunnel for insect movement in a water or sand gradient. By recording either the locations of multiple insects after some amount of time or the locations of individual insects over some length of time, one creates a distribution of  $T_p$ . Some metric of that distribution (usually mean or median, see Dewitt and Friedman, 1979) is then taken as the  $T_p$  of the insect.

Several problems with this approach may bias or even invalidate such estimates of  $T_p$ . Firstly, many of these gradients used a lamp as a heat source (e.g. Prince and Parsons, 1977), thereby creating a photo-thermal gradient rather than just a thermal gradient. Given the well known phototactic behavior of *Drosophila* and other insects (Manning and Markow, 1981; Markow, 1979a; Markow and Fogleman, 1981; Rieger et al., 2007), such gradients may tell you little about  $T_p$ . Further, surprisingly few authors have recognized their implicit null expectation that in the absence of a temperature gradient or if the subjects themselves have no  $T_p$ , then the insects should be uniformly distributed on the apparatus. We may or may not expect this, dependent on the model used to describe organismal movement (see Anderson et al., 2007, for an example in *Caenorhabditis elegans*). A number of experiments have found that insects aggregate at the edges of a gradient apparatus at uniform temperature (Deal, 1941; *Drosophila*: Waddington et al., 1954; *D. melanogaster*: Fogleman, 1979; *Periplaneta americana*: Murphy and Heath, 1983), whereas others have found a uniform distribution in the absence of a temperature gradient (Hong et al., 2006).

Additionally, small ectothermic insects experience a  $Q_{10}$  effect on thermal gradients. Their metabolic rates and rates of movement depend on environmental temperature (e.g. Crill et al., 1996). This may mean that insects will tend to spend more time at the cold end of the gradient, simply because they move more slowly (or stop moving altogether) in colder temperatures. These null “wall” and  $Q_{10}$  effects (i.e. effects not dependent on  $T_p$ ) have only recently begun to be appreciated (for examples in *C. elegans*, see Yamada and Ohshima, 2003; Anderson et al., 2007), but they could profoundly affect interpretations of insect distributions on gradients, including biasing estimates of  $T_p$ . Ignoring the null expectation in these tests can cause difficulties of interpretation analogous to those described in Heath (1964). In particular, the choice of model (see Anderson et al., 2007, for one possibility) and whether the experimental distributions reach equilibrium will strongly affect subsequent interpretation.

How does one address these issues to accurately assess thermal preference in the lab? Several approaches would seem appropriate. A simple theoretical model of flies under Brownian motion would allow prediction of a null distribution in the absence of  $Q_{10}$  effects or temperature preference (Anderson et al., 2007). Further, existing data on walking speed of flies as a function of temperature could be used to incorporate the  $Q_{10}$

effect into the model (see Yamada and Ohshima, 2003; Anderson et al., 2007, for examples applied to *C. elegans* thermotaxis). Determination of fly distributions under a range of gradient conditions (different constant temperatures, shifting gradients) could be combined with the model to determine the relative importance of wall effects,  $Q_{10}$  effects, and  $T_p$  in determining insect distributions on gradients. Estimates of  $T_p$  could then be adjusted to address these potential biases.

### 3. How do *Drosophila* sense temperature?

Temperature sensation can be divided into at least two main categories: the detection of noxious or painful temperatures that cause rapid tissue damage (i.e. nociception) and the detection of innocuous temperatures. Anatomically, *Drosophila* larvae likely detect painfully high temperatures ( $\geq 39^\circ\text{C}$ ) in both the central nervous system and in highly arborized peripheral neurons beneath the epidermis (Tracey et al., 2003). At the molecular level, the Painless protein is critical for high-temperature nociception (Tracey et al., 2003). Painless is a member of the TRPA subfamily of transient receptor potential (TRP) cation channels and is expressed both centrally and in the peripheral nervous system. Both larvae and adults lacking Painless function show significantly increased latencies in responding to high-temperature nociceptive stimuli (Tracey et al., 2003; Xu et al., 2006). Furthermore, the peripheral nerves of Painless mutants show diminished responses to high temperature ( $42^\circ\text{C}$ ) stimulation, consistent with a role for Painless in the detection of high temperatures. In a separate line of studies, tolerance to high temperatures ( $>40^\circ\text{C}$ ) was found to partially depend on another TRPA channel, Pyrexia (Lee et al., 2005). Adults lacking Pyrexia function paralyzed more rapidly and at higher frequency upon exposure to  $40^\circ\text{C}$  than their wild-type counterparts (Lee et al., 2005). Whether there is significant overlap in the requirements for Painless and Pyrexia in high-temperature responses has not been examined. However, both channels are thought to encode temperature-regulated TRP channels (thermoTRPs) activated in the  $37\text{--}42^\circ\text{C}$  range.

Responses to more innocuous warmth appear to involve distinct molecules and neurons from those required for high-temperature responses. This has been most extensively analyzed in larvae, where larval avoidance of moderately elevated temperatures ( $\geq 30^\circ\text{C}$ ) requires dTRPA1, another TRPA family member, rather than Painless or Pyrexia (Rosenzweig et al., 2005, 2008). dTRPA1 encodes a warmth-activated ion channel that responds at lower temperatures ( $\sim 25\text{--}27^\circ\text{C}$ ) than Painless or Pyrexia (Viswanath et al., 2003; Hamada et al., 2008), consistent with the differential requirements for these proteins. At the neural circuit level, avoidance of moderately elevated temperatures is also distinct from the response to high-temperature mechanical stimulation (Rosenzweig et al., 2005). Inhibiting the function of multiple dendritic neurons in the larval body wall diminishes responses to high-temperature mechanical stimulation but does not affect warm avoidance. Conversely, ablation of dTRPA1-expressing neurons in the central brain decreases warm avoidance but does not affect responses to high-temperature mechanical stimulation.

Responses to cool temperatures also appear to involve molecules and neurons distinct from those involved in warmth avoidance. dTRPA1, Painless and Pyrexia are all dispensable for avoidance of moderately cool temperatures (below  $\sim 20^\circ\text{C}$ ) by first-instar larvae, suggesting that these TRPA channels are specifically involved in warm avoidance (Rosenzweig et al., 2008). Instead, cool avoidance requires TRPL and TRP, two members of a different family of TRP channels, the TRPCs

(Rosenzweig et al., 2008). TRP and TRPL are well-characterized proteins famous for their essential roles in fly phototransduction, where they mediate photoreceptor depolarization downstream of fly opsins. Interestingly, the functions of TRP and TRPL in vision and cool avoidance are distinct as neither the larval photoreceptors nor the phototransduction molecules NORPA and INAF are required for cool avoidance (Rosenzweig et al., 2008). Whether TRP and TRPL encode thermoTRPs that respond to temperature directly or participate in cool responses more indirectly remains to be determined. At the neural circuit level, neurons within the larval terminal are selectively activated by cooling (Liu et al., 2003). Consistent with the terminal organ functioning as the larval cool sensor, inactivation of these neurons completely eliminates cool avoidance without affecting warm avoidance (Rosenzweig et al., 2008).

Like larvae, *Drosophila* adults possess distinct sensors for innocuously cool and warm temperatures. At the molecular level, the basis of adult cool sensing is unknown, but warm sensing requires dTRPA1. In the adult, loss of dTRPA1 function disrupts both physiological and behavioral responses to warmth, eliminating heat responses in critical thermosensory neurons and causing flies to accumulate in warmer than normal regions on a thermal gradient (Hamada et al., 2008). Interestingly, even a twofold reduction in dTRPA1 function increases  $T_p$  from  $\sim 25$  to  $\sim 27^\circ\text{C}$ . At the neural circuit level, initial ablation studies led to the proposal that both warm and cool sensors were housed within the *Drosophila* third antennal segment (Sayeed and Benzer, 1996). However, recent work indicates that the warm and cool sensors are actually anatomically distinct: the third antennal segment is specifically required for cool avoidance, but the critical warmth sensors are in the brain and they correspond to four warmth-activated neurons (the AC neurons) that express dTRPA1 (Hamada et al., 2008). In these studies, surgical removal of the third antennal segment selectively reduced avoidance of cooler gradient regions, while the loss of dTRPA1 function in the AC neurons selectively reduced avoidance of warmer gradient regions (Hamada et al., 2008). Disrupting both these sensors simultaneously by removing the third antennal segments from flies lacking dTRPA1 function created flies that distributed relatively evenly across the thermal gradient, suggesting that these sensors act additively to drive flies toward their normal  $T_p$  of  $\sim 25^\circ\text{C}$  (Hamada et al., 2008).

Beyond the periphery, the processing of thermosensory information remains largely terra incognita. The AC neurons send processes to three different brain regions, the subesophageal ganglion, the superior lateral protocerebrum, and the VL2a and VL2p glomeruli within the antennal lobe (Hamada et al., 2008). Which of these regions contribute to thermal preference and the nature of their contributions remain unknown. The mushroom body region of the fly brain is also required for proper thermal preference behavior (Hong et al., 2008). The contribution of the mushroom body to temperature preference is also not yet clear, but it appears that appropriate levels of cAMP signaling within this structure are required for flies to choose an appropriate  $T_p$ .

The advent of such molecular and physiological analysis of thermal preference behavior in *Drosophila* provides an opportunity to begin to address the molecular and neural strategies that underlie the rich thermal preference literature. Naively, one can imagine at least two alternative strategies that could drive flies toward the preferred temperature,  $T_p$ . In one simplistic model, flies could use thermosensory neurons that are maximally active near  $T_p$ . In such a comfort receptor model, flies would reach  $T_p$  by moving toward wherever the activity of these sensors was maximal. Alternatively, flies could use thermosensory neurons that are maximally active at temperatures above or below  $T_p$ , but are inactive or minimally active at  $T_p$ . In such a discomfort

receptor model, flies would accumulate near  $T_p$  as a result of avoiding temperatures above or below  $T_p$ . Current data, though limited, conform best to the latter view. In warm gradient regions, dTRPA1-expressing AC neurons would act as discomfort receptors. Consistent with this view, calcium-imaging experiments show that AC neurons are initially activated near or just above 25 °C, and their activity increases as the temperature rises (Hamada et al., 2008). It will be interesting to further test this model by examining how molecular and physiological manipulations that alter the temperature threshold of the AC neurons affect  $T_p$ .

Although an AC neuron-based discomfort model for heat avoidance would explain why flies do not accumulate above  $T_p$ , it would not explain what prevents flies from accumulating below  $T_p$ . One possibility is that a separate discomfort receptor relying on cold-activated neurons functions at cooler temperatures. This model predicts that the third antennal segment should contain cool sensing neurons activated at or below  $T_p$ . An alternative possibility is that behavior at cool temperatures involves a comfort receptor strategy, in which the third antennal segment contains a population of neurons inhibited by cooling below  $T_p$ . The former possibility seems more likely because the antennae of many species of non-blood-sucking insects have been characterized electrophysiologically and usually contain cold-activated but not warm-activated thermoreceptors (Tichy and Gingl, 2001; Tichy, 2007; Fischer and Tichy, 2002). Confirmation of this speculation awaits the identification and physiological characterization of thermosensory neurons in the *D. melanogaster* antenna.

While still speculative, such a dual-discomfort model for  $T_p$  behavior is consistent with the current data in the field. Such a model also suggests that the shifts in  $T_p$  observed over the course of development and in response to changing conditions could arise from shifts in the temperature thresholds for these thermal sensors. This is an attractive possibility, given the well-established ability of signaling through receptor tyrosine kinases and G-protein coupled receptors to significantly (sometimes by >10 °C) alter the temperature thresholds of mammalian thermoTRP proteins (Huang et al., 2006).

Together these data suggest that *Drosophila* larvae take the temperature of their environments by using a series of distinct molecular and cellular sensors that are tuned to respond to different portions of the thermal spectrum. This is strikingly analogous to thermosensation in mammals, where different thermoTRPs and thermosensory neurons respond to different temperatures (Jordt et al., 2003; Dhaka et al., 2006).

#### 4. $T_p$ in oviposition site selection

Eggs cannot move and are therefore forcibly subjected to the thermal environment into which they are laid. Females could increase their fitness by depositing eggs in thermally favorable locations that not only prevent exposure of eggs to deleterious or lethal extreme temperatures (Feder et al., 2000), but also expose eggs to temperatures optimal for development. Those eggs that develop more quickly may be at a competitive advantage as larvae because they will have first access to food. For a seed beetle (*Stator limbatus*), oviposition temperature in combination with rearing temperature strongly affected fitness (Stillwell and Fox, 2005). Females may be able to not only choose egg location, but to effect differences in egg hatching and development success via maternal effects while laying in different temperatures (Stillwell and Fox, 2005). Additionally, developmental temperature can have strong effects on future territorial success of males (Zamudio et al., 1995). To choose appropriate sites for egg development, females must use current environmental cues as indicators of future thermal regime (Levins, 1969). However, which, if any, current environ-

mental cues accurately predict future thermal regime is presently unclear (Huey, 1991; Padilla and Adolph, 1996; Feder et al., 1997; Kingsolver and Huey, 1998).

To date, studies of oviposition site selection in *Drosophila* have suffered from several problems. First, some studies count progeny emerged rather than counting the number of eggs laid at different temperatures (e.g. Nevo et al., 1998). Any observed differences could therefore reflect both variation in oviposition site selection and variation in survival of eggs, larvae, or pupae at the different temperatures. Also, this methodology ignores the ability of larvae to behaviorally thermoregulate (i.e. if eggs are laid in suboptimal temperatures, the larvae can still move to more ideal temperatures; see Section 5).

Nevo et al. (1998) introduced *D. melanogaster* and *D. simulans* into a temperature gradient with five food vials distributed along its length. After 40 h, they removed adults, transferred the vials to 25 °C, and counted the emerged progeny. Flies captured at warmer, drier sites had more progeny emerge in hotter, drier vials (28 and 32 °C) than in cooler, wetter vials (14 and 19 °C). However, whether these differences were due to oviposition preference or differential mortality (of eggs, larvae, or pupae) is unclear. Similarly, Jones et al. (1987) indirectly estimated developmental temperatures of *D. melanogaster* across an altitudinal gradient using mutant flies for which adult eye color depended on temperature experienced by the pupal stage. Mutant flies recaptured at high and low altitude showed very similar eye colors, indicating that they experienced similar developmental temperatures, despite the very different environmental temperatures measured across the altitudinal gradient. Jones et al. (1987) argued that this pattern reflected oviposition site selection by females: i.e. high altitude females might have chosen relatively warmer sites for egg deposition. However, the result may also reflect differential larval mortality: oviposition was random with respect to temperature, but only eggs at permissive temperatures survived to adulthood (Huey, 1991). Another possibility is that oviposition was random with respect to temperature, but larvae were able to move to select favorable pupation temperatures (see Section 5).

Few studies have actually measured oviposition thermal preference in *Drosophila*. Fogleman (1979) allowed female *D. melanogaster* to oviposit in a laboratory thermal gradient and reported the resulting spatial distribution of eggs. He found significant among-strain differences in oviposition  $T_p$ , but these differences were easily overridden by developmental temperature (see Section 7 for more examples).

Female flies may ignore substrate temperature during oviposition because current temperature is not a good indicator of future substrate temperature (Huey, 1991; Feder et al., 1997). Simple measurements of temperatures of natural oviposition substrates in the field over time would indicate the degree to which temperatures are temporally and spatially correlated (see e.g. Padilla and Adolph, 1996; Kingsolver and Huey, 1998; Gibbs et al., 2003); if such correlations exist, it would strengthen the idea that flies could use temperature as a reliable cue for oviposition site selection. Alternatively, females may use other cues as indicators of future temperature regime. For example, biological, physical, and chemical differences between sites may indicate their past and future temperatures. Remarkably, female *D. melanogaster* did not avoid fruit that had been previously heated to high temperatures or that contained heat-killed larvae (Feder et al., 1997), suggesting that *Drosophila* do not use these cues to assess the suitability of oviposition sites. Another possibility is that females interpret the presence of living conspecific larvae as indicative of a suitable oviposition site (del Solar and Palomino, 1966; Rockwell and Grossfield, 1978; Kiester and Slatkin, 1974), despite potential competition from the larvae already present

(Gilpin, 1974). The color of the substrate may indicate its suitability as well, and *Drosophila* may be able to discriminate among oviposition sites based on color (Volpe et al., 1967). *D. nigrospiracula* and *D. mettleri* chose oviposition sites based on substrate (soil or cactus), vertical position, and light (Fogleman et al., 1981; see also Srivastava and Singh, 1993). Adult distributions of these species were distinctly different from where they laid eggs (Markow and Fogleman, 1981), clearly indicating oviposition site choice by females. Temperature of the substrate may interact with ethanol levels to affect female oviposition preference, as well (Hougouto et al., 1982). Other oviposition cues include substrate texture (Chess and Ringo, 1985) and shading.

Females may prevent exposure of eggs to adverse temperatures by choosing when, rather than where, they lay their eggs. *D. buzzatii* collected from lowland sites tended to oviposit later in the day (in the laboratory) than did those collected from highland sites (Dahlgard et al., 2001). These diel differences in oviposition preferences may reflect temperature differences between sites, with lowland flies avoiding the hottest times of day. Note that peak oviposition activity in many flies in nature occurs in the afternoon, after temperatures have peaked. Eggs can hatch in less than 24 h, and emergent larvae can thermoregulate (see Section 5). These considerations may mean that selection of oviposition sites by females is somewhat irrelevant.

An unresolved issue is whether oviposition thermal preference differs from female thermal preference. In thermally variable environments, preferred oviposition temperatures may be lower than adult temperature preference, because eggs cannot move to avoid occasional exposure to damaging or lethally high temperatures (Huey et al., 2002, this is likely exacerbated by asymmetry of thermal performance curves). Some evidence suggests that oviposition thermal preference may closely match female thermal preference. When forced to live in a food vial for 24 h at a set temperature, female *Drosophila* of several species laid similar numbers of eggs across a wide range of temperatures (Schnebel and Grossfield, 1986), indicating that egg-production rates were remarkably insensitive to temperature. For example, females of *D. melanogaster*, *D. simulans*, and *D. ananassae* all laid the same number of eggs in temperatures ranging from 16 to 28 °C (Schnebel and Grossfield, 1986). This temperature insensitivity of egg production combined with the known thermal preferences of adults (see Section 6), could lead to highly congruent oviposition and adult thermal preferences.

## 5. Larval $T_p$

Unlike eggs, *Drosophila* larvae can move to select thermally favorable microenvironments. Temperatures of larval substrates such as fruit and cactus rots can vary widely in space and time (Feder, 1997; Gibbs et al., 2003), and can exceed larval physiological tolerances. Larvae that cannot physiologically compensate for, or escape from high temperatures will die.

Some work suggests that *Drosophila* larvae can increase their survival of exposure to high temperatures (41 °C) for short periods of time (30 min to 1 h) through changes in heat shock protein expression (Welte et al., 1993; Feder et al., 1996). However, these treatments are less extreme than temperature profiles of larval substrates in the field (Feder, 1997; Gibbs et al., 2003). Furthermore, even when temperatures are not life-threatening, they can strongly determine individual fitness through effects on development time and resultant adult size (David and Clavel, 1967, 1969; Cossins and Bowler, 1987; Chown and Nicolson, 2004). Behavioral thermoregulation may therefore play an important role in determining fitness of *Drosophila* larvae in the field but remains largely unstudied.

Recent work suggests that, among populations, *Drosophila* larvae vary in their thermal preference and in their response to increasing temperatures. Further, these differences match predictions from their thermal ecology (e.g. desert vs. temperate species Wang et al., 2008). Similarly, sister species *D. yakuba* and *D. santomea* and their hybrids differ significantly in both preferred temperatures and in behavioral responses to increasing temperatures (Wang and Kim, in preparation). The high elevation species, *D. santomea*, prefers warmer temperatures and initiates escape responses at lower temperatures. This apparent contradiction (high preferred temperature but initiation of escape response at a relatively lower temperature) highlights the potential richness of thermoregulatory behaviors. Preferred temperatures and escape responses may be driven by different evolutionary forces. Population and species-level variation in thermal behavior may arise from differences in expression patterns and thresholds of activation of *Painless*, *Pyrexia*, *dTRPA1*, or other, as yet unidentified temperature-responsive molecules. As such, these studies may provide a unique opportunity to link the neural and molecular bases of thermal sensation to thermal ecology (see Section 10).

Third-instar *Drosophila* larvae wander prior to pupating and potentially assess the thermal suitability of pupation sites. Choosing thermally favorable locations for pupation may be particularly important given that pupae, like eggs, are immobile and therefore may be particularly susceptible to lethal and/or suboptimal microhabitats. In *Musca domestica*,  $T_p$  decreases by >10 °C as larvae move from early feeding stages to later non-feeding stages (Deal, 1941) and recent data suggest a similar transition in *D. melanogaster*. First-instar *D. melanogaster* larvae robustly avoid temperatures below ~23 °C (Rosenzweig et al., 2008), but third-instar larvae prefer temperatures near 18 °C (Liu et al., 2003; Kwon et al., 2008). The preference of third instars for significantly cooler temperatures than either first instars or adults indicates significant developmental regulation of  $T_p$ . At all these stages, warm avoidance relies on *dTRPA1*, suggesting that regulation of *dTRPA1* threshold could be a critical element in determining these developmental transitions in  $T_p$ .

Sokal et al. (1960) found no effect of temperature (range 19–31 °C) on *D. melanogaster* pupation site within laboratory vials (on media or on the walls). However, individual larvae were not given the chance to choose a pupation site based on temperature, as, within a vial, temperature was relatively constant. Fogleman and Markow (1982) spaced third-instar *Drosophila* larvae evenly on a temperature gradient, and recorded locations of pupae after 48 h. They reported a small (0.7 °C) but significant difference in  $T_p$  between two cactophilic *Drosophila* (*D. nigrospiracula* and *D. mettleri*). However, they did not account for differential mortality between the species larvae on the temperature gradient. From their data, it appears that the higher reported pupal  $T_p$  of *D. nigrospiracula* likely reflects reduced mortality of this species at higher temperatures, not a shift in  $T_p$ , per se.

Pandey and Singh (1993) found that temperature determined how high larvae pupate above the medium. In most strains of four species (*D. ananassae*, *D. bipectinata*, *D. melerkotiana*, and *D. biarmipes*), larvae pupated close to the food surface at 20 °C, far above the surface at 24 °C, and mostly on the medium at 30 °C. Similarly, Schnebel and Grossfield (1992) found that 12 species/subspecies of *Drosophila* tended to pupate close to the food when temperatures were high and that a subset of these pupated high on the vial sides at lower temperatures. However, these pupation height differences may not depend on temperature per se, but rather on correlates of temperature, such as the moisture content of the food or the humidity of the air. Many studies have documented the effects of moisture on pupation height (Sokal et al., 1960; Sameoto and Miller, 1968; Sokolowski et al., 1986; Pandey and Singh, 1993), and water content of the food is strongly

correlated with temperature, unless explicitly controlled experimentally.

Pupation site may be influenced by movement predilections of the larvae, not just by their thermal or other preferences (Markow, 1979b). In a series of papers, Sokolowski and colleagues determined that larvae that tend to wander long distances (“rovers”) also tend to pupate on or in the soil whereas those that move little (“sitters”) tend to pupate on or in the fruit (Bauer and Sokolowski, 1985; Sokolowski et al., 1986). These behavioral differences are genetically influenced, are evident in both laboratory strains and field-collected flies (Sokolowski et al., 1986; Bauer and Sokolowski, 1985; Sokolowski and Hansell, 1983), and may influence fitness depending on soil moisture (Sokolowski et al., 1986; Sameoto and Miller, 1968). Sitters (which pupate on fruit) are likely favored when the soil is dry whereas rovers (which pupate in soil) are likely favored when the soil is moist (Sokolowski et al., 1986).

The combination of genetic control of wandering behavior (Sokolowski et al., 1986) and other factors including time of pupation (Sokal et al., 1960; Pandey and Singh, 1993), larval density (Pandey and Singh, 1993; Ringo and Wood, 1983), light (Manning and Markow, 1981; Pandey and Singh, 1993), and sex (Bauer and Sokolowski, 1985, but see Pandey and Singh, 1993) complicates (but does not rule out) the existence of  $T_p$  for wandering *Drosophila* larvae. Perhaps because they cannot behave, pupae tend to be the most heat resistant life stage of *Drosophila* (Krebs and Loeschcke, 1995). Clearly, many factors could influence choice of pupation site. Future studies will need to consider all of these confounding factors to clearly demonstrate the importance of temperature in pupation site selection.

## 6. $T_p$ of adult *Drosophila*

Thermoregulatory behavior of adult *Drosophila* has received the most attention. In general, flies do not seem to have a single  $T_p$ , but modify  $T_p$  somewhat in response to various circumstances. Moreover,  $T_p$  may at times be overridden altogether for reproduction, digestion, escape from predators, or other more immediate concerns (see Regal, 1966, 1967, for examples in lizards). These findings highlight the need for cautious interpretation of  $T_p$  for an individual fly or for fly populations. Here we summarize what is known about the many potential moderators of adult  $T_p$ .

Studies conflict on whether *Drosophila*  $T_p$  differs between the sexes. *D. virilis* males selected significantly lower temperatures on a thermal gradient than did females (Yamamoto, 1994b). These flies had a bimodal distribution, with peaks near 14 and 32 °C. Males were more likely to be in the low temperature peak and females were relatively prevalent in the high temperature one (Yamamoto, 1994b). However, for *D. immigrans* (Yamamoto, 1994b), *D. simulans* (Krstevska and Hoffmann, 1994), and *D. melanogaster* (Yamamoto and Ohba, 1984; Sayeed and Benzer, 1996) males and females had similar  $T_p$ . Although males and females of *D. simulans* and of *D. melanogaster* do not differ in  $T_p$ , they do differ in the way that rearing temperature affects  $T_p$ . Female  $T_p$  of both species responded strongly to rearing temperature, with those reared in cooler temperatures having higher  $T_p$ . Male  $T_p$  did not respond to rearing temperature (Krstevska and Hoffmann, 1994).

$T_p$  may also change with adult age. For *D. melanogaster*, mean  $T_p$  did not change from 0 to 14 days after eclosion; however, variance of  $T_p$  declined from day 0 to day 5 before stabilizing thereafter (Yamamoto and Ohba, 1984; Sayeed and Benzer, 1996). Similarly, for *D. virilis* and *D. immigrans*, variance in  $T_p$  stabilized a week after emergence (Yamamoto and Ohba, 1982).

Humidity may also strongly influence  $T_p$ , given known humidity preferences (Sayeed and Benzer, 1996) and the tight relationship between temperature and relative humidity. For example, some insects preferred cooler temperatures when kept in a dry environment prior to testing (*Adesmia clothroata*: Deal, 1941; *Blatta orientalis*: Gunn and Cosway, 1938). However, other authors have found no effect of gradient humidity on  $T_p$  (*Blatta orientalis*: Gunn, 1933; *P. americana*: Chapman, 1965).

Prince and Parsons (1977) monitored three *Drosophila* species on a non-linear thermal gradient (16.5–36.5 °C) for 12 h at 0% and 100% relative humidity (RH). At 0% RH flies began accumulating in the cold end of the gradient after 6 h, whereas at 100% RH, flies tended to maintain stable distributions centered around 29–32 °C. They concluded that flies in dry air move to lower temperatures to minimize water loss (Prince and Parsons, 1977), and thereby increase survival (Parsons, 1979). In any case, humidity clearly affects  $T_p$ , suggesting the need to control humidity when measuring  $T_p$ .

For several *Drosophila* species, temperature affects phototaxis (Markow, 1979b); and circadian rhythms in activity are, in part, determined by temperature cycles (Yoshii et al., 2002). Consequently, light levels and circadian rhythms might modify  $T_p$ . For example, high light levels in nature would likely increase the thermal load on an insect making it seek cooler operative environments. Circadian (light/dark) cycles indicate changing seasons, which in turn involve temperature changes. Flies may acclimate accordingly such that light/dark cycles drive changes in  $T_p$  in the absence of changing ambient temperature (see Regal, 1974, for an example in a lizard).

Thermal preferences can differ depending on whether ectotherms are fed or fasting (Regal, 1966; Huey, 1982). In most cases, ectotherms prefer higher temperatures when fed and lower temperatures when fasting (mosquitoes: Thomson, 1938; fish: Angilleta et al., 2002; cockroaches: Chapman, 1965), likely because this strategy maximizes growth efficiency (Brett, 1971). However, there are exceptions. For example, three beetle species preferred lower temperatures on a gradient with food than on one without food (Deal, 1941). Also, starved *C. elegans* dispersed uniformly along a gradient whereas fed worms had a strong  $T_p$  (Hedgecock and Russell, 1975). Anderson et al. (2007) found that one strain of *C. elegans* preferred lower temperatures on a gradient with food as compared to one without food.

Insect  $T_p$  may also change in response to the presence of conspecifics. Dominance interactions involving controlling access to heat sources have been documented in lizards (Regal, 1971; Downes and Shine, 1998): if general, these might modify distributions of  $T_p$  if insects are measured in groups. Results are mixed for insects. Cockroaches (*Schistocerca gregaria*) congregated less in the cold when placed on a gradient together (Chapman, 1965). Conversely, for *Drosophila*, the number of flies tested at once (150–300) did not affect mean  $T_p$  or its variance (Krstevska and Hoffmann, 1994, but these were preliminary experiments—no data presented).

Few of the many potential moderators of  $T_p$  (or their interactions) have been rigorously investigated in *Drosophila*, though they may be very important. Further, the extent to which the abiotic conditions interact with the physiological state of an insect to determine its  $T_p$  in the wild is largely unknown and likely complex. For example, Taylor (1986) used a maze apparatus to test how three main factors influenced habitat choice of *D. pseudoobscura* (light vs. dark, 15 °C vs. 25 °C, and maltose vs. lactose as food), and found complex interactions among these factors. For example, flies preferred the cool habitat under illumination, but did not differentiate between cool and hot habitats in the dark. This experiment and evidence that many factors may influence  $T_p$  argue that future work combining detailed, controlled laboratory

studies with field experiments will be necessary to identify the extent to which adult *Drosophila* regulate  $T_p$  in the context of multiple and shifting abiotic and biotic variables.

## 7. Effects of developmental and acclimation temperatures on $T_p$

Many phenotypic traits of adult *Drosophila* are sensitive both to “developmental” temperature (temperature experienced from oviposition to eclosion, see David et al., 1983) and to “acclimation” temperature (temperature experienced prior to testing, usually within the same life stage), yet relatively few studies have systematically investigated the impact of either developmental or acclimation temperatures on thermal preferences of *Drosophila*.

The effects of developmental temperature may vary among species and even between sexes of the same species. Both male and female *D. melanogaster* preferred lower temperatures when reared at 28 °C than when reared at 19 or 25 °C (Krstevska and Hoffmann, 1994). In the same study, *D. simulans* females had significantly higher  $T_p$  when reared at 19 °C than when reared at 25 and 28 °C, but male  $T_p$  did not respond to developmental temperature.

Yamamoto and Ohba (1984) reared 10 *Drosophila* species at 15, 20, and 25 °C and then measured thermal preference. In general, species had higher  $T_p$  when reared at 25 °C relative to 20 °C. Rearing flies at 15 °C resulted in thermal preferences that were both lower (4 species) and higher (6 species) than corresponding thermal preferences at 20 °C. Good (1993) found that rearing temperature could reverse effects of long-term laboratory natural selection at different fixed temperatures. Flies that had evolved at 30 °C for 10 generations preferred temperatures ~3 °C warmer than flies that had evolved at 25 °C. However, this difference disappeared when 30 °C strains were reared from egg to adult at 25 °C.

Acclimation studies have also shown mixed results, with insect  $T_p$  showing no response (Murphy, 1986), a positive trend (Murphy and Heath, 1983), or more complicated patterns (Chapman, 1965). For *Drosophila*, acclimation of *D. tripunctata* males and females to 26 °C rather than 20 °C for 2 days caused them to prefer significantly higher temperatures on a thermal gradient; and *D. robusta* males and females showed a similar, albeit non-significant trend (McDaniel et al., 1995). In another study, neither *D. melanogaster* nor *D. simulans* shifted  $T_p$  in response to acclimation temperature (Krstevska and Hoffmann, 1994).

The available evidence, though sparse, suggests mixed effects of rearing and acclimation temperatures on  $T_p$ . The degree to which a species  $T_p$  responds to acclimation may reflect how stressful temperatures are (McDaniel et al., 1995), and may depend on a multitude of other factors. Given the important effects of rearing and acclimation temperatures on other aspects of *Drosophila* thermal physiology (see, e.g. David et al., 1983; Gibert et al., 2000, 2001; Petavy et al., 2001), future work on the effects of these on  $T_p$  may prove fruitful.

## 8. Heritability of $T_p$

In addition to the marked phenotypic plasticity in  $T_p$  described above (Sections 6 and 7), heritable variation in  $T_p$  has also been documented. Yamamoto (1994b) found significant variation in  $T_p$  among isofemale lines of wild-caught *D. immigrans* and *D. virilis*. These differences in  $T_p$  remained stable even after 6 months of rearing in identical lab conditions, indicating marked heritability of  $T_p$  in these populations. Similarly, a diallel analysis revealed that *D. immigrans*  $T_p$  had a high heritability (81%) (Yamamoto, 1994a). In this species,  $T_p$  was primarily controlled by additive

genetic effects, followed by dominance (of low temperature preference), and maternal effects (Yamamoto, 1994a).

Laboratory natural selection can indirectly indicate heritability of a trait (by revealing the potential for the trait to evolve). Good (1993) reared *D. melanogaster* at 25, 27, or 30 °C for 10 generations, transferred them to 25 °C for 1 week, and then measured  $T_p$ . Females showed a stronger response to selection than did males, with  $T_p$  increasing with population temperature, suggesting heritability in the trait among the selected populations. However, as mentioned previously, plasticity overcame this evolutionary response, at least for the 30 °C population. Flies kept at constant temperatures in the laboratory for long periods of time (inadvertent laboratory natural selection) may also show heritable shifts in  $T_p$  away from the ancestral value. *D. tripunctata* and *D. robusta* had significantly higher thermal preferences when kept in the laboratory (at 20 °C, near their  $T_p$ ) for 4 or 7 years vs. just 1 year (McDaniel et al., 1995). Such experiments may also lead to pathologies in thermal sensation and regulation (due to mutation accumulation, for example; see Anderson et al., 2007, for a possible example with *C. elegans*).

Artificial selection is a powerful technique for investigating the heritability and underlying genetic architecture of traits, as well as for searching for potential physiological factors underlying the evolutionary shifts in traits (Gibbs, 1999; Rose et al., 1992; Bennett and Lenski, 1999; Bradley et al., 1999; Harshman and Hoffmann, 2000; Zera and Harshman, 2001; Garland, 2003). For  $T_p$ , only two artificial selection experiments have been published to date. Richmond and Finkel (1973) selected up and down (9 generations) on  $T_p$  of adult *D. melanogaster*. Mean  $T_p$  increased by ~5 °C in the hot line (average of generations 8 and 9), and it dropped ~2 °C in the cold line, suggesting significant heritability in  $T_p$ . However, this experiment was unreplicated and details have never been reported. In contrast, Fogleman (1979) found no response to 10 generations of selection on oviposition temperature preference in *D. melanogaster*; but he noted that his strain had been evolving at a fixed temperature in the laboratory (25 °C) for over 25 years. As such, his results could indicate little heritability in this trait in general, or they could indicate loss of heritable variance for this line that had adapted to laboratory conditions.

## 9. $T_p$ and fitness

Ultimately,  $T_p$  matters only in the context of its effect on organism fitness. The importance of  $T_p$  to Darwinian fitness could be indicated by a number of different patterns. A large body of literature has documented correlations between  $T_p$  and  $T_{opt}$  (temperature where some metric of performance, and by extension, fitness is optimal) in many ectotherms (for reviews see Huey and Bennett, 1987; Angilleta et al., 2002; Martin and Huey, 2008). Documentation of this classic “co-adaptation” hypothesis in *Drosophila* would reinforce the importance of behavioral thermoregulation (i.e. having a  $T_p$ ) to fitness. However, having high performance does not guarantee high fitness (Martin and Huey, 2008).

For *Drosophila* in particular, some data suggest that preferred temperatures are close to temperatures that maximize performance measures important to fitness (e.g. walking speed and fecundity). For example, the effects of temperature on relative fitness of three *Drosophila* species matched prediction from their thermal ecology (Fogleman, 1979). The warm-adapted species (*D. affinis*) had a higher relative fitness at 25 °C, whereas the cold-adapted species (*D. athabasca*) had higher relative fitness at 18 °C. Unfortunately,  $T_p$  was inferred from geographic and temporal distribution, not explicitly measured. In another study, *D. melanogaster* preferred higher temperatures than *D. simulans*,

consistent with differences in their resistance to climate extremes and in their optimal rearing temperatures (Krstevska and Hoffmann, 1994). Some fitness components may be relatively insensitive to environmental temperature (at least at intermediate temperatures), minimizing the effects of  $T_p$  on fitness. For example, mating success (percentage of female flies that are inseminated) of 10 *Drosophila* species was essentially constant across wide temperature ranges (Schnebel and Grossfield, 1984), suggesting that temperature preference likely does not affect the mating success component of fitness for these flies.

The importance of  $T_p$  to fitness would also be indicated by systematic shifts in  $T_p$  among populations and species in response to changes in environmental temperature. Interestingly, *D. melanogaster* and *D. simulans* from the south-facing and therefore hotter slope of Evolution Canyon preferred to lay eggs at higher temperatures than did those from the north-facing, cooler slope (in a laboratory gradient; Nevo et al., 1998). Further, flies from higher up the slope (where conditions were hotter due to increased sun exposure) tended to prefer warmer temperatures for oviposition. These flies had been kept in the laboratory at 25 °C prior to testing (the number of generations was not reported), suggesting that the reported differences were due to evolutionary divergence and not plasticity. Conversely,  $T_p$  of 11 *Drosophila* species was negatively correlated with temperature of appearance (the mean ambient temperature at the collection site across all days the species was collected; Yamamoto and Ohba, 1984). Thus, in contrast to flies from Evolution Canyon, species active in hot weather tended to prefer the coolest temperatures and vice versa.

Other studies have found little to no correlation between geography and  $T_p$  (Krstevska and Hoffmann, 1994). For both *D. immigrans* and *D. virilis*, within population (among isofemale line) variation was far greater than among population variation (populations from Taiwan to Sapporo; Yamamoto, 1994b). Further, although  $T_p$  varied significantly among populations of *D. virilis*, it did not show the expected correlation with latitude. Yamamoto (1994b) did not, however, report any local temperature data for the collection localities, so it is not clear whether differences in microclimate between localities disrupted the expected latitudinal differences in temperature.

## 10. The evolution of thermal sensors and thermal sensing

Taken together, the current molecular and genetic data suggest that *Drosophila* take the temperature of their environments by using a series of distinct molecular and cellular sensors that are tuned to respond to different portions of the thermal spectrum. This is strikingly analogous to thermosensation in mammals, where different thermoTRPs and thermosensory neurons respond to different temperatures (Jordt et al., 2003; Dhaka et al., 2006). Whether this superficial similarity reflects a common evolutionary origin among temperature sensing cells and circuits in animals remains an open question.

*Drosophila* thermosensation also shares clear similarities with mammalian thermosensation at the molecular level, as both flies and mammals use thermoTRPs. However, the known fly and mammalian thermoTRPs are drawn from different TRP subfamilies. For example, mammals use a series of TRPV channels for warm sensing, but flies use a series of TRPA channels. This distinction correlates with the differential expansion of TRPV and TRPA subfamilies in the mammalian and fly genomes. While mammals have six TRPVs, flies have only two TRPVs, neither of which is implicated in warm transduction (Rosenzweig et al., 2005; M.R. and P.G., unpublished). While flies have four TRPAs, mammals and most other chordates have only a single TRPA channel (TRPA1). These mammalian TRPA1s are not heat-

activated, but are rather implicated in cold sensing, although the latter finding remains controversial (Story et al., 2003; Bautista et al., 2006; Kwan et al., 2006). Thus, while thermoTRP-based mechanisms are central to thermosensation in both flies and mammals it remains an open question whether this similarity reflects a common evolutionary origin of thermosensory mechanisms or evolutionary convergence upon the TRP family of channels as a solution to thermal sensing.

Sequence relationships among the *Drosophila* TRPA channels suggest a common evolutionary origin for the fly's warmth sensors. Among the four *Drosophila* TRPAs, dTRPA1 is the only one with extensive similarity to chordate TRPA1s. Insect and chordate TRPA1s are homologous from their N to C-termini and share a signature feature: a tandem array of 17 conserved ankyrin repeats in their intracellular N-terminus (Hamada et al., 2008). Such an extended array of ankyrin repeats has the potential to form an elongated coil with the properties of a mechanical spring, a structure that could participate in channel gating (Gaudet, 2008). The two other putative TRPA thermoTRPs, Painless and Pyrexia, are arthropod-specific and bear limited sequence homology to TRPA1s or to one another outside their transmembrane regions. Interestingly, the TRPA family is further expanded in the malaria mosquito *Anopheles gambiae*, which has three Painless orthologs and two Pyrexia orthologs in addition to a single dTRPA1 ortholog, agTRPA1, which has been demonstrated to encode a warmth-activated thermoTRP (Hamada et al., 2008). It is tempting to speculate that the three warmth-sensing *Drosophila* TRPA channels arose by duplication and divergence from a single TRPA1-like ancestor, with the three channels evolving to respond to distinct temperature ranges. Reflecting their evolutionary kinship to dTRPA1, Painless and Pyrexia also contain multiple N-terminal ankyrin repeats, although their ankyrin repeats are smaller in number (4 and 9, respectively) and divergent in sequence from those in TRPA1. As the molecular basis of thermal sensing by thermoTRP channels in general remains mysterious, it will be interesting to uncover whether common elements within dTRPA1, Pyrexia and Painless contribute to temperature sensing and to identify the mechanisms responsible for the apparent divergence in temperature threshold among these three channels.

## 11. Future directions in *Drosophila* thermal physiology

*Drosophila* have been productive model systems in genetics, physiology, neurobiology, and evolutionary biology, among others (Kornberg and Krasnow, 2000). Their short generation times, the ease with which they can be maintained in the lab, and their increasingly well understood genomes make these small flies ideal candidates for understanding evolutionary physiology of thermoregulation in ectotherms. Surprisingly little is known about the thermal biology of *Drosophila*. Temperature preferences for oviposition and thermal behavior of early and late stage larvae are largely unstudied (Sections 4, 5). We know the most about thermoregulatory behavior of adult *Drosophila*, but conclusions there are mixed, both because results seem to depend in part on the particular methodologies used to estimate thermal preferences (Section 2), and because of the many potential moderators of thermal preference that have been inadequately controlled in most studies (Section 6). There is also currently no general consensus on the plasticity of thermal preference in response to developmental and acclimation temperature, which have been well studied with regard to other traits in *Drosophila* (Section 7). Finally, perhaps the largest gap in our knowledge is in understanding the relationship between thermal preference and fitness. Despite some evidence that thermal preference is heritable, few studies have demonstrated evolved divergence in thermal

preference in the lab or in the field. In particular, we know next to nothing about the thermal ecology of *Drosophila* in the wild (but see e.g. Parsons, 1975; Taylor and Powell, 1977; Jones et al., 1987; Feder et al., 2000).

Now is an exciting time for advancement of the field, not only because of how little we know about thermal preference in *Drosophila*, but also because recent advances in understanding the neuromolecular underpinnings of thermal sensation raise an intriguing possibility. We are perhaps on the verge of understanding a exceedingly complex trait from the level of membrane ion channels (Hamada et al., 2008) to molecular pathways within cells in the fly brain (Hong et al., 2008) to behavior of individual organisms (Sayeed and Benzer, 1996) and the fitness effects of that behavior in the lab to evolutionary divergence of the behavior in the field.

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