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# Effects of natural temperature variation on male perception of female scents in *Drosophila melanogaster*

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Ecological factors can shape the processes and mechanisms underlying mate attraction. Growing evidence shows that temperature influences the biosynthesis, transmission and perception of semiochemicals involved in the transfer of information during reproduction (e.g. pheromones). However, little is known about how natural variation in temperature may affect chemically mediated mate attraction. In this study, whether and how temperature variation influences male attraction to female scents was examined in flies from a wild *Drosophila melanogaster* population. Chemosensory trials were conducted at a common temperature arena of 24 °C giving males a choice between a nonscented swab (control) versus a swab impregnated with female scents. Female scents were collected by allowing females to interact with cotton swabs for 6 h prior to tests at 20 °C or 28 °C, while control swabs were maintained at the same temperatures but without any females. Results indicated that males were only attracted to swabs bearing female scents maintained at 20 °C. In addition, chromatography–mass spectrometry was used to investigate whether female cuticular hydrocarbons, which are key molecules in mate attraction, changed depending on temperature (20 °C, 24 °C or 28 °C) and exposure time (6 or 12 h). Temperature and treatment duration showed a strong influence on female cuticular hydrocarbons. Furthermore, previously identified sexual pheromones with a role in mate attraction were not affected in a way that is consistent with the results from chemosensory trials. The results indicate that high temperatures may impair mate attraction, and this interaction may be partially mediated by the effects of temperature on female cuticular hydrocarbons. We discuss how these constraints on chemically mediated mate attraction may affect sexual selection processes and reproductive trade-offs.



Sexual selection is central to understanding the evolution of sexual adaptations and life-history strategies (Andersson, 1994), as well as the speciation and fate of species and populations (Candolin & Heuschele, 2008; Janicke et al., 2018; Servedio & Boughman, 2017). Given its fundamental role in shaping biological diversity, identifying the reason why the strength and direction of sexual selection vary markedly across species and environments is of great importance. In recent decades, growing evidence has highlighted the important role of ecological factors in modulating sexual selection (Emlen & Oring, 1977; Maan & Seehausen, 2011; Miller & Svensson, 2014), with ecological variation and environmental lability influencing sex-specific selection (De Lisle et al., 2018), sexual conflict (Londoño-Nieto et al., 2023; 2025; Perry et al., 2017) and the coevolution of male and female traits and life histories (Arnqvist et al., 2022). Yet, disentangling the mechanisms through which ecology shapes sexual selection is necessary to understand how sexual selection operates in nature and responds to environmental changes.

Temperature affects the physiology, morphology and behaviour of organisms, especially in ectotherms (Barbarossa et al., 2021; Kristensen et al., 2008; Monteiro et al., 2017; Nowakowski et al., 2018), generating strong selective pressures (Worm & Tittensor, 2018) that can shape animal communication (Bradbury & Vehrencamp, 2011). In particular, chemical communication, which is the widespread sensory channel for sexual signalling (Johansson & Jones, 2007), is sensitive to environmental temperature (Boullis et al., 2016; Groot & Zizzari, 2019; Roggatz et al., 2022). First, semiochemicals mediate the chemical interaction among organisms (Wyatt, 2014), and they are considered as metabolic products that can be generated de novo or synthesized from dietary precursors (Henneken et al., 2017; Johnston & Del Barco-Trillo, 2009). Therefore, temperature-induced effects on metabolism (Brown et al., 2004) or enzymatic activities (e.g.

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oxidases, reductases and desaturases) may alter their biosynthesis or release to the environment (Heathcote et al., 2014; Sentis et al., 2015). Second, temperature can degrade the molecular structure of secreted semiochemicals, thereby affecting long, heavy-chain compounds associated with short-distance interactions or xeric habitats, as well as short, light and highly volatile compounds that are used in long-distance communication or mesic habitats (Baeckens et al., 2018; Wyatt, 2014). Third, temperature may affect the perceptual processes involved in chemoreception (Boullis et al., 2016; Sentis et al., 2015). For example, temperature changes may impede the alignment between a pheromone and its binding protein, thereby affecting its transfer to olfactory receptor neurons (Weng et al., 2015). Given the crucial role of chemical communication in the interaction of many species (Buchinger & Li, 2023; Johansson & Jones, 2007), determining whether temperature alters the structure of semiochemicals and disrupts chemical communication is necessary to understand the dynamics of sexual selection in nature.

In general, insects produce scents made of a complex blend of chemicals (Blomquist & Bagnères, 2010; Howard, 1993), with hydrocarbons secreted onto the cuticle to prevent dehydration (Hadley, 1984; Harrison et al., 2012). Some of these cuticular hydrocarbons have been co-opted as chemical signals (Blomquist & Bagnè;res, 2010; Howard, 1993) such as sexual pheromones, which play a key role in sexual interactions (Johansson & Jones, 2007). In the wild, *Drosophila melanogaster* flies congregate around food patches, where they engage in courtship, mating and egg laying (Reaume & Sokolowski, 2006). Males must recognize conspecific females (Stocker, 1994) and strategically allocate their investment in reproduction as a function of female quality and sociosexual context (Byrne & Rice, 2006; Edward & Chapman, 2013). Chemical communication plays a central role throughout this process (Averhoff & Richardson, 1974; Everaerts, Lacaille, & Ferveur, 2010; Grillet et al., 2012; Savarit & Ferveur, 2002; Sharon et al., 2010). Males use visual stimuli and volatile pheromones to detect a potential female at a distance. Once at a closer range, they rely mainly on female cuticular hydrocarbons, which act as sexual pheromones or cues, to assess reproductive potential (e.g. fecundity) and to detect whether the female has previously mated (Byrne & Rice, 2006; Gowaty et al., 2003). Female 'attractiveness' is a critical factor in male mate selection (Laturney & Billeter, 2016; Pischeda et al., 2014). Thus, male assessment of female 'attractiveness' is critical for reproduction (Laturney & Billeter, 2016; Pischeda et al., 2014), and female cuticular hydrocarbons play an important role in male attraction at short and long ranges. In this context, several pheromones have been termed aphrodisiacs or courtship pheromones, if they stimulate courtship, or antiaphrodisiacs, if they inhibit it (Billeter & Wolfner, 2018; Ferveur, 1997, 2005, 2005). For example, 7,11-heptacosadiene, 7-pentacosene and 9-pentacosene are sexual pheromones produced by females that stimulate male courtship (Ferveur, 1997; Siwicki et al., 2005). By contrast, 5-tricosene and 7-tricosene are pheromones secreted by both sexes (although virgin females produce them in low quantities) that are transferred from males to females during mating to inhibit courtship of competitor males (Billeter & Wolfner, 2018; Ferveur, 1997, 2005). Given that changes in temperature can alter cuticular hydrocarbons in *D. melanogaster* (Bontonou et al., 2013; Savarit & Ferveur, 2002), cascading effects on chemically mediated mate attraction may affect reproduction in this species.

Here, whether environmental temperature can influence male attraction to female scents in *D. melanogaster* was investigated. Donor females were exposed to either 20 °C or 28 °C for 6 h inside vials containing swabs to collect their scent. These temperatures all fall within the natural thermal range experienced in the wild by the source population from which these laboratory flies originate (Londoño-Nieto et al., 2023). This procedure was repeated in vials with swabs but without females (i.e. control). Then, a choice experiment was conducted to assess male attraction to scented versus nonscented (control) swabs. In addition, gas chromatography–mass spectrometry (GC/MS) was used to assess whether and how female cuticular hydrocarbon profiles were affected by temperature (20 °C, 24 °C or 28 °C) at different exposure times (6 or 12 h).

## METHODS

### *Fly Stocks*

In October 2018, wild *D. melanogaster* were collected using banana traps in three wineries located in Requena, Spain: Bodegas Hispano + Suizas (GPS coordinates: 39.466128–1.149642), Pago de Tharsys (39.497834–1.122781) and Dominio de la Vega (39.515079–1.143757). Traps were set up inside the wineries and in nearby vineyards. After collection, flies were anaesthetized with $CO_2$, and field-collected females were isolated in vials with standard food. Females were allowed to lay eggs for 48 h, and then their eggs were incubated at 24 °C, with 60% humidity and a 12:12 h dark:light (LD) cycle, for 14 days to allow adult flies to emerge. Subsequently, the genital arch of F1 males from each of these isofemale lines was examined to rule out potential *D. simulans*, in which the size and exposure of the genital arch are greater than those in *D. melanogaster* (Chyb & Gompel, 2013; Faria & Sucena, 2017). Then, a permanent laboratory stock was set up by collecting three males and three females from each *D. melanogaster* isofemale line (a total of 276 flies from 46 isofemale lines) and releasing them into a population cage with a surplus of food medium supplemented with live yeast (i.e. population Vega-Libre [VG]). This population was maintained under overlapping generations, at an average temperature of 24 °C with daily preprogrammed fluctuations (±4 °C) approximating natural circadian temperature fluctuation in spring, 60% humidity, and a LD 12:12 h cycle (Pol Eko ST 1200 incubator). Our experiment was conducted from March to April 2019, using individuals from this VG population. In obtaining experimental flies, yeasted grape juice agar plates were initially introduced into the VG stock culture to induce female oviposition. Then, eggs were collected and incubated at 24 °C under controlled density conditions, by placing approximately 200 eggs on 50 ml of food in 250 ml bottles (Clancy & Kennington, 2001). Ice anaesthesia was used to collect emerging virgin flies within 6–7 h of eclosion. These experimental flies were used for chemosensory trials (focal males and donor females) and cuticular hydrocarbon analyses (donor females).

### *Chemosensory Trials*

In investigating whether temperature affects male attraction to female scents, behavioural assays were conducted in which focal males were given a choice between two target chemical stimuli: a nonscented cotton swab (i.e. control) and a cotton swab impregnated with scents from donor females (i.e. treated; Fig. 1).

Donor females and focal males were virgin and sexually mature (2–3 days posteclosion). The swabs used in our experiment were placed in a vial alone (i.e. control) or with 10 living females (i.e. treated). Both swabs were placed in an incubator (Pol Eko ST 1200) at either 20 °C or 28 °C for 6 h, a short-term thermal shift similar to that experienced by flies in the field. A 6 h treatment was selected to assess the potential effects of temperature change over at least half of the flies' active daily period (where 'activity' here is defined as the time outside their nocturnal rest phase; in spring, flies of this population experience approximately

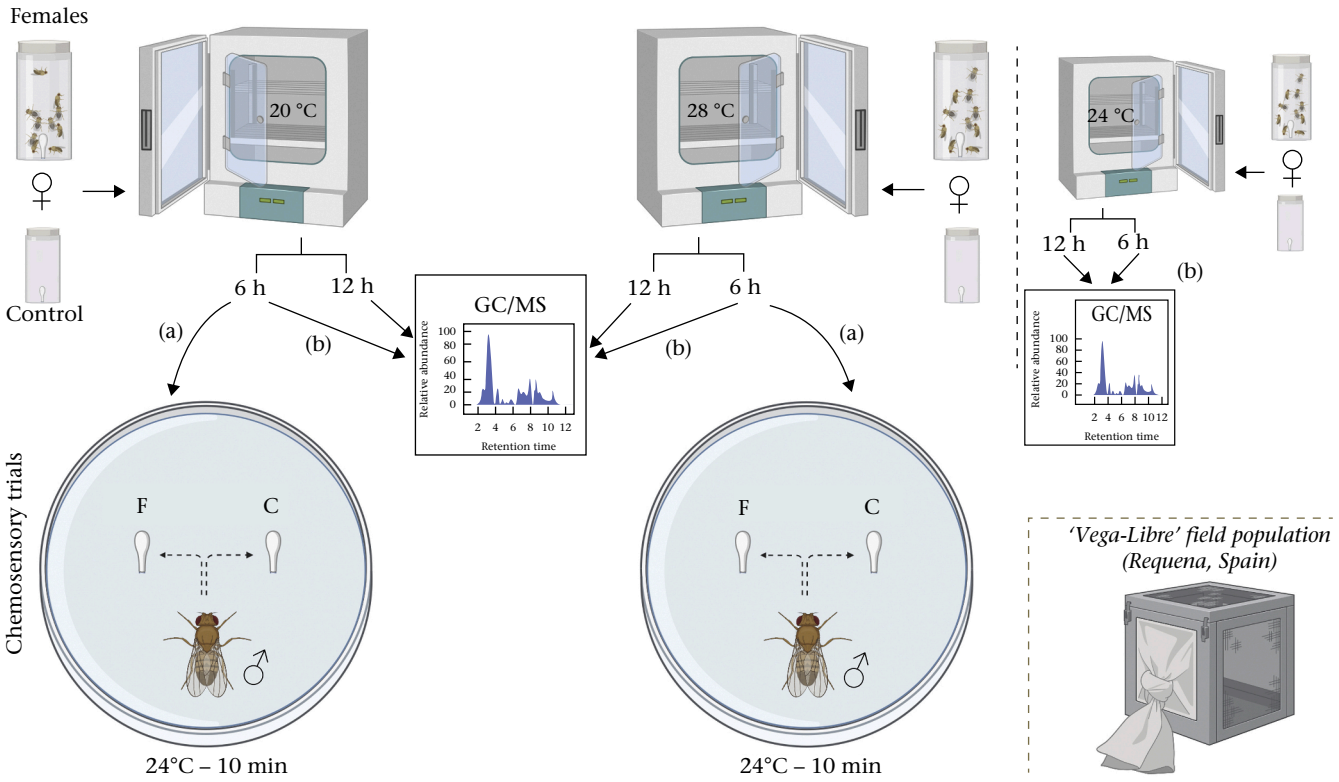


**Figure 1.** Overview of the experimental design. Virgin males and females were collected from a wild population of *Drosophila melanogaster* (Vega-Libre, Requena, Spain), and a population was established at the Ethology laboratory at the University of Valencia (Spain). (a) For the first experiment, females were exposed to two thermal environments (20 °C or 28 °C for 6 h) along with swabs to collect their odours. After this period, chemosensory trials were conducted at a common garden at 24 °C (mean temperature of the original population), where males were given a choice between a nonscented control swab ('C') and a swab impregnated with female scents ('F'). (b) In the second experiment, females were exposed to three thermal environments (20 °C, 24 °C or 28 °C) for 6 or 12 h, after which chromatography–mass spectrometry was used to study the mechanism by which the composition of female cuticular hydrocarbons varied across thermal environments.

12 h of daylight). Behavioural trials were conducted immediately after the end of the temperature treatment. The experimental arena consisted of a 50 mm nonsealed glass Petri dish inverted over a piece of filter paper. Petri dishes provide a flat, symmetrical and transparent surface that facilitates the accurate recording of fly behaviour (Nichols et al., 2012; Simon et al., 2012). Immediately before chemosensory trials, the swabs were placed in the centre of the arena equidistant from each other (with a separation of 2 cm) and from the sides of the dish. Each focal male was provided with its own, previously unused, pair of swabs (control versus treated), which prevented potential cross-contamination and degradation over time between trials. Immediately after introducing the swabs, the focal male (which was maintained at the same temperature as the stock population, that is, 24 °C) was introduced into the arena, and the assay started. All assays were conducted in a temperature-controlled room at 24 °C for 10 min, during which time an observer (A.C.) recorded the time in contact, the number of contacts and latency to the first contact to each swab in accordance with a blind protocol. Based on prior pilot tests with *D. melanogaster*, 10 min is a sufficient amount of time for males to detect and court females and female-scented swabs. Assays were carried out from 1700 to 2000 hours (afternoon peak of activity; Helfrich-Förster, 2000) for 10 days. Overall, swabs containing female scent rather than decapitated or immobilized females (Grossfield, 1972) were used to eliminate potential confounding effects from other sensory channels, such as visual cues related to body size, which in *Drosophila* can bias mate choice (Bontonou & Wicker-Thomas, 2014; Byrne & Rice, 2006). Furthermore, this method prevents chemical cues associated with female death from influencing male behaviour, as such cues have been shown to induce remarkable behavioural changes in conspecifics (Corbel & Carazo, 2022).

### *Cuticular Hydrocarbon Analyses*

In assessing temperature effects on female scents, GC/MS was used to analyse cuticular hydrocarbons in females that had been previously exposed (Pol Eko ST 1200 incubator) to different thermal environments: 20 °C, 24 °C or 28 °C for 6 or 12 h (Fig. 1). Here, a 12 h treatment was added to explore whether extended exposure to different treatment temperatures had a more pronounced/cumulative effect on cuticular hydrocarbons. Donor females were virgin and sexually mature (2–3 days posteclosion) at the time of temperature treatment. Immediately after the treatment, the flies were preserved in hexane, in groups of five females per sample, for subsequent analyses. A total of 100 µl of hexane spiked with 1 µg/ml of n-docosane D66 was used as internal standard in an orbital agitator (Reax 2, Heidolph, Germany) for 15 min/sample. The liquid phase was collected in a Pasteur pipette and transferred into a conical glass insert for GC/MS. For GC/MS analyses, an Agilent 6890N gas chromatograph with a HP-5MS UI Agilent capillary column (30 mm × 0.25 mm × 0.25 µm) coupled to a mass spectrometer equipped with a triple quadrupole analyser (TSQ Quantum XLS, Thermo Fisher Scientific; Bremen, Germany) was used for quantification. Using an Agilent 7683B auto injector, 1 µl of sample was introduced into a 270 °C inlet operated in splitless mode. The split valve was turned on after 2 min. The following oven temperature program was used: 50 °C for 1 min, increased at 40 °C/min to 150 °C, then increased at 5 °C/min to 320 °C, followed by a 9 min hold. Helium was used as the carrier gas at a constant flow (1.4

mL/min). Positive electron ionization at 70 eV with default temperature settings (ion source at 150 °C, quadrupole at 230 °C) was used for the mass selective detection system. Ions were detected in scan mode in the range of 33–450 m/z. The transfer line was maintained at 300 °C. Compounds were identified by comparing the retention times of chromatographic peaks with those of authentic standards (when available) run under the same conditions and by matching mass spectra with the data system library (score of over 80% from the library; NIST, 2017 software, Mass Spectral Search Program). Kovat's retention index values with a C16–C44 (ASTM D5442 standard) n-alkanes series were used and compared with those reported in the literature for similar columns (Farine et al., 2012). The analyses were conducted at the Advanced Research Facilities of the University of the Basque Country (SGIker, UPV-EHU, Spain).

### *Statistics*

In analysing the data from chemosensory trials, a model with 'time in contact', 'number of contacts' and 'latency' as response variables; temperature (20 °C or 28 °C), swab treatment ('female scent' or 'control') and their interaction as fixed factors and 'individual' and 'day' as random factors was built. In estimating the significance of terms, likelihood ratio tests were performed using ANOVA with a chi-square test comparing full versus reduced models, which is a standard approach for mixed-effect models when accounting for random factors such as 'individual' and 'day' (Zuur et al., 2009). Sample sizes were $N_{20\ °C} = 26$ males (260 donor females) and $N_{28\ °C} = 31$ males (310 donor females). R.3.2.4 software (Team, 2018) and package lmer (Bates et al., 2007, 2015) were used for these analyses. In testing for differences in cuticular hydrocarbon profiles among temperatures, duration of treatments and their potential interaction, the canonical coordinate scores of cuticular hydrocarbon profiles were estimated by using PRIMER V6.1.13, which is implemented in the PERMANOVA þ V1.0.3 add-on package (Anderson et al., 2008; Anderson & Willis, 2003M. J. Anderson & Willis, 2003). PERMANOVA þ V1.0.3 was also used to check the clustering of the samples in accordance with temperature and duration. Sample sizes were $N_{20\ °C–6\ h} = 28$ samples (140 females), $N_{20\ °C–12\ h} = 27$ samples (135 females), $N_{24\ °C–6\ h} = 28$ samples (140 females), $N_{24\ °C–12\ h} = 26$ samples (130 females), $N_{28\ °C–6\ h} = 27$ samples (135 females) and $N_{28\ °C–12\ h} = 26$ samples (130 females).

### *Ethical Note*

*Drosophila melanogaster* is not under ethical regulation in Spain and the ASAB Ethical Committee/ABS Animal Care Committee Guidelines for the ethical treatment of nonhuman animals in behavioural research and teaching were followed for the treatment of flies during the study. During the experiments, we followed well-established protocols in our laboratory that try to minimize stress to the greatest extent possible.

## RESULTS

### *Effects of Temperature on Attractiveness of Female Scents*

A marginally nonsignificant interaction was detected between temperature and swab treatment (female scents versus control) for the time in contact with each stimulus ($\chi^2_1 = 3.796$, $P = 0.051$; Fig. 2), along with a main effect of swab treatment ($\chi^2_1 = 7.964$, $P = 0.005$) but not of temperature ($\chi^2_1 = 0.458$, $P = 0.498$). Given the likelihood of an interaction, separate analyses were run for each temperature. A clear swab treatment effect was observed at 20 °C,

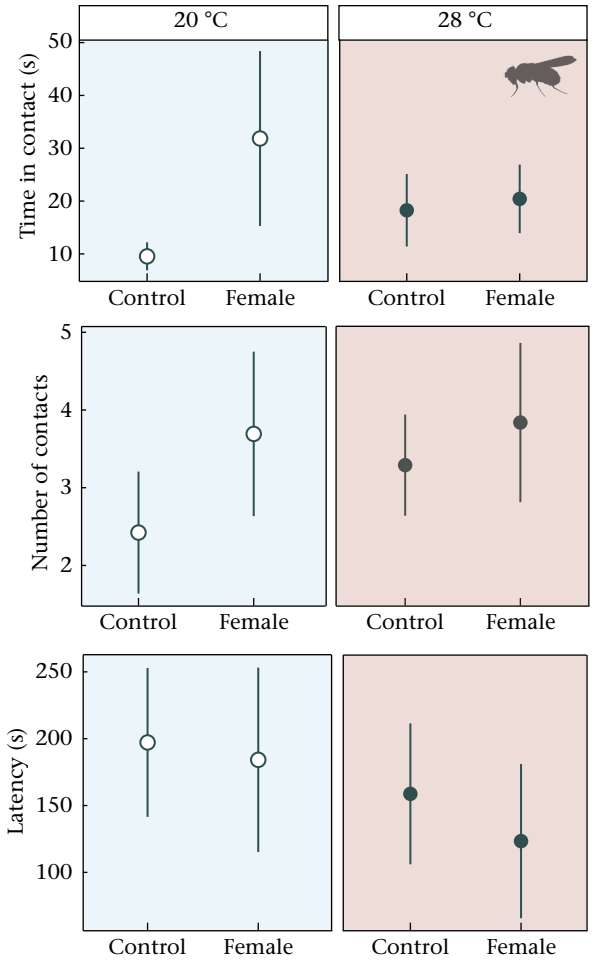


**Figure 2.** Results (mean ± SEM) from chemosensory trials conducted at 24 °C where males could choose between a nonscented control swab and a swab impregnated with odours from donor females kept at either 20 °C or 28 °C for 6 h. The time that males spent in contact with each stimulus (herein referred to as 'time in contact'), the number of interactions with each stimulus (herein referred to as 'number of contacts') and the time that males lasted to make their first contact with each stimulus (herein referred to as 'latency' in seconds) were measured.

such that males spent more time in contact with swabs bearing female scents ($\chi^2_1 = 10.781$, $P = 0.001$), but no significant differences were observed at 28 °C ($\chi^2_1 = 0.764$, $P = 0.382$). In addition, separate analyses were performed on the number of contacts with each stimulus for each temperature, and a significant effect was found at 20 °C ($\chi^2_1 = 5.313$, $P = 0.021$) but not at 28 °C ($\chi^2_1 = 0.481$, $P = 0.487$). However, in this case, no significant temperature × swab treatment interaction was detected in the full model ($\chi^2_1 = 2.117$, $P = 0.145$). Finally, neither a significant interaction for latency to the first contact ($\chi^2_1 = 0.147$, $P = 0.70$), nor main effects of either swab treatment ($\chi^2_1 = 1.307$, $P = 0.252$) or temperature ($\chi^2_1 = 0.147$, $P = 0.7007$) were detected (Fig. 2).

### *Effects of Temperature on Cuticular Hydrocarbons*

PERMANOVA showed a clear effect of temperature (Pseudo $F_{2,159} = 9.210$, $P = 0.001$) and treatment duration (Pseudo $F_{3,159} = 3.143$, $P = 0\ 0.001$) on cuticular hydrocarbon profiles (Table S1). When the clustering of the samples was tested on the basis of temperature and treatment duration, 77.16% and 70.37% of the samples, respectively, were accurately classified (temperature: permutational test, $\delta^2_1 = 0.585$, $P = 0.001$; duration: permutational

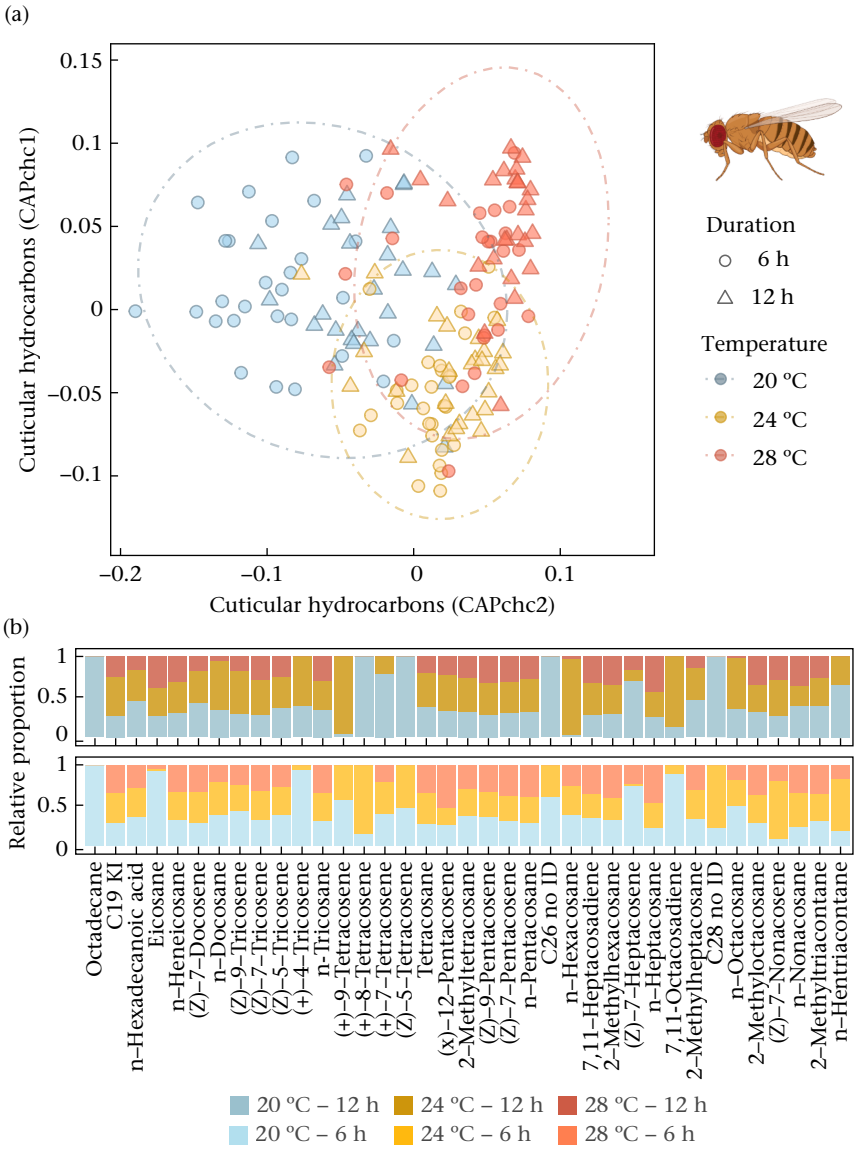


**Figure 3.** Effects of temperature on the cuticular hydrocarbon profiles of female wild *Drosophila melanogaster*. (a) Two-dimensional score plot illustrating the clustering of canonical coordinate values (CAP1 and CAP2, herein referred to as 'CAPchc1' versus 'CAPchc2'), which result from the analyses of the cuticular hydrocarbon profiles of females exposed at 20 °C, 24 °C or 28 °C for 6 or 12 h. (b) Detected semiochemicals through chromatography–mass spectrometry and their relative proportion (against n-docosane D66 as internal standard) across various temperatures and treatment durations.

test, $\delta^2_1 = 0.433$, $P = 0.001$; Fig. 3). LM analyses performed using the canonical variables extracted from PERMANOVA showed a clear interaction between temperature and duration ($CAP_{chc1}$; $F_{2,156} = 9.23$, $P < 0.001$; $CAP_{chc2}$; $F_{2,156} = 4.044$, $P = 0.019$). Main effects of temperature ($CAP_{chc1}$; $F_{2,156} = 153.54$, $P < 0.001$; $CAP_{chc2}$; $F_{2,156} = 62.052$, $P < 0.001$) and treatment duration ($CAP_{chc1}$; $F_{1,156} = 42.985$, $P < 0.001$; $CAP_{chc2}$; $F_{2,156} = 5.862$, $P = 0.016$) on cuticular hydrocarbon profiles were also detected. Post hoc tests showed significant differences among temperatures, as well as among treatment durations (Table 1). Using principal component analysis (PCA), similar results were obtained. A significant interaction was observed between temperature and duration for PC1 ($F_{2,156} = 9.23$, $P = 0.025$; 12.68%) but not for PC2 ($F_{2,156} = 0.073$, $P = 0.93$; 10.78%). A heatmap illustrates the relative weight of each chemical to the axis (Fig. S1; Table S2).

After analysing the potential interaction between temperature and treatment duration for compounds previously suggested to mediate male courtship as 'aphrodisiacs' (7,11-heptacosadiene, 7-pentacosene and 9-pentacosene) and 'antiaphrodisiacs' (7-tricosene and 5-tricosene), no significant interaction effects were found for any of the compounds (Fig. S2 and S3). However, main effects of temperature on 5-tricosene and 7-tricosene, as well as effects of treatment duration on 7-pentacosene and 9-pentacosene, were detected. In particular, neither an interaction for 7,11-heptacosadiene ($F_{2,156} = 0.403$, $P = 0.214$), nor main effects (temperature; $F_{2,\ 156} = 1.572$, $P = 0.211$; duration; $F_{1,\ 156} = 1.863$, $P = 0.174$) were found. For 7-pentacosene, no significant interaction ($F_{2,156} = 2.389$, $P = 0.095$) or temperature effect ($F_{2,\ 156} = 2.192$, $P = 0.115$) was detected, but an effect for treatment duration was found ($F_{1,\ 156} = 7.277$, $P = 0.007$), with higher abundances at longer treatment durations. This was also the case for 9-pentacosene (temperature × treatment duration: $F_{2,156} = 2.279$, $P = 0.105$; temperature: $F_{2,156} = 2.59$, $P = 0.078$; duration: $F_{1,\ 156} = 5.982$, $P = 0.015$). In the case of 5-tricosene, no significant interaction was detected ($F_{2,156} = 2.628$, $P = 0.075$), but in this case both main effects were significant (temperature; $F_{2,156} = 12.086$, $P < 0.001$;

**Table 1**
Results from post hoc tests assessing potential differences in the two canonical coordinate values (CAP1 and CAP2, herein referred to as 'CAPchc1' versus 'CAPchc2') obtained from the canonical analyses of the cuticular hydrocarbon profiles of wild *Drosophila melanogaster* that were exposed to different thermal environments (20 °C, 24 °C or 28 °C) for 6 or 12 h

| Treatments | CAPchc1 | | | $CAP_{chc2}$ | | |
|---|---|---|---|---|---|---|
| | *Z* | *P* | Estimate | *Z* | *P* | Estimate |
| 20 °C/28 °C | −16.795 | <0.001 | −0.108 ± 0.006 | −3.113 | 0.0062 | −0.022 ± 0.007 |
| 20 °C/24 °C | −12.430 | <0.001 | −0.079 ± 0.006 | 7.762 | <0.001 | 0.056 ± 0.007 |
| 28 °C/24 °C | 4.399 | 0.001 | 0.028 ± 0.006 | 10.788 | <0.001 | 0.079 ± 0.007 |
| 12 h/6 h | −6.510 | <0.001 | −0.034 ± 0.005 | −2.464 | 0.0148 | −0.014 ± 0.00596 |

duration; $F_{1, 156} = 9.066$, $P = 0.003$), with a larger abundance of this compound at higher temperatures and after shorter treatment duration. Finally, for 7-tricosene, no interaction between temperature and duration ($F_{2,156} = 0.403$, $P = 0.337$) or for treatment duration ($F_{1, 156} = 0.195$, $P = 0.659$) was detected, but temperature effects were significant (temperature; $F_{2, 156} = 4.503$, $P = 0.012$). The results of post hoc tests are presented in Table S3, where significant differences varied depending on the compounds and treatments.

## DISCUSSION

This study shows that temperature can affect male attraction to female scents in *D. melanogaster* within the natural reproductive thermal range for the studied population. In an experimental arena maintained at 24 °C, males were more attracted to female scents versus controls when female donors and cotton swabs had been kept at 20 °C but not at 28 °C. In addition, female cuticular hydrocarbons differ in their composition depending on the thermal environment they had been exposed to.

In *D. melanogaster*, reproduction is guided by chemical, visual, tactile and acoustic signals. This study focused on chemical communication, which plays a major role in triggering and stimulating courtship in this species (Ferveur, 1997; Savarit et al., 1999). Overall, the thermal environment experienced by females can influence male attraction, such that males were attracted (spent more time versus control) to female scents at 20 °C but not at 28 °C. As shown in previous studies (Kuo et al., 2012; Markow & Toolson, 1990), time in contact with a chemical stimulus is a suitable proxy for assessing male attraction in *D. melanogaster*, as it correlates closely with male efforts in courtship and harassment to females (Bastock & Manning, 1955). Similarly, temperature can affect the number of contacts to female scents. Overall, our results indicate the potential role of environmental temperature in shaping chemically mediated male attraction. These findings are relevant for two main reasons.

First, these results indicate that temperature may act as a modulator of mate attraction through chemical communication, with potential consequences on sexual selection (Andersson, 1994; Andersson & Simmons, 2006). If female scents are less effective in attracting mates at warmer temperatures, then males may struggle to locate females, and when they do, they may invest less time and suboptimal energy in the process, with cascading effects on the intensity of intersexual (and potentially intrasexual) competition. At a precopulatory level, impaired mate attraction may hamper the efficient evaluation of potential candidates for mating (Edward, 2015; Johansson & Jones, 2007), thereby reducing the relative fitness of high-quality individuals and weakening sexual selection (Candolin, 2019; Cotton et al., 2006). In addition, reduced mate attractiveness might reduce sexual conflict by decreasing harassment to females, which can impact female fitness (Rice et al., 2006). At a postcopulatory level, if mate attraction is impaired at hot temperatures, then the number of matings/rematings would likely decrease, thereby lowering sperm competition levels (Bretman et al., 2009, 2010) and the strength of sexual selection (Andersson & Simmons, 2006).

Second, our results indicate that temperature may influence the cost of reproduction through its effects on chemical communication. In particular, weaker mate attraction can increase search and interaction costs (Bateson, 1983), leading to higher allocation of time and energy in mate searching, concomitantly higher reproduction costs or a potential decrease in the number of interactions with potential mating partners. This interaction may also affect mate choice by restricting the options to choose from (Andersson & Simmons, 2006). Thus, high temperatures may impose constraints on chemically mediated mate attraction, which may affect sexual selection processes in *D. melanogaster*. Further research must investigate whether flies can plastically modulate their reproductive behaviour to overcome/compensate for such constrains. In our experiment, female scent donors were in direct contact with the swab during temperature treatment. Consequently, temperature could influence the production and deposition of chemical compounds, via its effects on female metabolism, and the chemical compounds already present on the swab throughout the 6 h treatment. Both effects could be relevant, as temperature-induced changes in females and their scents likely occur together in nature. Moreover, the scents captured in the swabs contain not only cuticular hydrocarbons but also alternative sources of semiochemicals, such as faeces, which have been shown to play a role in social aggregation (Duménil et al., 2016; Keesey et al., 2016).

Finally, as temperature has been shown to influence the composition of cuticular hydrocarbons in *D. melanogaster* (Krupp et al., 2020; Rajpurohit et al., 2021), this study aimed to determine whether and how the range of temperatures used in our experiment might affect female scents, either by modulating their production or by altering the molecular structure of cuticular hydrocarbons. This objective is particularly relevant given the role of some cuticular hydrocarbons as sexual pheromones, for example, by attracting potential mates, stimulating courtship and mating or inducing female oviposition (Dweck et al., 2015; Ferveur, 1997, 2005). Our analyses of female cuticular hydrocarbons indicate that their composition varied depending on temperature and the duration of exposure to different thermal environments. However, the potential disruption of mate attraction at warm temperatures (i.e. 28 °C) could not be explained by temperature effects on some previously identified sexual pheromones (Billeter & Wolfner, 2018; Ferveur, 1997, 2005). Although the presence of 9-pentacosene (an aphrodisiac pheromone that serves as a synergistic stimulator of 7-pentacosene) decreased at 28 °C, temperature did not induce substantial changes in either 7,11-heptacosadiene or 7-pentacosene. The lack of temperature effects on these two stimulators of courtship could be due to the fact that these stimulators are long-chain heavy cuticular

hydrocarbons with a relatively high melting temperature (Bontonou et al., 2013; Gibbs et al., 1997). A temperature effect was detected on antiaphrodisiacs 7-tricosene and 5-tricosene (short-chained compounds) but not in the expected direction, that is their abundance decreased at 28 °C, where mate attraction was disrupted in our assays. Given their known effects as inhibitors of courtship (Billeter & Wolfner, 2018; Ferveur, 1997, 2005), their lower abundance at warmer temperatures should increase male attraction, not the opposite (as we observed). Nevertheless, virgin females (such as those in our experiment) produce relatively low amounts of these two antiaphrodisiacs, with a significant increase of their levels (and function) only after mating, because of a mechanical exchange from males to females (Everaerts et al., 2010a, 2010b).

Although the role of the abovementioned sexual pheromones is well established in *D. melanogaster* (Everaerts et al., 2010a, 2010b; Ferveur, 1997, 2005), the function of most cuticular hydrocarbons remains uncertain. The results indicate that most cuticular hydrocarbon profiles respond to thermal variation. We hypothesize that some of them, alone or in combination, might also participate in mate attraction. In addition, GC/MS techniques can capture a wide spectrum of chemicals present in *D. melanogaster* cuticle (and other insects). However, not all of them, such as solid-phase microextraction or laser desorption/ionization mass spectrometry, can identify another range of chemicals, and some of them are invisible for GC/MS (Everaerts et al., 2010; Kuo et al., 2012). These other chemicals, if affected by temperature, could also shape male attraction by female scents.

This study shows that temperature changes, even within the natural reproductive range of a wild population, thereby affecting chemical communication in *D. melanogaster* and mate attraction. In nature, such changes in temperature can determine how and when mate attraction unfolds across different temporal (circadian rhythms, seasonal cycles or between seasons) and spatial scales, including micro- and macrogeographical variation. Consequently, the dynamics of sexual selection could be modulated. This work highlights the importance of understanding the mechanism by which the complexity of naturally changing environments may impact chemical communication and sexual selection processes in nature.

## Author Contributions

**Roberto García-Roa:** Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Almudena Castro:** Investigation. **Enrique Font:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Pau Carazo:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

## Data Availability

Data associated with this article are available in the Dryad digital repository at https://doi.org/10.5061/dryad.fbg79cp6r.

## Declaration of Interest

The authors declare no competing financial interests.

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## Supplementary Material

Supplementary material associated with this article is available at https://doi.org/10.1016/j.anbehav.2025.123352.

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