Interspecies variation of larval locomotion kinematics in the genus *Drosophila* and its relation to habitat temperature

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Abstract

Speed and trajectory of locomotion are characteristic traits of individual species. During evolution, locomotion kinematics is likely to have been tuned for survival in the habitats of each species. Although kinematics of locomotion is thought to be influenced by habitats, the quantitative relation between the kinematics and environmental factors has not been fully revealed. Here, we performed comparative analyses of larval locomotion in 11 *Drosophila* species. We found that larval locomotion kinematics are divergent among the species. The diversity is not correlated to the body length but is correlated instead to the minimum habitat temperature of the species. Phylogenetic analyses using Bayesian inference suggest that the evolutionary rate of the kinematics is diverse among phylogenetic trees. The results of this study imply that the kinematics of larval locomotion has diverged in the evolutionary history of the genus *Drosophila* and evolved under the effects of the minimum ambient temperature of habitats.

Keywords: animal locomotion, larval crawling, the genus Drosophila, kinematics, evolution

Introduction

Kinematics of animal locomotion is a critical trait enabling each species to survive in their habitats (Alexander, 2006). Movement patterns have been sculpted during their evolution by adaptation to their environments, and could have diverged among species (Kappeler, 2010). Comparative analyses have identified several examples of differences in the kinematics of locomotion within a group of related species, including insects (Theunissen et al., 2015), reptiles (Fuller et al., 2011), birds (Stoessel & Fischer, 2012), and primates (Thompson et al., 2018). In general, differences in locomotion among species have been attributed to the morphological differences and/or the ecological diversity among species. An example of morphological differences is found in three stick insect species with distinct body proportions but living in similar feeding environments, which show different kinematics in the

legged locomotion (Theunissen et al., 2015). In contrast, as an example of ecological diversity, two gecko species inhabiting either sandy or rocky environments have been shown to exhibit distinct postures (Fuller et al., 2011). Whereas the interspecies divergence in locomotion patterns can be observed in various phylogenetic branches of the animal kingdom, quantitative comparative analyses of locomotion kinematics remain limited.

Flies of the genus *Drosophila* have long been used as a model to study interspecific diversification and evolution (Markow & O'Grady, 2005). One remarkable trait diversified in the genus *Drosophila* is the food they eat: larvae in the subgenus *Sophophora* in the genus *Drosophila* eat fruit, whereas larvae in the subgenus *Drosophila* in the genus *Drosophila* feed on vegetables (Markow, 2015; Watada et al., 2020; Watanabe et al., 2019). Some eat multiple kinds ("generalists" including *Drosophila melanogaster*) while others have strong preferences in food ("specialists" including *Drosophila sechellia*) (Markow, 2015; Watada et al., 2020; Watanabe et al., 2019). These diverged species in the genus *Drosophila* offer an opportunity to perform interspecific comparisons of locomotion. Fly larvae, or maggots, have been widely used in the study of the kinematics of locomotion (Berrigan & Lighton, 1993; Berrigan & Pepin, 1995; Fox et al., 2006; Green et al., 1983; Ruiz-Dubreuil et al., 1996; Sokolowski, 1985; Sokolowski et al., 1997). Among the fly species, *Drosophila melanogaster* (*Dmel*) is one of the most examined species, especially by virtue of the availability of resources in genetics and connectomics (Ohyama et al., 2015; Schneider-Mizell et al., 2016; Simpson & Looger, 2018; Venken et al., 2011). *Dmel* larvae locomote by a sequence of forward crawling, and changes of crawling direction achieved by bending their bodies (Green et al., 1983). The kinematics of the larval crawling can be adaptively changed. Natural populations of *Dmel* larvae possess a polymorphism in the crawling behaviour: larvae of "rover" strains crawl a large area in feeding, whereas larvae of "sitter" strains stay in a patch of food during feeding (Sokolowski, 1980). The gene responsible for this polymorphism is mapped to the foraging gene, which encodes a cyclic guanosine monophosphate-dependent protein kinase (Osborne et al., 1997). Ambient temperature is another factor affecting the kinematics of

68 larval locomotion (Kwon et al., 2010; Liu et al., 2003; Ohyama et al., 2013; Rosenzweig et
69 al., 2005a). In thermotaxis behaviour in temperature gradient environments, larvae regulate
70 the length of crawling runs between turns and the size and direction of turns (Luo et al., 2010),
71 and the probability of turns is also affected by ambient temperature gradients (Klein et al.,
72 2015). Cellular mechanisms in larval temperature sensation have been clarified. Different
73 temperatures are detected by specialized sensory organs and receptor proteins (Dillon et al.,
74 2009). Cold temperatures are sensed by two ganglia (the dorsal organ ganglion and the
75 terminal organ ganglion) (Klein et al., 2015; Liu et al., 2003; Ni et al., 2016a), and warm
76 temperatures are detected by dTRPA1-positive neurons in the brain (Rosenzweig et al.,
77 2005a). Some multi-dendritic (md) neurons in the body wall detect cold temperatures, while
78 other md neurons are sensitive to warm temperatures (Liu et al., 2003). Compared with this
79 remarkable level of understanding of the regulation of crawling behaviour in *Dmel* larvae,
80 locomotion kinematics in the larvae of sister species in the genus *Drosophila* remains unclear.
81 Here we conducted an interspecies comparison of the kinematics of larval
82 locomotion in the genus *Drosophila*. We address two questions in this study: are locomotion
83 kinematics of larvae similar among *Drosophila* species?; and, if the kinematics are diverged,
84 are morphological characteristics and/or ecological differences related to the diversity? To
85 this aim, we recorded locomotion of larvae of 11 *Drosophila* species and extracted kinematic
86 parameters using the tracking software FIMTrack (Risse et al., 2017). Clustering analysis
87 with Jensen-Shannon divergence and statistical analyses show that two kinematics
88 parameters (bend probability and crawling speed) are diverged among the *Drosophila*
89 species. We found that the minimum habitat temperature of each species is related to the
90 kinematics rather than the size of larvae. The relationship between the kinematics and
91 minimum habitat temperature held at two distinct ambient temperatures: 24°C and 32°C.
92 Phylogenetic analyses of these kinematics, based on Bayesian inference (Hohna et al.,
93 2016), suggests that the rate of evolution of the kinematics is diverged among phylogenetic
94 branches. Among eight kinematics parameters we tested, the crawling speed at 24°C and

32°C were suggested to have correlatively evolved. Consequently, our results suggest that the kinematics of larval locomotion in the genus *Drosophila* evolved under the effects of minimum ambient temperature in their habitats, and diverged in the evolutionary history.

Results

Kinematic analysis of crawling and bending behaviour in *Drosophila* larvae

In this study, we analyzed the kinematics of larval locomotion. To this aim, we recorded the locomotion of fly larvae that were crawling freely on an agarose substrate stage on a temperature-controlled plate (Figure 1A; see Method section for details). We placed eight to ten larvae at the centre of the stage, which was kept at 24°C, illuminated them with infrared light, which is invisible to fly larvae and does not affect their behaviour, and recorded larval locomotion for three minutes at five frames per second (Figure 1B). Maximum projection of the time-series images shows traces of larval locomotion as multiple curves of multiple larvae (Figure 1C). In an example of *Drosophila melanogaster* (Figure 1D), the traces show smooth curved lines interconnected with angles where larvae exhibit turning behaviour and change crawling direction, which is consistent with previous studies (Berni et al., 2012; Gershow et al., 2012; Gomez-Marin et al., 2011; Gomez-Marin & Louis, 2014; Green et al., 1983; Sims et al., 2019; Tastekin et al., 2015). Larvae under these conditions predominantly exhibit forward locomotion in which muscular contraction is propagating from the posterior to anterior segments. Characteristics of larval locomotion can be described by two measures: the bend angle and centroid speed (Gershow et al., 2012; Gomez-Marin et al., 2011). The bend angle measures the angle of body axis bending (Figure 1E) and the centroid speed is the speed of the position of the larval centroid (Figure 1F). To obtain these values for each larva at each time frame, we used the object tracking software for small animals, FIMTrack (Risse et al., 2017). The turning behaviour can be detected by the change in the angle of the body axis (Figure 1E) and the reduction in the centroid speed (Figure 1F), as reported previously (Gershow et al., 2012; Gomez-Marin et al., 2011). Accordingly, we

used the bend angle and centroid speed for quantitative analysis of larval locomotion in this study.

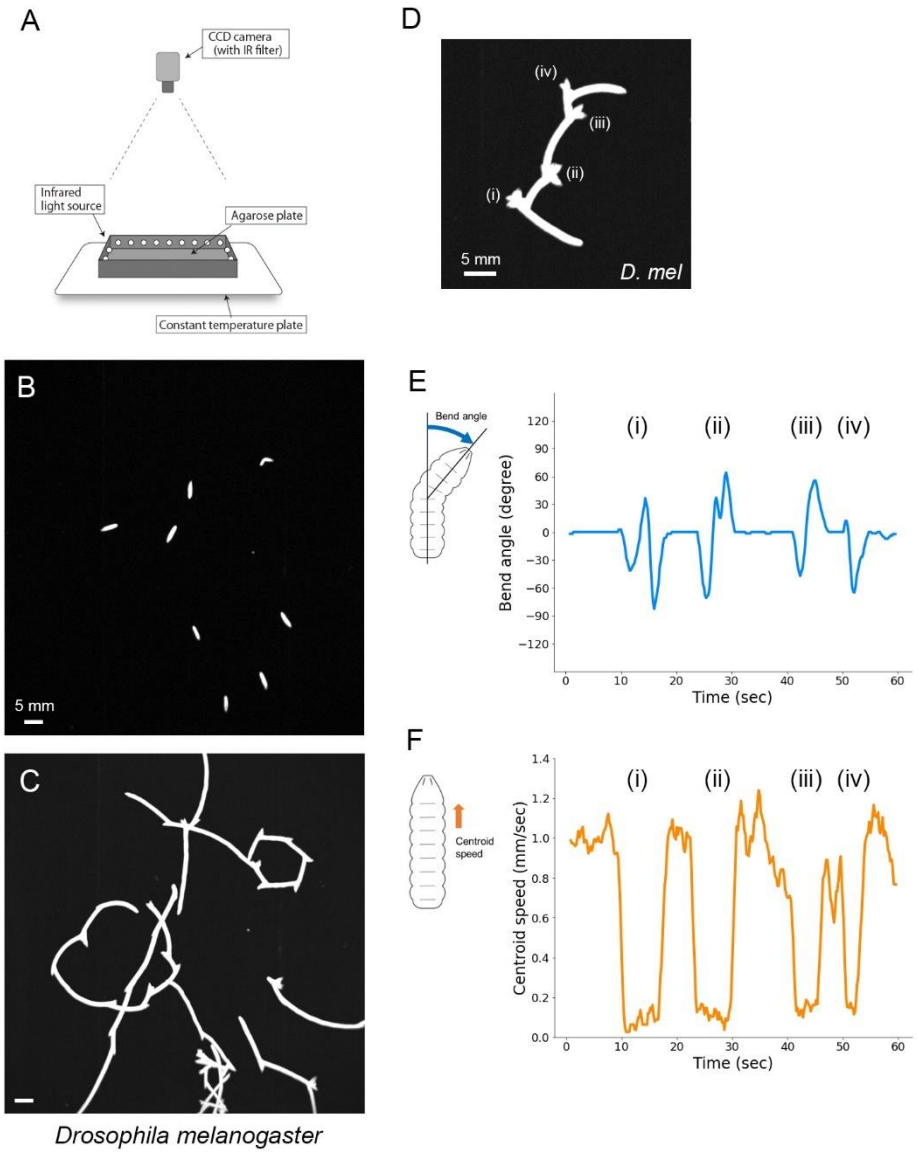
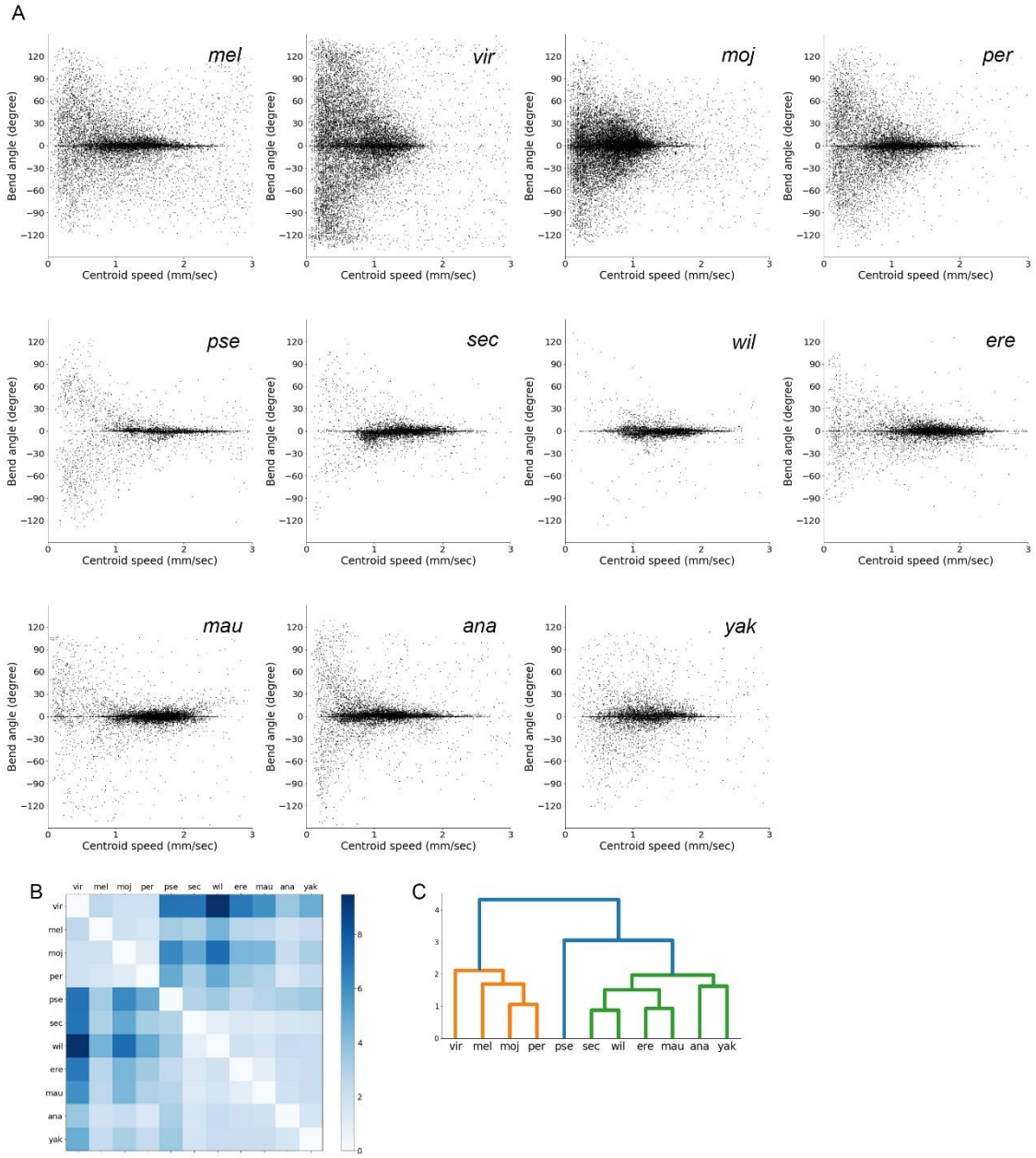


Figure 1. Measurement of larval crawling. (A) Setup of the recording of larval crawling with infrared light and temperature control plate. (B) An example image of multiple larvae recorded by the setup (A). (C) The trajectories of larval locomotion of *Drosophila melanogaster* recorded for two minutes. (D) A trajectory of single *Drosophila melanogaster* locomotion.

130 Locations where the larva changes its direction are labelled (i) to (iv). (E and F). Bend angle
131 (E) and centroid speed (F) of the larva in (D). Labels (i) to (iv) correspond to those in (D).
132
133 **Classification of locomotion properties in the genus *Drosophila***
134 For interspecies comparison of *Drosophila* larval locomotion, we collected 11 fly
135 species in the genus *Drosophila*, whose genome sequences have been read (A. G. Clark et
136 al., 2007; Miller et al., 2018), and for which living individuals are available from fly stock
137 centres (KYORIN-Fly, Fly Stocks of Kyorin University; KYOTO Stock Center (DGRC) at the
138 Kyoto Institute of Technology). The 11 *Drosophila* (*D.*, hereafter) species consist of *D.*
139 *ananassae* (*Dana*)*, D. erecta* (*Dere*)*, D. mauritiana* (*Dmau*)*, D. melanogaster* (*Dmel*)*, D.*
140 *mojavensis* (*Dmoj*)*, D. persimilis* (*Dper*)*, D. pseudoobscula* (*Dpse*)*, D. sechellia* (*Dsec*)*, D.*
141 *willistoni* (*Dwil*) *and D. yakuba* (*Dyak*). We plotted the centroid speed and bend angle of freely
142 crawling larvae of each species (Figure 2A). In all the cases, data points accumulated around
143 a region where the bend angle is zero (Figure 2A), which reflects an observation that larvae
144 do not bend during the majority of the time (Figure 1D). We noticed that the deviations from
145 zero in bend angle axis are different among species. For example, data points in *Dvir* are
146 scattered along the bend angle axis more than those in *Dwil* (Figure 2A). To quantify
147 similarities in the distribution of the two-dimensional plots among the species, we calculated
148 Jensen-Shannon divergence, which measures the similarity of two probability distributions
149 (Lin, 1991). We classified the 11 species based on the similarity in the probability distribution
150 of the crawling speed and bend angle plots by hierarchical clustering (Figure 2B and 2C).
151 The classification analysis shows that four species (*Dmel, Dvir, Dmoj and Dper*) form a cluster.
152 These four species show scattered data points along the bend angle axis compared with the
153 other seven species (Figure 2A). This observation suggests that the kinematics of larvae in
154 the genus *Drosophila* is diverse among species.



155

156 **Figure 2. Interspecific comparison of kinematics of larval crawling in the genus**
157 ***Drosophila*.** (A) Plots of the speed of the centroid of larvae and the bend angle in the 11
158 *Drosophila* species. Each point corresponds to a datum of a single larva of a species in a
159 single time frame. (B) Jensen-Shannon divergences of the probability density of (A). (C)
160 Hierarchical clustering of the kinematics of larval locomotion of the 11 *Drosophila* species.

161

162 **Definition of the bend probability and crawling speed**

163 The clustering analysis suggests that the kinematics is divergent among species.
164 To interpret the diversity in terms of locomotion behaviour, we defined two indices: the bend
165 probability and crawling speed. The bend probability measures how often larvae bend their
166 body laterally. We set the minimum angle of bend as 20 degrees, which was used previously
167 (Risse et al., 2013), and labelled larvae that bent more than this threshold angle to the right
168 or left side as "Bending" (Figure 3A and 3B). This threshold allowed us to extract the
169 difference in the frequency of bend among the species we examined, and we found that while
170 *Dmel* larvae exhibit larger bend angles than this threshold (Figure 3A), the bend angles in
171 *Dwil* larvae are mostly less than the threshold (Figure 3B). We defined the bend probability
172 of each single larva as a ratio of the number of time frames labelled "Bending" to the total
173 frame number. To define the second index crawling speed, we labelled larvae that bent less
174 than the threshold as "Crawling" (Figure 3A and 3B). We defined the median of centroid
175 speeds of larvae that were labelled "Crawling" as the crawling speed for each single larva.
176 By these definitions, we calculated the bend probability and crawling speed of each larva of
177 the species.

178

179 **Diversity in the kinematics of larval locomotion among the *Drosophila* species**

180 We plotted the bend probability of each species (Figure 3C). A statistical analysis
181 shows that the bend probability of the species is diverse ($p = 6.8 \times 10^{-25}$, Kruskal-Walls test).
182 While *Dvir* larvae exhibited frequent bending (bend probability: 0.355 ± 0.045, n = 24), *Dwil*
183 larvae rarely bend (bend probability: 0.003 ± 0.004, n = 18) (Figure 3C). We also plotted the
184 crawling speed of the *Drosophila* species (Figure 3D). The statistical analysis shows that the
185 speed is also diverse among the species ($p = 9.3 \times 10^{-24}$, Kruskal-Walls test). For example,
186 *Dwil* larvae crawl faster than *Dvir* larvae do (crawling speed: 1.46 ± 0.07 mm/sec, n = 24 in
187 *Dwil*; crawling speed: 0.95 ± 0.05 mm/sec, n = 18 in *Dvir*). These analyses indicate that the
188 bend probability and crawling speed are differentiated in the genus *Drosophila*.

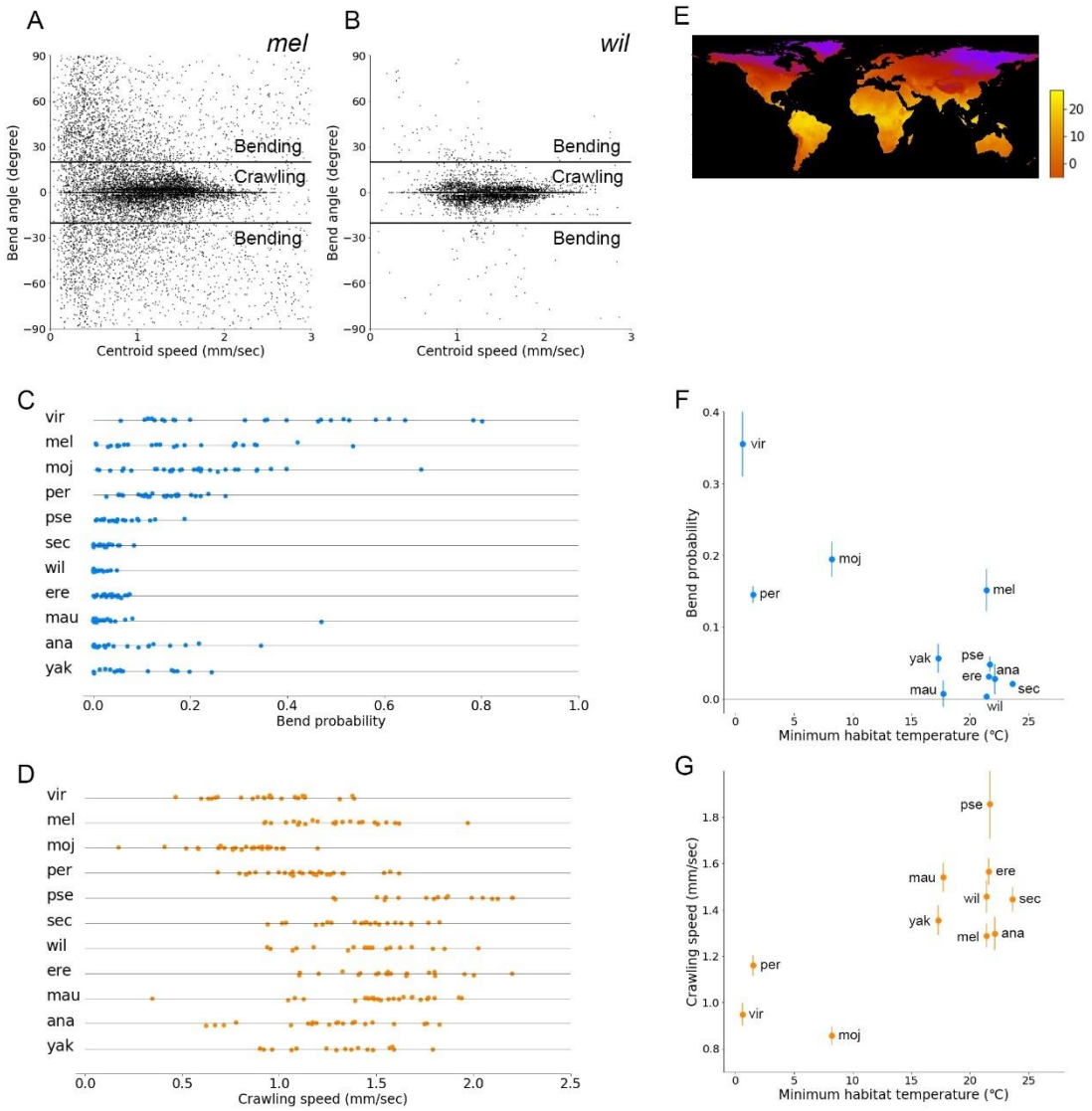


Figure 3. Bend probability and crawling speed of larval locomotion in the 11 *Drosophila* species at 24°C and their relationship to habitat temperature of each species. (A and B) Plots of the speed of the centroid of larvae and the bend angle of *D. melanogaster* (A) and *D. willistoni* (B). Each point corresponds to a datum of a single larva of a species at a single time frame. Thick horizontal lines denote the threshold between crawling and bending. (C) Bend probability of individual larvae of each species. (D) Crawling

speed of individual larvae of each species. Sample numbers in (C) and (D) are the following: *Dvir*: n=24; *Dmel*: n=24; *Dmoj*: n=30; *Dper*: n=27; *Dpse*: n=19; *Dsec*: n=21; *Dwil*: n=18; *Dere*: n=22; *Dmau*: n=26; *Dana*: n=22; *Dyak*: n=16. (E) A world map of minimum habitat temperature. (F) Scatter plot of bend probability at 24°C against the minimum habitat temperature Tmin. (G) Scatter plot of crawling speed at 32°C against the minimum habitat temperature Tmin. Median ± sem is shown in F and G.

Relationship between the kinematics of larval locomotion and minimum habitat temperature of the *Drosophila* species

Next, we tried to find factors that relate to the diversification of larval kinematics among species. Among the factors in the morphological differences and the ecological diversity that might be involved in the kinematics diversity (Theunissen et al., 2015) we examined the body length of larvae as a morphological factor and habitat temperature as an ecological factor. A previous study reported an allometric relationship between the body size and the crawling speed in *Diptera* larvae (Berrigan & Pepin, 1995). The authors analyzed larvae in the order *Diptera*, the average size of which spans from 3.7 mm (*Dmel*) to 15.9 mm (*Sarcophaga bullata*). We tested whether the relationship between the body length and the crawling speed also holds within the genus *Drosophila*, a subgroup of the order *Diptera*. The length of the *Drosophila* larvae we used spanned from 3.49 ± 0.05 mm (*Dyak*) to 5.68 ± 0.16 mm (*Dpse*) (Supplementary Figure 1A). We found no significant relationship between the body length and crawling speed (Supplementary Figure 1B; Pearson correlation = -0.099). The bend probability shows a positive correlation to body length (Supplementary Figure 1C; Pearson correlation = 0.66). However, when omitting the data of *Dvir*, which has the highest values in both the larva length and bend probability, the correlation became weak (Pearson correlation = 0.28) suggesting that larval length is not a significant factor for larval kinematics variation within the genus *Drosophila*.

Habitat temperature is one of the critical factors influencing species traits (Schmidt-

224 Nielsen, 1997). A previous study showed that variability of habitat temperatures of the genus
225 *Drosophila* is related to the proportions of duplicated genes in the genome (Makino & Kawata,
226 2012). To test the possible roles of habitat temperature in the evolution of *Drosophila* larval
227 locomotion, we examined the relationship between habitat temperatures and the locomotion
228 kinematics in the genus *Drosophila*. The habitat regions of the 11 species were obtained from
229 the literature (Brake & Baechli, 2008; Makino & Kawata, 2012; Markow & O'Grady, 2005),
230 and climate temperature data were obtained from a global climate dataset, WORLDCLIM
231 (Hijmans et al., 2005) (Figure 3E, Supplementary Figure 2). We examined the relation
232 between larval kinematics (bend probability or crawling speed) and indices of habitat
233 temperatures (Figure 3, Supplementary Figure 2 and 3). We found that the bend probability
234 and crawling speed are both correlated with minimum habitat temperature, Tmin (Figure 3F
235 and 3G. Pearson correlation: bend probability vs Tmin = -0.81, crawling speed vs Tmin =
236 0.74; for the definition of Tmin, see Materials and Methods). Even after omitting extreme
237 values (*Dvir* in the bend probability data and *Dpse* in the crawling speed data), the correlation
238 remained high (Pearson correlation: the bend probability vs Tmin (without *Dvir*) = -0.69, the
239 crawling speed vs Tmin (without *Dpse*) = 0.78). In species inhabiting moderate environments
240 (where Tmin is 15 to 25°C), larvae showed low bend probability and fast crawling (Figure 3F
241 and 3G). On the other hand, larvae of species inhabiting cold environments (where Tmin is
242 0 to 10°C) exhibited frequent bending and slow crawling (Figure 3F and 3G). This observation
243 implies that minimum temperature in the habitat may be one of the leading factors in sculpting
244 the kinematics of larval locomotion in the genus *Drosophila*.
245

246 **Correlation between bend probability and minimum habitat temperature among**
247 ***Drosophila* species at high ambient temperature**

248 So far we had analyzed larval locomotion at 24°C. Larvae of *Dmel* are known to
249 crawl faster at 32°C than at 25°C (Ohyama et al., 2013). So we next examined whether the
250 relation between the kinematics indices and minimum habitat temperature hold or not, and

how the kinematics indices are changed, at a higher ambient temperature. We performed the same set of measurements and analysis of larval locomotion at 32°C (Figure 4 and 5). The interspecies comparison showed that the bend probabilities at 32°C were correlated with minimum habitat temperature as they did at 24°C (Figure 4B; Pearson correlation = -0.85). To examine the effects of ambient temperature on the bend probability in detail, we compared the differences of bend probability at 24°C and 32°C among the species (Figure 4C). We found no significant relationship between the change in bend probability and the minimum habitat temperature (Figure 4C; Pearson correlation = 0.40). Seven species showed no significant changes in the bend probability at 32°C (*Dmoj*, *Dper*, *Dpse*, *Dsec*, *Dmau*, *Dana* and *Dyak* in Figure 4A - 4D), while *Dvir* and *Dmel* showed a decrease in the bend probability at 32°C, and *Dwil* and *Dere* exhibited an increase (Figure 4A - 4C, 4E and 4F). Accordingly, we concluded that while the shift of the bend probability between the distinct temperatures is diverse among the *Drosophila* species, the overall trend between the bend probability in relation to minimum habitat temperature holds at the higher ambient temperature of 32°C.

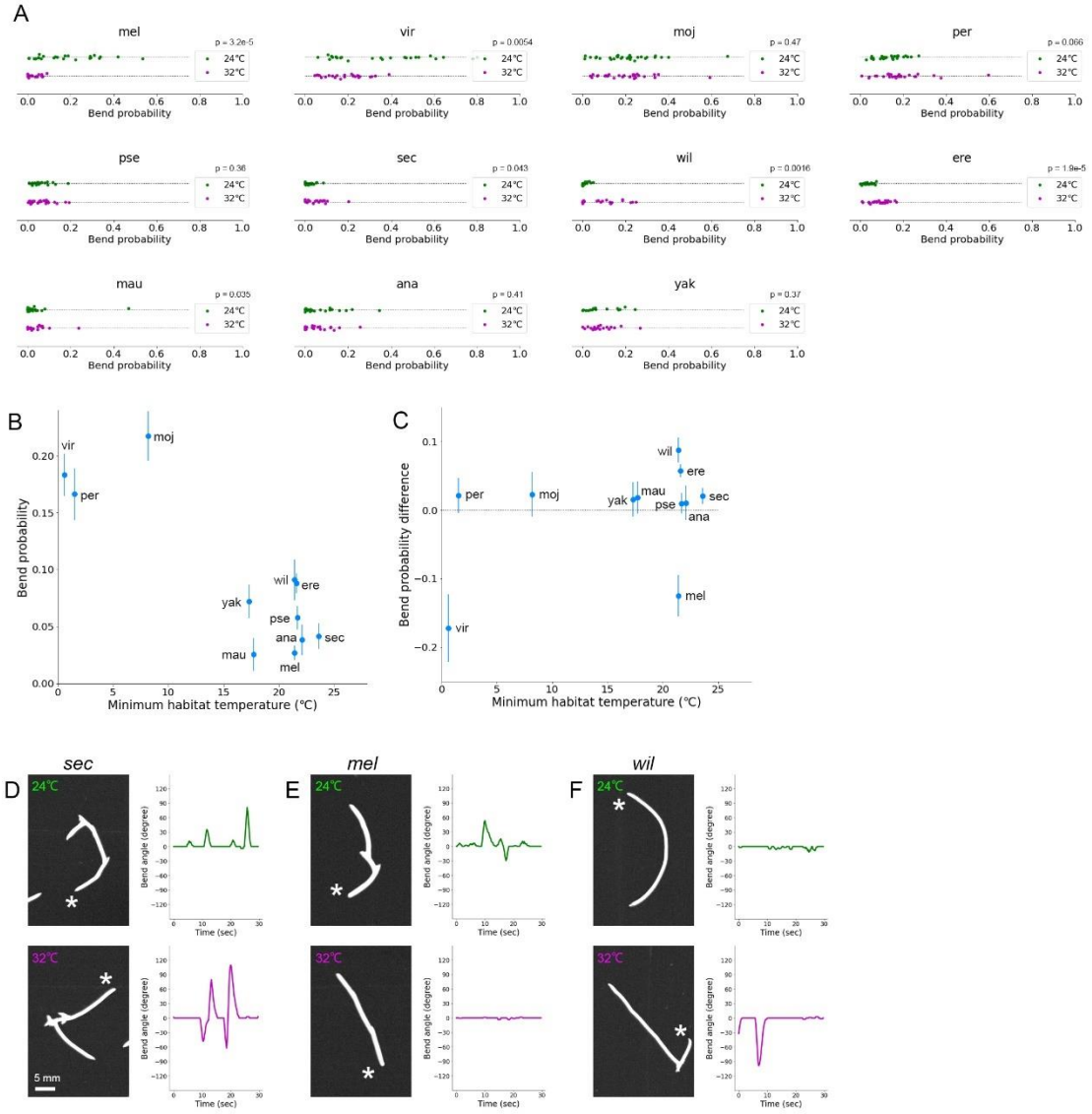


Figure 4. Bend probability in larval locomotion in the 11 *Drosophila* species at 32°C. (A) Bend probability of the 11 species at 24°C and 32°C. The data at 24°C are the same as in Figure 3C. Sample numbers at 32°C are the following: *Dvir*: n=25; *Dmel*: n=20; *Dmoj*: n=29; *Dper*: n=27; *Dpse*: n=25; *Dsec*: n=20; *Dwil*: n=21; *Dere*: n=24; *Dmau*: n=17; *Dana*: n=23; *Dyak*: n=19. (B) Scatter plot of bend probability at 32°C against the minimum habitat temperature, Tmin. (C) Scatter plot of the difference in bend probability between 32°C and at 24°C (bend probability at 32°C – bend probability at 24°C) against the minimum habitat temperature Tmin. (D-F) Examples of trajectories (left) and the bend angle (right) at 24°C

(top) and 32°C (bottom) of larval locomotion of each species (D: *Dsec*, E: *Dmel*, and F: *Dwil*). Median ± sem is shown in B and C.

Correlation between crawling speed and minimum habitat temperature among *Drosophila* species at high temperature

Next, we analyzed the crawling speed at 32°C (Figure 5A). Similarly to our analysis of the bend probability, we found that the crawling speed at 32°C was correlated with the minimum habitat temperature among the species, as it was at 24°C (Figure 5B; Pearson correlation = 0.67). To examine the effects of ambient temperature on the crawling speed in detail, we compared the difference in the crawling speed at 24°C to 32°C among the species (Figure 5C). In contrast to the case in the bend probability, shifts in the crawling speed among the species were common: ten species (all the species but *Dper*) showed a significant increase in crawling speed at 32°C (Figure 5A). *Dper* also exhibited an increase in the crawling speed but it was not statistically significant (Figure 5A). Accordingly, at 32°C, larvae of all the species we tested increased their speed of crawling, which might reflect a general demand to avoid malfunctions in metabolic reactions and dehydration of the body. We also tested the relationship between the change in the speed at 32°C and the minimum habitat temperature for each species (Figure 5C). We found no significant correlation between the speed change and the habitat temperature (Figure 5C; Pearson correlation = 0.07), which also implies that the speeding-up at high temperature is a general requirement among the species for the larval survival. Accordingly, whereas the details of shifts are different (diverse shift in the bend probability and common shift in the crawling speed among the species), the overall relationship between the kinematic indices and minimum habitat temperature also holds at the higher ambient temperature of 32°C.

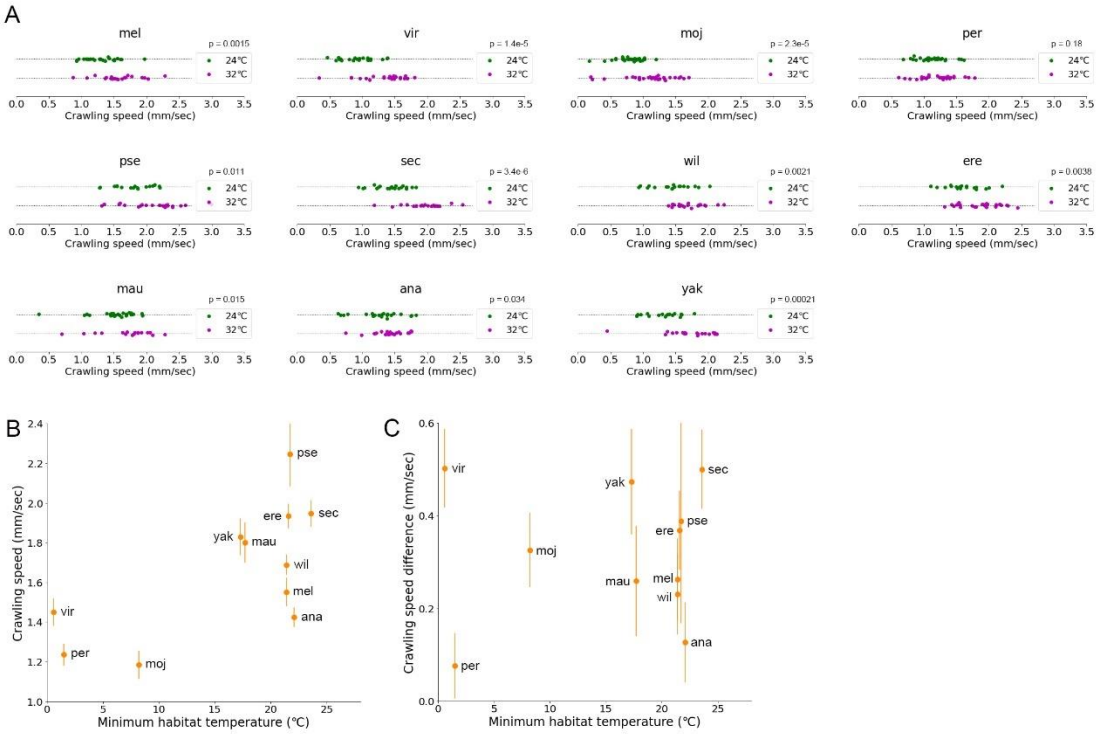


Figure 5. Crawling speed of larval locomotion in the 11 *Drosophila* species at 32°C. (A) Crawling speed of the 11 species at 24°C and 32°C. The data at 24°C are the same as in Figure 3D. Sample numbers at 32°C are the following: *Dvir*: n=25; *Dmel*: n=20; *Dmoj*: n=29; *Dper*: n=27; *Dpse*: n=25; *Dsec*: n=20; *Dwil*: n=21; *Dere*: n=24; *Dmau*: n=17; *Dana*: n=23; *Dyak*: n=19. (B) Scatter plot of the crawling speed at 32°C against the minimum habitat temperature Tmin. (C) Scatter plot of the difference of the crawling speed between at 32°C and at 24°C (crawling speed at 32°C – crawling speed at 24°C) against the minimum habitat temperature Tmin. Median ± sem is shown in B and C.

A kinematic trend of larval locomotion in the genus Drosophila

To see any gross trend in the shift of larval kinematics in the different species at distinct ambient temperatures, we plotted the kinematics of individual larvae of all the species (Figure 6). At the ambient temperature of 24°C, a trend of kinematics was observed in which the data points were distributed from low crawling speed / high bend probability (the top-left

corner in the plot) to high crawling speed / low bend probability (the bottom-right corner in the plot) as the minimum habitat temperature increased (Figure 6A). Intriguingly, the kinematics indices between distinct species were not segregated but rather overlapped. This continuum property can also be observed in the kinematics data at the ambient temperature of 32°C (Figure 6B). According to the modern phylogenetic concept (Markow & O'Grady, 2005), the subgenera *Sophophora* and *Drosophila* (both belonging to the genus *Drosophila*) are ancestral and descendant, respectively. The 11 genus *Drosophila* species in our study consisted of nine ancestral subgenus *Sophophora* species (all but *Dvir* and *Dmoj*) and two descendant subgenus *Drosophila* species (*Dvir* and *Dmoj*). In our plots of kinematics, species in the descendant subgenera *Drosophila* (*Dvir* and *Dmoj*) were located at the top-left corner in the continuum (Figure 6). This observation implies that the kinematics indices of larvae in ancestral *Drosophila* species took values in this continuum and kinematics indices in descendant species have gradually diverged along this continuum by adaptation to minimum habitat temperature during evolution.

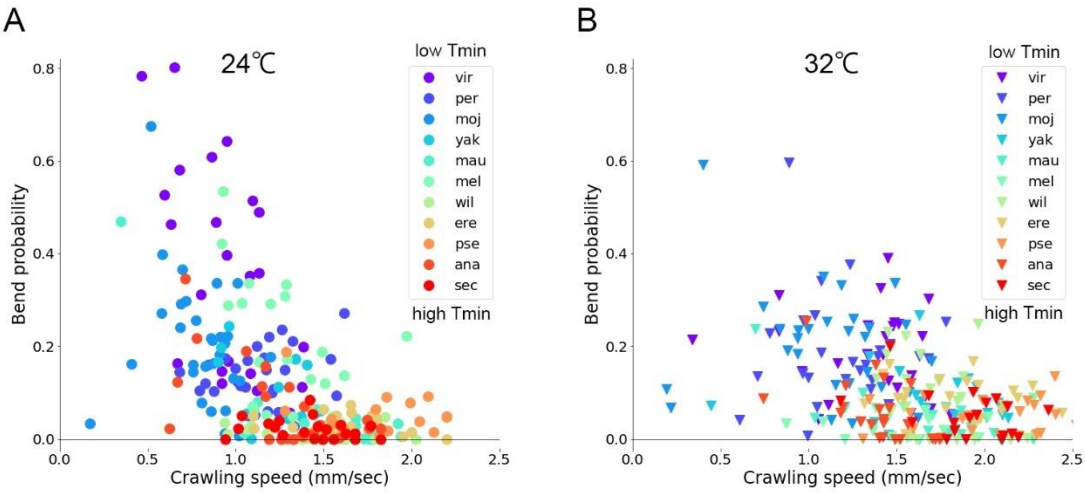


Figure 6. Scatter plot of kinematics in the 11 species at 24°C and 32°C. (A and B) Two dimensional plots of the crawling speed and bend probability. Each point corresponds to data of a single larva of the species. The colour of markers denotes minimum habitat temperature

333 (Tmin) as shown in the legend. A: 24°C, B: 32°C.

334

335 **No correlation between crawling speed and habitat temperature among *Drosophila***
336 **species at an extreme ambient temperature of 40°C**

337 Temperatures of 24°C and 32°C are within the range of natural habitat temperatures
338 of most of the species we tested (Supplementary Figure 2). We next examined larval crawling
339 at an extreme temperature and investigated the relation between the kinematics indices and
340 minimum habitat temperature. Too high a temperature can be noxious for many animals,
341 including *Drosophila* larvae. When *D. melanogaster* larvae are stimulated with a probe
342 heated to 42°C, they exhibit a stereotyped rolling motion as an escape behaviour (Dason et
343 al., 2019; Petersen et al., 2018; Tracey et al., 2003). At below 40°C, on the other hand, larvae
344 do not exhibit rolling behaviour (Petersen et al., 2018), which allows us to study crawling
345 kinematics in this semi-noxious extreme environment. We recorded larval locomotion of the
346 *Drosophila* species at the ambient temperature of 40°C, which corresponds to the highest
347 edge of maximum habitat temperature histograms for many of the species (Supplementary
348 Figure 2). We measured the bend probability and crawling speed of the 11 *Drosophila*
349 species (Supplementary Figure 4 and 5). At the ambient temperature of 40°C, neither the
350 bend probability nor crawling speed was correlated with minimum habitat temperature
351 (Figure 7A and 7B; Pearson correlation: bend probability at 40°C vs Tmin = 0.17, crawling
352 speed at 40°C vs Tmin = -0.17). Since 40°C is close to the maximum habitat temperature
353 rather than the minimum habitat temperature, we analyzed the relation between the
354 kinematics indices and maximum habitat temperature instead of minimum habitat
355 temperature. However, neither the bend probability nor crawling speed was correlated with
356 maximum habitat temperature (Pearson correlation: bend probability at 40°C vs maximum
357 habitat temperature = -0.12, crawling speed vs maximum habitat temperature = -0.10).
358 Accordingly, the relation between the kinematic indices and habitat temperature does not
359 hold at the extreme ambient temperature of 40°C. This observation suggests that the

influence of minimum habitat temperature on the evolution of the locomotion kinematics is restricted within a specific range of ambient temperatures. To examine the effects of the extreme ambient temperature on the kinematics indices in detail, we examined the shift in the bend probability at the distinct temperatures of 40°C and 32°C among the species (Supplementary Figure 4). In seven species, bend probability increased at 40°C. Three species that inhabit moderate temperature areas (*Dvir*, *Dper* and *Dmoj*) and one species that lives on an isolated island (*Dsec*) showed little change in the bend probability, which may reflect a distinct strategy in evolution to cope with the semi-noxious environment. We also examined the shift in the crawling speed at the distinct temperatures of 40°C and 32°C among the species (Supplementary Figure 5). We found the crawling speed was reduced at 40°C in all the species (Supplementary Figure 5), which might be a common adaptation of the *Drosophila* larval locomotion at the extreme ambient temperature and/or due to an abnormal physiological reaction at the semi-noxious temperature. Intriguingly, in some species, backward crawling, which seldom occurs at 32°C, can be observed at 40°C (Figure 7C). Consequently, these observations show that at the extreme ambient temperature of 40°C, the relation between the kinematics indices and habitat temperature does not hold and larvae exhibit common (increase in the crawling speed) and diverse (changes in the bend probability and generation of backward crawling) shifts in the locomotion kinematics among the *Drosophila* species.

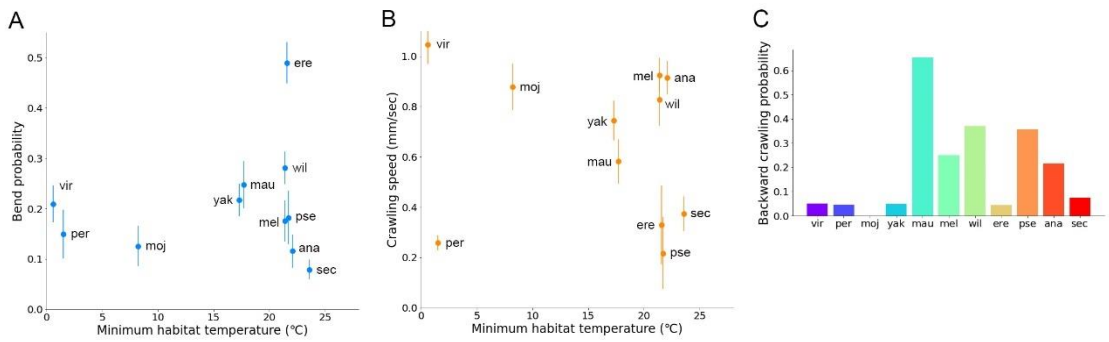


Figure 7. Larval behaviour in the 11 species at 40°C. (A) Scatter plot of the bend

probability at 40°C against the minimum habitat temperature Tmin. (B) Scatter plot of the crawling speed at 40°C against the minimum habitat temperature Tmin. Median ± sem is shown in (A) and (B). Sample numbers in (A) and (B) are the following: *Dvir*: n=20; *Dmel*: n=28; *Dmoj*: n=23; *Dper*: n=21; *Dpse*: n=16; *Dsec*: n=27; *Dwil*: n=27; *Dere*: n=22; *Dmau*: n=26; *Dana*: n=23; *Dyak*: n=20. (C) Backward crawling probability at 40°C of each species. Sample numbers in (C) are the following: *Dvir*: n=20; *Dmel*: n=28; *Dmoj*: n=23; *Dper*: n=22; *Dpse*: n=14; *Dsec*: n=27; *Dwil*: n=27; *Dere*: n=23; *Dmau*: n=26; *Dana*: n=23; *Dyak*: n=21. Colouring of the species follows that in Figure 6A.

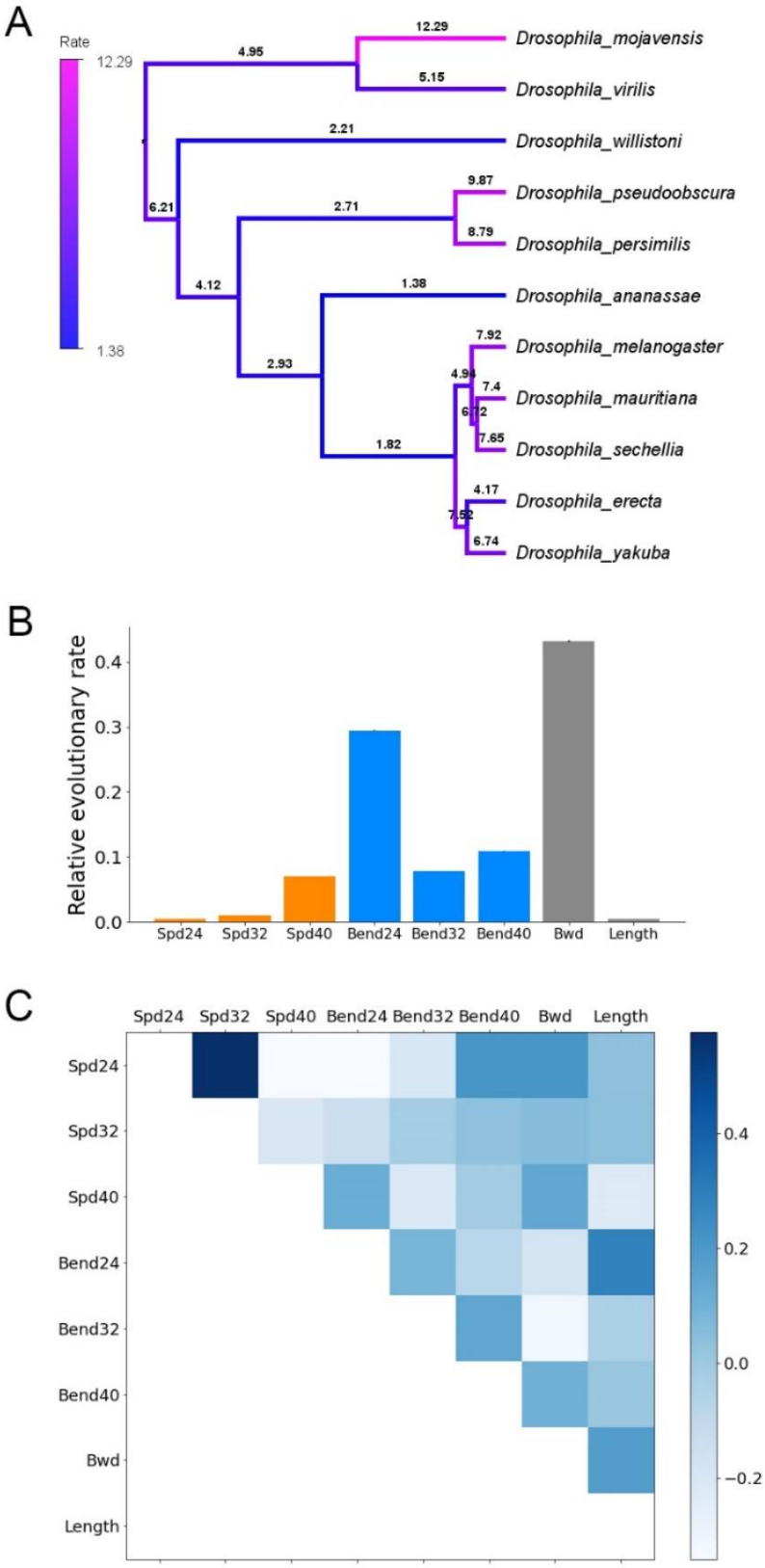
Phylogenetic analyses of interspecies differences in kinematics

The kinematics measurements of 11 *Drosophila* species at three temperatures have demonstrated interspecies differentiation in the crawling kinematics. To obtain a hint on how these kinematics parameters have developed during the evolutionary history of the genus *Drosophila*, we conducted Bayesian phylogenetic analyses using RevBayes v. 1.0.7 (Hohna et al., 2016; Höhna et al., 2017). At first, we inferred phylogenetic trees of the 11 *Drosophila* species based on eight nuclear genes used previously (Turelli et al., 2018) (See Methods for detail). Then by using our kinematics dataset, we estimated the following three factors in the phylogenetic tree by Bayesian inference: the rate of evolution at each branch of the phylogenetic trees, relative rates of evolution among the kinematic parameters, and the correlation between the kinematic parameter evolutions (Kalay et al., 2020). To perform the inference, we constructed a data matrix of eight parameters (the bend probability at 24, 32 and 40°C, the crawling speed at 24, 32 and 40°C, backward crawling probability at 40°C and the body length), for each of the 11 *Drosophila* species. We assumed that these parameters evolve under a multivariate Brownian-motion model (Huelsenbeck & Bruce, 2003; Kalay et al., 2020; Lartillot & Poujol, 2011) (See Methods for detail). We estimated the three factors (the evolution rates at phylogenetic trees, relative evolution rates among the kinematic parameters, and the correlation between the parameter evolutions) by running a Markov

408 Chain Monte Carlo simulation.

409 The phylogenetic analyses indicated that the rates of evolution in the kinematics are
410 highly diverse over branches in the phylogenetic tree (Figure 8A). In addition, the
411 evolutionary rates are distinct among the eight kinematics parameters (Figure 8B). The bend
412 probability at 24°C and the probability of backward crawling at 40°C have a relatively high
413 evolution rate, which might reflect large diversification of these two parameters among the
414 species (Figure 6A and 7C).

415 Some kinematics traits might have evolved cooperatively. To test this possibility, we
416 calculated the correlation of the evolutionary changes between the kinematic parameters. In
417 the correlation analyses of all pairs of the parameters, the crawling speed at 24°C and the
418 crawling speed at 32°C were the most correlated (Figure 8C). On the other hand, the
419 evolutions of the bend probability between at 24°C and 32°C were less correlated. This
420 observation is consistent with the notion that while changes in the bend probability from 24°C
421 to 32°C among the species are diverse (Figure 4C), all the species show an increase in the
422 crawling speed by the temperature shift (Figure 5C). Consequently, the phylogenetic
423 analyses imply that the kinematics indices of larval locomotion have evolved differently in
424 distinct branches of a phylogenetic tree with keeping a correlation between specific
425 locomotion traits such as crawling speed at distinct temperatures.


A
Rate
12.29
1.38
12.29
4.95
5.15
Drosophila_mojavensis
Drosophila_virilis
2.21
Drosophila_willistoni
9.87
6.21
2.71
Drosophila_pseudoobscura
8.79
Drosophila_persimilis
4.12
1.38
Drosophila_ananassae
7.92
Drosophila_melanogaster
2.93
4.94
7.4
Drosophila_mauritiana
7.65
1.82
Drosophila_sechellia
4.17
Drosophila_erecta
6.74
Drosophila_yakuba
B
Relative evolutionary rate
0.0
0.1
0.2
0.3
0.4
Spd24
Spd32
Spd40
Bend24
Bend32
Bend40
Bwd
Length
C
Spd24
Spd32
Spd40
Bend24
Bend32
Bend40
Bwd
Length
0.4
0.2
0.0
−0.2

427 **Figure 8. Phylogenetic analyses of larval locomotion in the genus *Drosophila*.** (A)
428 Phylogenetic tree estimated from Bayesian inference. The topology is inferred by coding
429 sequences of eight genes of the 11 *Drosophila* species. As a prior distribution for the
430 Bayesian inference, an uncorrelated exponential (UCED) relaxed-clock model was used.
431 Colour and a value of each branch denote the relative rate of the evolution of the eight
432 kinematic traits. (B) Relative evolution rates of the eight kinematics parameters estimated by
433 the Bayesian inference. (C) Correlation between the evolution of eight kinematics parameters.
434 Abbreviations in (B) and (C) are the following: Spd24: crawling speed at 24°C, Spd32:
435 crawling speed at 32°C, Spd40: crawling speed at 40°C, Bend24: bend probability at 24°C,
436 Bend32: bend probability at 32°C, Bend40: bend probability at 40°C, Bwd: probability of
437 backward crawling, Length: axial body length of larvae.
438
439 **Discussion**
440 In this work, we investigated interspecies differences in larval locomotion in the
441 genus *Drosophila*. We used the bend probability and crawling speed as measures to examine
442 larval locomotion. Despite the similar appearance of larval bodies in the different species, the
443 kinematics of larval locomotion is diverged (Figure 2). The body length is not a leading factor
444 for the diversity of kinematics (Supplementary Figure 1). In contrast, minimum habitat
445 temperature correlates with both the bend probability and crawling speed at both 24°C and
446 32°C ambient temperature (Figure 9), which implies the kinematics indices are adapted to
447 ambient temperature in evolution. Phylogenetic analysis by Bayesian inference suggests that
448 the rates of evolution are divergent among the branches of the phylogenetic tree (Figure 8A).
449 Among the kinematic parameters, the bend probability at the ambient temperature of 24°C
450 and the backward crawling probability at 40°C have relatively higher evolution rates than the
451 others and the evolution of the crawling speed at 24°C and 32°C are correlated (Figure 8B
452 and 8C). Regarding the questions raised in the Introduction section, our results suggest the
453 following: (1) Locomotion kinematics of larvae is divergent among *Drosophila* species. (2)

The minimum habitat temperature is more related to the kinematics indices (the bend probability and crawling speed) than the body length (Figure 9).

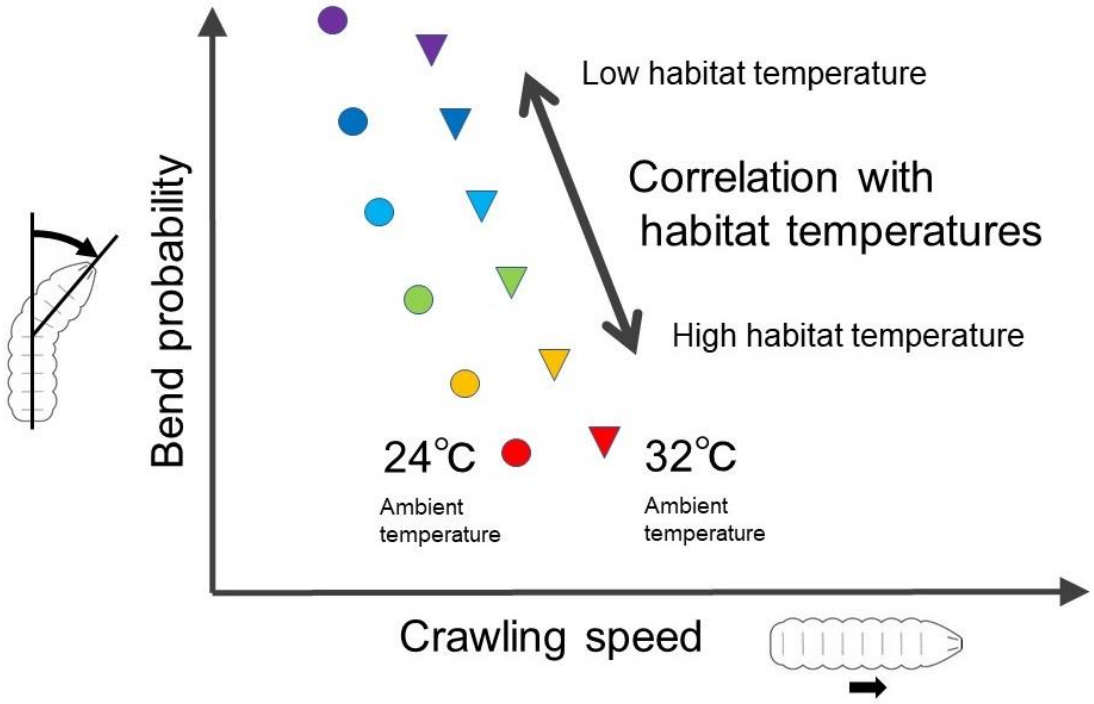


Figure 9. Summary of this study. Disks and triangles denote kinematics of each species at 24°C and 32°C, respectively. Colour of markers indicates distinct minimum habitat temperatures as shown in Figure 6A.

The correlation between habitat temperature and the kinematics raises a question about the underlying mechanisms of adaptive change in kinematics to the minimum habitat temperature. We presume that the adaptive change was likely to be driven by two factors: the pupation positioning and competition over food. Regarding the pupation behaviour, a previous study showed a correlation between path lengths of crawling and heights of pupation location in *D. melanogaster* (Sokolowski, 1985). Since pupae are immobile, adequate selection of pupation position is vital for their survival. Moisture is one of the key factors that affects the selection of pupation site, since dry environments can cause pupal

469 desiccation, while soaked media might lead to the drowning of pupae (Sokolowski, 1985;
470 Sokolowski et al., 1984). It is reasonable to assume that habitat temperature is a leading
471 factor in determining the moisture levels of microenvironments of *Drosophila* larvae.
472 Accordingly, divergent temperatures in the habitats of flies leads to variability in moisture
473 levels, the ambient moisture affects the pupation position (Sokolowski et al., 1984), and the
474 pupation position is related to the path length of crawling (Sokolowski, 1985). These three
475 links might underlie the relationship between the larval locomotion kinematics and minimum
476 habitat temperature of each species.

477 The second possible mechanism underlying the relation between minimum habitat
478 temperature and locomotion kinematics is related to competition over food. It is suggested
479 that larval feeding is competitive between individuals (Rohlfs & Hoffmeister, 2003). There is
480 a positive relation between fitness and density (called Allee effects) in *D. melanogaster* larvae
481 (Wertheim et al., 2002) and so female flies aggregate eggs when laying them (Rohlfs &
482 Hoffmeister, 2003). Consequently, the larval population tends to be overcrowded (Burnet et
483 al., 1977) and larvae compete over the limited resources to meet their need to consume
484 sufficient amount of nutrient within limited larval periods (Nicholson, 1954). If the available
485 food is limited and larval density is high, larvae need to crawl further (Sokolowski et al., 1997),
486 which can be an evolutionary driving force to increase the crawling speed. In contrast, if there
487 is plenty of food in the habitat, the crawling speed remains unchanged or decreases during
488 evolution, because crawling behaviour is energetically costly (Berrigan & Lighton, 1993).
489 Accordingly, feeding conditions, especially the choice of which foods to eat, can affect
490 kinematics of larval locomotion in evolutionary processes so that the divergence of food to
491 eat can lead to the divergence in locomotion kinematics. In regard to this point, food for larvae
492 of the genus *Drosophila* is divergent and related to their living environments (Markow, 2015;
493 Watada et al., 2020; Watanabe et al., 2019). So, the nutritional conditions (nutrient balances
494 and fermentation etc.) and physical properties (hardness and wetness etc.) of food to eat
495 vary among the *Drosophila* species, which can drive evolutionary divergence in the

496 kinematics of larval locomotion. Then, the growth of plants, including fruits and vegetables,
497 for larvae is strongly affected by habitat temperature. Consequently, divergence in minimum
498 habitat temperature may affect the locomotion kinematics during evolution through
499 divergence in the foods that larvae feed on, and divergence in requisite feeding behaviour
500 for larval growth. Comprehensive kinematics studies of other *Drosophila* species and
501 quantitative analyses of microenvironments of wild larvae in nature will give us insights on
502 the relationships between ambient temperature and diverse larval locomotion kinematics.

503 How can we approach the neural circuit mechanisms underlying the interspecies
504 divergence in larval locomotion? Circuit mechanisms in larval locomotion have been
505 examined intensively in *Drosophila melanogaster*. Recent connectomics studies have
506 identified several key neurons for larval locomotion in *Drosophila melanogaster* (Carreira-
507 Rosario et al., 2018; M. Q. Clark et al., 2018; Fushiki et al., 2016; Heckscher et al., 2015;
508 Hughes & Thomas, 2007; Kohsaka et al., 2014, 2017, 2019; Schneider-Mizell et al., 2016;
509 Tastekin et al., 2015; Zwart et al., 2016). Regarding bending, the thoracic neuromere was
510 shown to be important in bending in chemotaxis (Tastekin et al., 2015). Especially, the
511 commissural connection is crucial for bending (Berni, 2015). In addition, a signal from the
512 chordotonal sensory organ is also required to generate bending (Ainsley et al., 2003).
513 Regarding the crawling speed, a group of inhibitory premotor neurons were identified to be
514 critical (Kohsaka et al., 2014). Proprioceptive feedback and neuromodulation are both
515 important for the normal crawling speed (Fox et al., 2006; Hughes & Thomas, 2007).
516 Furthermore, kinematics of the larval locomotion have been measured and investigated
517 quantitatively in detail (Gepner et al., 2015; Gershow et al., 2012; Gomez-Marin et al., 2011;
518 Gomez-Marin & Louis, 2014; Heckscher et al., 2012; Hernandez-Nunez et al., 2015; Lahiri
519 et al., 2011; Luo et al., 2010) and analysed by mathematical modelling (Gjorgjieva et al.,
520 2013; Loveless et al., 2019; Loveless & Webb, 2018; Pehlevan et al., 2016; Sun et al., 2020).
521 Regarding temperature sensing, neuronal and molecular mechanisms on temperature-
522 guided behaviour have been clarified (Dillon et al., 2009; Klein et al., 2015; Kwon et al., 2008,

523 2010; Liu et al., 2003; Ni et al., 2016b; Rosenzweig et al., 2005b, 2008; Shen et al., 2011).
524 These extensive findings on the cellular and molecular mechanisms on larval crawling in
525 *Drosophila melanogaster* will be an ideal starting point to investigate the evolution of larval
526 behaviour in the genus *Drosophila*. For example, differences in commissure fibre tracts in
527 the central nervous system (Berni, 2015) among the species might underlie the divergence
528 in the bend probability. Interspecies comparison of a group of interneurons PMSIs (*period*-
529 positive median segmental interneurons), which are involved in the crawling speed (Kohsaka
530 et al., 2014), would reveal neural mechanisms on the evolutionary diversification in the
531 crawling speed. Comparative analyses of neural network architectures and gene expression
532 among the *Drosophila* species will relate the evolution of the nervous system to the adaptive
533 diversification in larval behaviour.
534
535 **Materials and Methods**
536
537 ***Drosophila* strains**
538 All animals were raised on standard cornmeal-based food. We used the following
539 fly stocks: *Drosophila ananassae* (k-s01), *Drosophila erecta* (k-s02), *Drosophila yakuba* (k-
540 s03), *Drosophila melanogaster* (k-s04), *Drosophila sechellia* (k-s10), *Drosophila persimilis*
541 (k-s11), *Drosophila pseudoobscura* (k-s12), *Drosophila willistoni* (k-s13), *Drosophila virilis* (k-
542 s14) and *Drosophila mojavensis* (k-s15) from KYORIN-Fly, Fly Stocks of Kyorin University
543 and *Drosophila mauritiana* (#900020) from KYOTO Stock Center (DGRC) at the Kyoto
544 Institute of Technology.
545
546 **Recording larval locomotion**
547 Third-instar wandering larvae of each strain were picked up and gently washed in
548 deionised water. Residual food on the larvae was brushed off with a paintbrush. An agarose
549 stage (size: 9cm x 9cm x 5mm; 1.5% agarose; RIKAKEN STAR agarose powder #RSV-

AGRP-100G) was placed on a temperature controlled plate (Cool Plate, AS ONE Corporation, Japan) and the temperature of the surface was kept at 24±1°C, 32±1°C or 40±1°C. Eight to ten larvae were placed on the agarose arena with a paintbrush. The larvae were illuminated with infrared light, which larvae cannot see (LDQ-150IR2-850, wavelength = 850 nm, CCS Inc., Japan) and recorded at five frames/sec with a CCD camera (CGE-B013-U, MIGHTEX, Canada; resolution: 1280 pixels x 960 pixels) and an infrared filter (775LP filter, Omega Optical, USA), and saved as a series of bitmap files. In our setting, the scale of image is 0.13 mm/pixel. For each of 11 species and at each of the three ambient temperatures, we repeated the measurement three times.

Larva tracking

We obtained a time series of bitmap image files by the procedure described above. The bitmap files were converted to tiff format by Fiji (https://imagej.net/fiji). In cases where larvae accumulated at the first frame, we removed the first several images until the larvae dispersed enough to be identified individually. The x and y coordinates of the centroid and the bend angle (Figure 1E) of individual larvae were obtained by using the FIMTrack software (Risse et al., 2017). We used tracking data of single larvae that were continuously tracked for more than 300 frames (which corresponds to one minute). In case a larva collided with another larva in the middle of the recording and the larva was traced differently before and after the collision with distinct labels, we treated the two traces as two larvae. The coordinates and bend angles were smoothed and plotted by Python 3.7.

Clustering analysis of kinematics

For clustering analysis of the plots in Figure 2A, we made a probability distribution. We discretised the centroid speed axis by an interval of 0.1 mm/sec between 0 and 4 mm/sec and the bend angle axis by an interval of two degrees between 0 and 140 degrees, then created 2800 bins in total (40 bins in the speed axis and 70 bins in the bend angle

axis). For each scatter plot, we counted the number of points in each bin and obtained probability density by normalisation with the total number of the points. We calculated the Kullback-Leibler divergence (KL) of probability distributions of two species p(bin) and q(bin) as:

$$\mathrm{KL}(\mathrm{p},\mathrm{q}) = \sum_{\mathrm{bin}=1}^{2800} \mathrm{p}(\mathrm{bin}) * \log \frac{\mathrm{p}(\mathrm{bin})}{\mathrm{q}(\mathrm{bin})}.$$

Since KL(p,q) is an asymmetric measure $(\mathrm{KL}(\mathrm{p},\mathrm{q}) \neq \mathrm{KL}(\mathrm{q},\mathrm{p}))$, we calculated the Jensen-Shannon divergence JS, which is a symmetric alternative to the Kullback-Leibler divergence:

$$\mathrm{JS}(\mathrm{p},\mathrm{q}) = \frac{1}{2}\{\mathrm{KL}(\mathrm{p},\mathrm{q}) + \mathrm{KL}(\mathrm{q},\mathrm{p})\} = \mathrm{JS}(\mathrm{q},\mathrm{p}).$$

Based on the Jensen-Shannon divergence, we conducted the hierarchical clustering using Python 3.7 and the SciPy library.

Habitat temperature of the *Drosophila* species

Global climate data were obtained from the WorldClim data website (https://www.worldclim.org/data/worldclim21.html). We used minimum, mean and maximum temperature in the world at a spatial resolution of five minutes (9.25 km x 9.25 km). Habitat regions of each *Drosophila* species were obtained from the literature (Brake & Baechli, 2008; Makino & Kawata, 2012; Markow & O'Grady, 2005) and the DrosWLD-Species database (https://bioinfo.museum.hokudai.ac.jp/db/modules/stdb/index.php?action=tbl&tbl_id=21) and traced them on the world map (2160 pixel x 4320 pixel) obtained from the WorldClim by using Adobe Illustrator software (Adobe Inc., USA). The maps in Supplementary Figure 2 were generated by Adobe Illustrator and the statistics of the temperature within each species' habitat were calculated by Python 3.7.

We examined the relation between the larval kinematics (the bend probability or crawling speed) and indices of habitat temperatures (Figure 3, Supplementary Figure 2 and 3). We first used three indices of habitat temperatures: the mode (or the most frequent value)

603 of maximum, minimum and average temperature indices within the habitats. We found no
604 significant relationship between the crawling speed and these three temperature indices
605 (Pearson correlation: crawling speed vs average temperature = 0.43, vs minimum
606 temperature = 0.35, vs maximum temperature = 0.19). Although there are significant
607 relationships between the bend probability and the mode of minimum and maximum
608 temperature (Pearson correlation: bend probability vs average temperature = -0.82, vs
609 minimum temperature = -0.80, vs maximum temperature = -0.41), when omitting the data of
610 *Dvir*, which had the highest values in bend probability and the lowest values in the
611 temperature, the correlation became weak (Pearson correlation: bend probability vs average
612 temperature = -0.40, vs minimum temperature = -0.43). These results collectively suggest
613 that the mode values of maximum, minimum and average habitat temperatures are not
614 significant factors for larval kinematics variation within the genus *Drosophila*. In the minimum
615 habitat temperature data of *Dvir* and *Dpse*, we noticed that the mode temperatures were
616 below 0°C, which cannot be a representative habitat temperature for them (Supplementary
617 Figure 2). To test a milder and more representative index, we used the warmest peaks in the
618 minimum habitat temperature histogram ("Tmin" in Supplementary Figure 2). Considering
619 uneven distribution of flies within the region demarcated in Supplementary Figure 2, the
620 temperature of the warmest peak in the minimum habitat temperature may represent the
621 coldest temperature the majority of the population of each species experiences over many
622 years.
623
624 **Statistical analysis**
625 Statistical calculation was conducted by Python 3.7 in the following analyses:
626 Kruskal-Walls test in Figure 3C and 3D, Pearson correlation in Figure 3F, 3G, 4B, 4C, 5B and
627 5C, Supplementary Figure 1B, 1C, 3A, 3B, 4B and 5B, and Mann-Whitney U-test in Figure
628 4A and 5A and Supplementary Figure 4A and 5A.
629

630 **Phylogenetic analyses**

631 We adopted Bayesian statistics for phylogenetic analyses. In all the phylogenetic
632 analyses, we used the software package, RevBayes v.1.0.7 (Hohna et al., 2016).

633 We estimated the *Drosophila* phylogenetic trees (Figure 8A) by using eight nuclear
634 loci from Turelli et al. (2018): *aldolase*, *bicoid*, *enolase*, *esc*, *transaldolase*, *white*, *wingless*,
635 and *yellow*. We used *Scaptodrosophila lebanonensis* (*S. lebanonensis*) as an outgroup.
636 Coding sequences of these genes for all the species except for *D. mauritiana* and *S.*
637 *lebanonensis* were obtained from Kalay et al., (2020). Coding sequences of the genes of *D.*
638 *mauritiana* and *S. lebanonensis* were obtained from the NCBI web site
639 (https://www.ncbi.nlm.nih.gov/). The genes were aligned with MAFFT version 7 (Katoh &
640 Standley, 2013). We selected the eight genes out of twenty genes described in Turelli et al.,
641 (2018) because homologous genes of the other twelve genes were not identified in the
642 genome assembly of *D. mauritiana*. Based on the eight coding sequences of the twelve
643 species, we performed chronogram analyses described in Turelli et al. (2018) and obtained
644 the phylogenetic tree in Figure 8A, where the root is the branch of *S. lebanonensis* and the
645 length of each branch indicates relative time in the evolution. We repeated the phylogeny
646 inference four times to obtain four trees to check the robustness of this inference in the
647 following analysis.

648 Based on the phylogenetic tree obtained above, we analyzed the evolution of
649 kinematics parameters. We used eight parameters (bend probability at 24, 32 and 40°C,
650 crawling speed at 24, 32 and 40°C, backward crawling probability at 40°C, and body length),
651 for each of the 11 *Drosophila* species. In this analysis, while the topology of the phylogenetic
652 tree is unchanged, the rates of evolution of the kinematic parameters and the rates of
653 evolution of the branches of the phylogenetic tree were estimated by Bayesian inference. We
654 assumed the kinematics parameters evolve under a multivariate Brownian-motion model
655 (Huelsenbeck & Bruce, 2003; Lartillot & Poujol, 2011). This model consists of two
656 components: the relative rates of evolution among the kinematics parameters and the

correlation between each pair of the kinematics parameters (Kalay et al., 2020). The Brownian model assumes that the changes in the kinematics parameters during evolution are additive, which has two consequences: 1) parameters can become negative; 2) evolution rates can depend on the scale of the kinematics parameters. To cope with these properties, we adopted log-transformation to the kinematics parameters as described previously (Kalay et al., 2020). This transformation guarantees that the original kinematics parameters (which are exponentials of the log-transformation) remain positive and controls the size issue by transforming additive changes to multiplicative ones (Kalay et al., 2020). For prior models for the evolution rates of the branches, we specified an uncorrelated exponential (UCED) relaxed-clock model, where the rate of each branch is drawn independently from an exponential distribution. We tested three other models (an uncorrelated gamma (UCG) relaxed-clock model, where the rate of each branch is drawn independently from a gamma distribution, an uncorrelated lognormal (UCLN) relaxed-clock model, where the rate of each branch is drawn independently from a lognormal distribution, and an autocorrelated lognormal (ACLN) relaxed-clock model), but since the estimated sample sizes, which are measures to check the quality of Markov-Chain Monte-Carlo (MCMC) simulation, were less than 500 in our calculation, we did not use these models in our analyses. We ran the MCMC simulation of UCED with each of the four phylogenetic trees described in the previous paragraph.

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Competing interests

We have no conflicts of interest with respect to the work.

References

Ainsley, J., Pettus, J., Bosenko, D., Gerstein, C., Zinkevich, N., Anderson, M., Adams, C., Welsh, M., & Johnson, W. (2003). Enhanced locomotion caused by loss of the Drosophila DEG/ENaC protein Pickpocket1. *Current Biology*, *13*, 1557–1563. https://doi.org/10.1016/S

Alexander, R. M. (2006). *Principles of Animal Locomotion*. Princeton University Press.

Berni, J. (2015). Genetic dissection of a regionally differentiated network for exploratory behavior in drosophila larvae. *Current Biology*, *25*(10), 1319–1326. https://doi.org/10.1016/j.cub.2015.03.023

Berni, J., Pulver, S. R., Griffith, L. C., & Bate, M. (2012). Autonomous circuitry for substrate exploration in freely moving drosophila larvae. *Current Biology*, *22*(20), 1861–1870. https://doi.org/10.1016/j.cub.2012.07.048

Berrigan, D., & Lighton, J. R. (1993). Bioenergetic and kinematic consequences of limblessness in larval Diptera. *The Journal of Experimental Biology*, *179*, 245–259.

Berrigan, D., & Pepin, D. J. (1995). How maggots move: Allometry and kinematics of crawling in larval Diptera. *Journal of Insect Physiology*, *41*(4), 329–337. https://doi.org/10.1016/0022-1910(94)00113-U

Brake, I., & Baechli, G. (2008). *Drosophilidae (Diptera)*. Apollo Books.

Burnet, B., Sewell, D., & Bos, M. (1977). Genetic analysis of larval feeding behaviour in Drosophila melanogaster: II. Growth relations and competition between selected lines. *Genetical Research*, *30*(2), 149–161. https://doi.org/10.1017/S0016672300017559

Carreira-Rosario, A., Zarin, A. A., Clark, M. Q., Manning, L., Fetter, R. D., Cardona, A., & Doe, C. Q. (2018). MDN brain descending neurons coordinately activate backward and inhibit forward locomotion. *ELife*, *7*, 1–28. https://doi.org/10.7554/eLife.38554

Clark, A. G., Eisen, M. B., Smith, D. R., Bergman, C. M., Oliver, B., Markow, T. A., Kaufman, T. C., Kellis, M., Gelbart, W., Iyer, V. N., Pollard, D. A., Sackton, T. B., Larracuente, A. M., Singh, N. D., Abad, J. P., Abt, D. N., Adryan, B., Aguade, M., Akashi, H., … MacCallum, I. (2007). Evolution of genes and genomes on the Drosophila phylogeny. *Nature*, *450*(7167), 203–218. https://doi.org/10.1038/nature06341

Clark, M. Q., Zarin, A. A., Carreira-Rosario, A., & Doe, C. Q. (2018). Neural circuits driving larval locomotion in Drosophila. *Neural Development*, *13*(1), 1–10. https://doi.org/10.1186/s13064-018-0103-z

Dason, J. S., Cheung, A., Anreiter, I., Montemurri, V. A., Allen, A. M., & Sokolowski, M. B. (2019). Drosophila melanogaster foraging regulates a nociceptive-like escape behavior through a developmentally plastic sensory circuit . *Proceedings of the National Academy of Sciences*, 201820840. https://doi.org/10.1073/pnas.1820840116

Dillon, M. E., Wang, G., Garrity, P. A., & Huey, R. B. (2009). Thermal preference in Drosophila. *Journal of Thermal Biology*, *34*(3), 109–119. https://doi.org/10.1016/j.jtherbio.2008.11.007

Fox, L. E., Soll, D. R., & Wu, C. F. (2006). Coordination and modulation of locomotion pattern

generators in Drosophila larvae: Effects of altered biogenic amine levels by the tyramine β hydroxlyase mutation. *Journal of Neuroscience*, *26*(5), 1486–1498. https://doi.org/10.1523/JNEUROSCI.4749-05.2006

Fuller, P. O., Higham, T. E., & Clark, A. J. (2011). Posture, speed, and habitat structure: Three-dimensional hindlimb kinematics of two species of padless geckos. *Zoology*, *114*(2), 104–112. https://doi.org/10.1016/j.zool.2010.11.003

Fushiki, A., Zwart, M. F., Kohsaka, H., Fetter, R. D., Cardona, A., & Nose, A. (2016). A circuit mechanism for the propagation of waves of muscle contraction in Drosophila. *ELife*, *5*, 1–23. https://doi.org/10.7554/elife.13253

Gepner, R., Skanata, M. M., Bernat, N. M., Kaplow, M., & Gershow, M. (2015). Computations underlying Drosophila photo- taxis, odor-taxis, and multi-sensory integration. *ELife*, *4*(MAY), 1–21. https://doi.org/10.7554/eLife.06229

Gershow, M., Berck, M., Mathew, D., Luo, L., Kane, E. A., Carlson, J. R., & Samuel, A. D. T. (2012). Controlling airborne cues to study small animal navigation. *Nature Methods*, *9*(3), 290–296. https://doi.org/10.1038/nmeth.1853

Gjorgjieva, J., Berni, J., Evers, J. F., & Eglen, S. J. (2013). Neural circuits for peristaltic wave propagation in crawling Drosophila larvae: analysis and modeling. *Frontiers in Computational Neuroscience*, *7*(April), 1–19. https://doi.org/10.3389/fncom.2013.00024

Gomez-Marin, A., & Louis, M. (2014). Multilevel control of run orientation in Drosophila larval chemotaxis. *Frontiers in Behavioral Neuroscience*, *8*(FEB), 1–14. https://doi.org/10.3389/fnbeh.2014.00038

Gomez-Marin, A., Stephens, G. J., & Louis, M. (2011). Active sampling and decision making in Drosophila chemotaxis. *Nature Communications*, *2*(1). https://doi.org/10.1038/ncomms1455

Green, C. H., Burnet, B., & Connolly, K. J. (1983). Organization and patterns of inter- and intraspecific variation in the behaviour of Drosophila larvae. *Animal Behaviour*, *31*(1), 282–291. https://doi.org/10.1016/S0003-3472(83)80198-5

Heckscher, E. S., Lockery, S. R., & Doe, C. Q. (2012). Characterization of Drosophila larval crawling at the level of organism, segment, and somatic body wall musculature. *Journal of Neuroscience*, *32*(36), 12460–12471. https://doi.org/10.1523/JNEUROSCI.0222-12.2012

Heckscher, E. S., Zarin, A. A., Faumont, S., Clark, M. Q., Manning, L., Fushiki, A., Schneider-Mizell, C. M., Fetter, R. D., Truman, J. W., Zwart, M. F., Landgraf, M., Cardona, A., Lockery, S. R., & Doe, C. Q. (2015). Even-Skipped+ Interneurons Are Core Components of a Sensorimotor Circuit that Maintains Left-Right Symmetric Muscle Contraction Amplitude. *Neuron*, *88*(2), 314–329. https://doi.org/10.1016/j.neuron.2015.09.009

Hernandez-Nunez, L., Belina, J., Klein, M., Si, G., Claus, L., Carlson, J. R., & Samuel, A. D. T. (2015). Reverse-correlation analysis of navigation dynamics in Drosophila larva using optogenetics. *ELife*, *4*(MAY), 1–16. https://doi.org/10.7554/eLife.06225

Hijmans, R. J., Cameron, S. E., Parra, J. L., Jones, P. G., & Jarvis, A. (2005). Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, *25*(15), 1965–1978. https://doi.org/10.1002/joc.1276

Höhna, S., Landis, M. J., & Heath, T. A. (2017). Phylogenetic inference using RevBayes. *Current Protocols in Bioinformatics*, *2017*(March), 6.16.1-6.16.34. https://doi.org/10.1002/cpbi.22

Hohna, S., Landis, M. J., Heath, T. A., Boussau, B., Lartillot, N., Moore, B. R., Huelsenbeck, J. P., & Ronquist, F. (2016). RevBayes: Bayesian phylogenetic inference using graphical models and an interactive model-specification language. *Systematic Biology*, *65*(4), 726–736. https://doi.org/10.1093/sysbio/syw021

Huelsenbeck, J., & Bruce, R. (2003). Detecting Correlation between Characters in a Comparative Analysis with Uncertain Phylogeny. *Evolution*, *57*(6), 1237–1247.

Hughes, C. L., & Thomas, J. B. (2007). A sensory feedback circuit coordinates muscle activity in Drosophila. *Molecular and Cellular Neuroscience*, *35*(2), 383–396. https://doi.org/10.1016/j.mcn.2007.04.001

Kalay, G., Atallah, J., Sierra, N. C., Tang, A. M., Crofton, A. E., Murugesan, M. K., Wykoff-Clary,

S., & Lott, S. E. (2020). Evolution of larval segment position across 12 Drosophila species*. *Evolution*, *74*(7), 1409–1422. https://doi.org/10.1111/evo.13911

Kappeler, P. (2010). *Animal Behaviour: Evolution and Mechanisms*. Springer.

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, *30*(4), 772–780. https://doi.org/10.1093/molbev/mst010

Klein, M., Afonso, B., Vonner, A. J., Hernandez-Nunez, L., Berck, M., Tabone, C. J., Kane, E. A., Pieribone, V. A., Nitabach, M. N., Cardona, A., Zlatic, M., Sprecher, S. G., Gershow, M., Garrity, P. A., Samuel, A. D. T., & Sternberg, P. W. (2015). Sensory determinants of behavioral dynamics in Drosophila thermotaxis. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(2), E220–E229. https://doi.org/10.1073/pnas.1416212112

Kohsaka, H., Guertin, P. A., & Nose, A. (2017). Neural Circuits Underlying Fly Larval Locomotion. *Current Pharmaceutical Design*, *23*(12), 1722–1733. https://doi.org/10.2174/1381612822666161208120835

Kohsaka, H., Takasu, E., Morimoto, T., & Nose, A. (2014). A group of segmental premotor interneurons regulates the speed of axial locomotion in drosophila larvae. *Current Biology*, *24*(22), 2632–2642. https://doi.org/10.1016/j.cub.2014.09.026

Kohsaka, H., Zwart, M. F., Fushiki, A., Fetter, R. D., Truman, J. W., Cardona, A., & Nose, A. (2019). Regulation of forward and backward locomotion through intersegmental feedback circuits in Drosophila larvae. *Nature Communications*, *10*(1), 2654. https://doi.org/10.1038/s41467-019-10695-y

Kwon, Y., Shen, W. L., Shim, H. S., & Montell, C. (2010). Fine thermotactic discrimination between the optimal and slightly cooler temperatures via a TRPV channel in chordotonal neurons. *Journal of Neuroscience*, *30*(31), 10465–10471. https://doi.org/10.1523/JNEUROSCI.1631-10.2010

Kwon, Y., Shim, H. S., Wang, X., & Montell, C. (2008). Control of thermotactic behavior via coupling of a TRP channel to a phospholipase C signaling cascade. *Nature Neuroscience*, *11*(8), 871–873. https://doi.org/10.1038/nn.2170

Lahiri, S., Shen, K., Klein, M., Tang, A., Kane, E., Gershow, M., Garrity, P., & Samuel, A. D. T. (2011). Two alternating motor programs drive navigation in Drosophila larva. *PLoS ONE*, *6*(8). https://doi.org/10.1371/journal.pone.0023180

Lartillot, N., & Poujol, R. (2011). A phylogenetic model for investigating correlated evolution of substitution rates and continuous phenotypic characters. *Molecular Biology and Evolution*, *28*(1), 729–744. https://doi.org/10.1093/molbev/msq244

Lin, J. (1991). Divergence Measures Based on the Shannon Entropy. *IEEE Transactions on Information Theory*, *37*(1), 145–151. https://doi.org/10.1109/18.61115

Liu, L., Yermolaieva, O., Johnson, W. A., Abboud, F. M., & Welsh, M. J. (2003). Identification and function of thermosensory neurons in Drosophila larvae. *Nature Neuroscience*, *6*(3), 267–273. https://doi.org/10.1038/nn1009

Loveless, J., Lagogiannis, K., & Webb, B. (2019). Modelling the neuromechanics of exploration and taxis in larval Drosophila. *PLoS Computational Biology*, 1–33. https://doi.org/10.5281/zenodo.1432637

Loveless, J., & Webb, B. (2018). A Neuromechanical Model of Larval Chemotaxis. *Integrative and Comparative Biology*, *58*(5), 906–914. https://doi.org/10.1093/icb/icy094

Luo, L., Gershow, M., Rosenzweig, M., Kang, K., Fang-Yen, C., Garrity, P. A., & Samuel, A. D. T. (2010). Navigational decision making in Drosophila thermotaxis. *Journal of Neuroscience*, *30*(12), 4261–4272. https://doi.org/10.1523/JNEUROSCI.4090-09.2010

Makino, T., & Kawata, M. (2012). Habitat variability correlates with duplicate content of drosophila genomes. *Molecular Biology and Evolution*, *29*(10), 3169–3179. https://doi.org/10.1093/molbev/mss133

Markow. (2015). The secret lives of Drosophila flies. *ELife*, *4*(JUNE), 1–9. https://doi.org/10.7554/eLife.06793

Markow, T. A., & O'Grady, P. M. (2005). *Drosophila: A guide to species identification and use*.

Academic Press.
Miller, D. E., Staber, C., Zeitlinger, J., & Hawley, R. S. (2018). Highly contiguous genome assemblies of 15 drosophila species generated using nanopore sequencing. *G3: Genes, Genomes, Genetics*, *8*(10), 3131–3141. https://doi.org/10.1534/g3.118.200160
Ni, L., Klein, M., Svec, K. V., Budelli, G., Chang, E. C., Ferrer, A. J., Benton, R., Samuel, A. D. T., & Garrity, P. A. (2016a). The ionotropic receptors IR21a and IR25a mediate cool sensing in Drosophila. *ELife*, *5*(APRIL2016), 1–12. https://doi.org/10.7554/eLife.13254
Ni, L., Klein, M., Svec, K. V., Budelli, G., Chang, E. C., Ferrer, A. J., Benton, R., Samuel, A. D. T., & Garrity, P. A. (2016b). The ionotropic receptors IR21a and IR25a mediate cool sensing in Drosophila. *ELife*, *5*(APRIL2016). https://doi.org/10.7554/eLife.13254
Nicholson, A. J. (1954). An outline of the dynamics of animal. *Australian Journal of Zoology*, *2*(1), 9–65.
Ohyama, T., Jovanic, T., Denisov, G., Dang, T. C., Hoffmann, D., Kerr, R. A., & Zlatic, M. (2013). High-Throughput Analysis of Stimulus-Evoked Behaviors in Drosophila Larva Reveals Multiple Modality-Specific Escape Strategies. *PLoS ONE*, *8*(8). https://doi.org/10.1371/journal.pone.0071706
Ohyama, T., Schneider-Mizell, C. M., Fetter, R. D., Aleman, J. V., Franconville, R., Rivera-Alba, M., Mensh, B. D., Branson, K. M., Simpson, J. H., Truman, J. W., Cardona, A., & Zlatic, M. (2015). A multilevel multimodal circuit enhances action selection in Drosophila. *Nature*, *520*(7549), 633–639. https://doi.org/10.1038/nature14297
Osborne, K. A., Robichon, A., Burgess, E., Butland, S., Shaw, R. A., Coulthard, A., Pereira, H. S., Greenspan, R. J., & Sokolowski, M. B. (1997). Natural behavior polymorphism due to a cGMP-dependent protein kinase of Drosophila. *Science*, *277*(5327), 834–836. https://doi.org/10.1126/science.277.5327.834
Pehlevan, C., Paoletti, P., & Mahadevan, L. (2016). Integrative neuromechanics of crawling in D. Melanogaster Larvae. *ELife*, *5*(2016JULY), 1–23. https://doi.org/10.7554/eLife.11031
Petersen, M., Tenedini, F., Hoyer, N., Kutschera, F., & Soba, P. (2018). Assaying Thermo-nociceptive Behavior in Drosophila Larvae. *Bio-Protocol*, *8*(4), 1–12. https://doi.org/10.21769/bioprotoc.2737
Risse, B., Berh, D., Otto, N., Klämbt, C., & Jiang, X. (2017). FIMTrack: An open source tracking and locomotion analysis software for small animals. *PLoS Computational Biology*, *13*(5), 1–15. https://doi.org/10.1371/journal.pcbi.1005530
Risse, B., Thomas, S., Otto, N., Löpmeier, T., Valkov, D., Jiang, X., & Klämbt, C. (2013). FIM, a Novel FTIR-Based Imaging Method for High Throughput Locomotion Analysis. *PLoS ONE*, *8*(1), 1–11. https://doi.org/10.1371/journal.pone.0053963
Rohlfs, M., & Hoffmeister, T. S. (2003). An evolutionary explanation of the aggregation model of species coexistence. *Proceedings of the Royal Society B: Biological Sciences*, *270*(SUPPL. 1), 33–35. https://doi.org/10.1098/rsbl.2003.0002
Rosenzweig, M., Brennan, K. M., Tayler, T. D., Phelps, P. O., Patapoutian, A., & Garrity, P. A. (2005a). The Drosophila ortholog of vertebrate TRPA1 regulates thermotaxis. *Genes and Development*, *19*(4), 419–424. https://doi.org/10.1101/gad.1278205
Rosenzweig, M., Brennan, K. M., Tayler, T. D., Phelps, P. O., Patapoutian, A., & Garrity, P. A. (2005b). The Drosophila ortholog of vertebrate TRPA1 regulates thermotaxis. *Genes and Development*, *19*(4), 419–424. https://doi.org/10.1101/gad.1278205
Rosenzweig, M., Kang, K. J., & Garrity, P. A. (2008). Distinct TRP channels are required for warm and cool avoidance in Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(38), 14668–14673. https://doi.org/10.1073/pnas.0805041105
Ruiz-Dubreuil, G., Burnet, B., Connolly, K., & Furness, P. (1996). Larval foraging behaviour and competition in Drosophila melanogaster. *Heredity*, *76*(1), 55–64. https://doi.org/10.1038/hdy.1996.7
Schmidt-Nielsen, K. (1997). *Animal Physiology: Adaptation and Environment*. Cambridge University Press.
Schneider-Mizell, C. M., Gerhard, S., Longair, M., Kazimiers, T., Li, F., Zwart, M. F., Champion,

A., Midgley, F. M., Fetter, R. D., Saalfeld, S., & Cardona, A. (2016). Quantitative neuroanatomy for connectomics in Drosophila. *ELife*, *5*(MARCH2016), 1–36. https://doi.org/10.7554/eLife.12059

Shen, W. L., Kwon, Y., Adegbola, A. A., Luo, J., Chess, A., & Montell, C. (2011). Function of rhodopsin in temperature discrimination in Drosophila. *Science*, *331*(6022), 1333–1336. https://doi.org/10.1126/science.1198904

Simpson, J. H., & Looger, L. L. (2018). *Functional Imaging and Optogenetics in Drosophila*. *208*(April), 1291–1309.

Sims, D. W., Humphries, N. E., Hu, N., Medan, V., & Berni, J. (2019). Optimal searching behaviour generated intrinsically by the central pattern generator for locomotion. *ELife*, *8*, 1–31. https://doi.org/10.7554/eLife.50316

Sokolowski, M. B. (1980). Foraging strategies of Drosophila melanogaster: a chromosomal analysis. *Behavior Genetics*, *10*(3), 291–302.

Sokolowski, M. B. (1985). Genetics and ecology of Drosophila melanogaster larval foraging and pupation behaviour. *Journal of Insect Physiology*, *31*(11), 857–864. https://doi.org/10.1016/0022-1910(85)90103-9

Sokolowski, M. B., Kent, C., & Wong, J. (1984). Drosophila larval foraging behaviour: Developmental stages. *Animal Behaviour*, *32*(3), 645–651. https://doi.org/10.1016/S0003-3472(84)80139-6

Sokolowski, M. B., Pereira, H. S., & Hughes, K. (1997). Evolution of foraging behavior in drosophila by density-dependent selection. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(14), 7373–7377. https://doi.org/10.1073/pnas.94.14.7373

Stoessel, A., & Fischer, M. S. (2012). Comparative intralimb coordination in avian bipedal locomotion. *Journal of Experimental Biology*, *215*(23), 4055–4069. https://doi.org/10.1242/jeb.070458

Sun, X., Liu, Y., Liu, C., Mayumi, K., Ito, K., Nose, A., & Kohsaka, H. (2020). A neuromechanical model and kinematic analyses for Drosophila larval crawling based on physical measurements. *BioRxiv*.

Tastekin, I., Riedl, J., Schilling-Kurz, V., Gomez-Marin, A., Truman, J. W., & Louis, M. (2015). Role of the subesophageal zone in sensorimotor control of orientation in drosophila larva. *Current Biology*, *25*(11), 1448–1460. https://doi.org/10.1016/j.cub.2015.04.016

Theunissen, L. M., Bekemeier, H. H., & Dürr, V. (2015). Comparative whole-body kinematics of closely related insect species with different body morphology. *Journal of Experimental Biology*, *218*(3), 340–352. https://doi.org/10.1242/jeb.114173

Thompson, N. E., O'Neill, M. C., Holowka, N. B., & Demes, B. (2018). Step width and frontal plane trunk motion in bipedal chimpanzee and human walking. *Journal of Human Evolution*, *125*, 27–37. https://doi.org/10.1016/j.jhevol.2018.09.006

Tracey, W. D., Tracey, W. D., Wilson, R. I., Wilson, R. I., Laurent, G., Laurent, G., Benzer, S., & Benzer, S. (2003). Gene Essential for Nociception. *Cell*, *113*, 261–273.

Turelli, M., Cooper, B. S., Richardson, K. M., Ginsberg, P. S., Peckenpaugh, B., Antelope, C. X., Kim, K. J., May, M. R., Abrieux, A., Wilson, D. A., Bronski, M. J., Moore, B. R., Gao, J. J., Eisen, M. B., Chiu, J. C., Conner, W. R., & Hoffmann, A. A. (2018). Rapid Global Spread of wRi-like Wolbachia across Multiple Drosophila. *Current Biology*, *28*(6), 963-971.e8. https://doi.org/10.1016/j.cub.2018.02.015

Venken, K. J. T., Simpson, J. H., & Bellen, H. J. (2011). Genetic manipulation of genes and cells in the nervous system of the fruit fly. *Neuron*, *72*(2), 202–230. https://doi.org/10.1016/j.neuron.2011.09.021

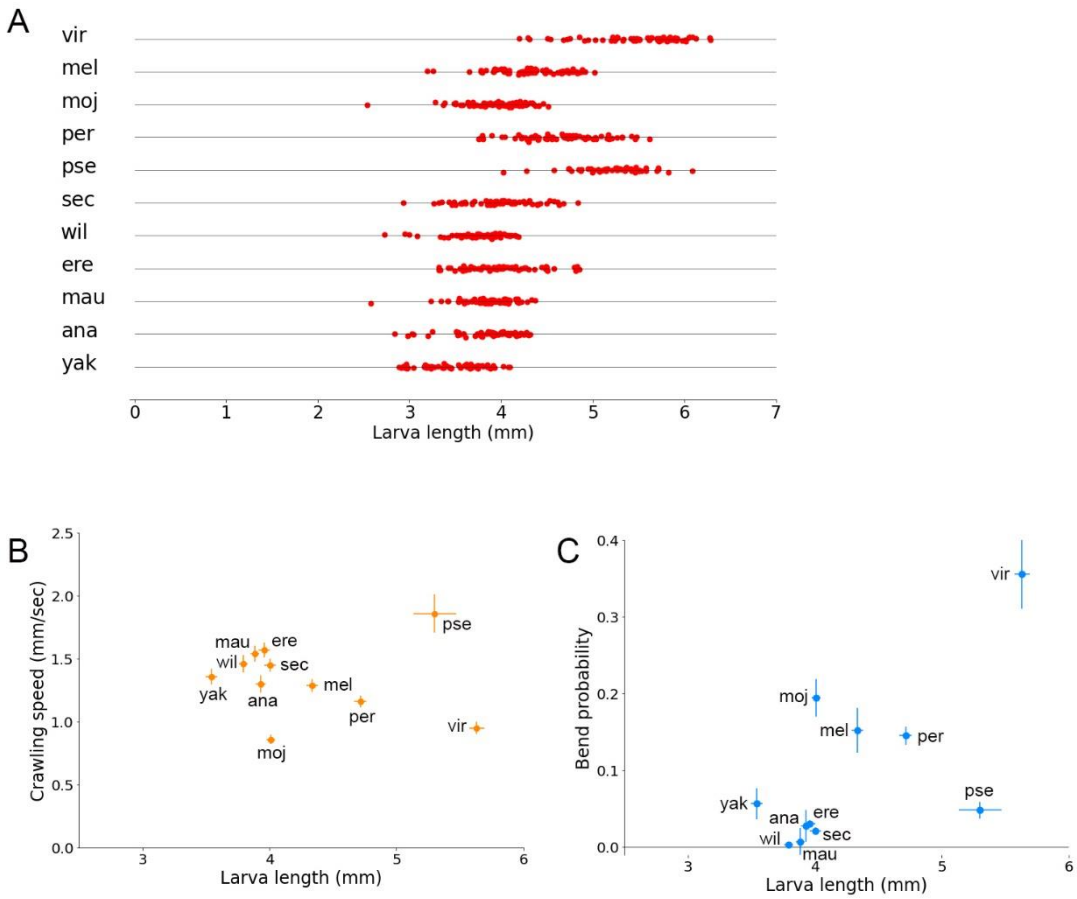
Watada, M., Hayashi, Y., Watanabe, K., Mizutani, S., Mure, A., Hattori, Y., & Uemura, T. (2020). Divergence of Drosophila species: longevity and reproduction under different nutrient balances . *Genes to Cells*, *June*. https://doi.org/10.1111/gtc.12798

Watanabe, K., Kanaoka, Y., Mizutani, S., Uchiyama, H., Yajima, S., Watada, M., Uemura, T., & Hattori, Y. (2019). Interspecies Comparative Analyses Reveal Distinct Carbohydrate-Responsive Systems among Drosophila Species. *Cell Reports*, *28*(10), 2594-2607.e7.

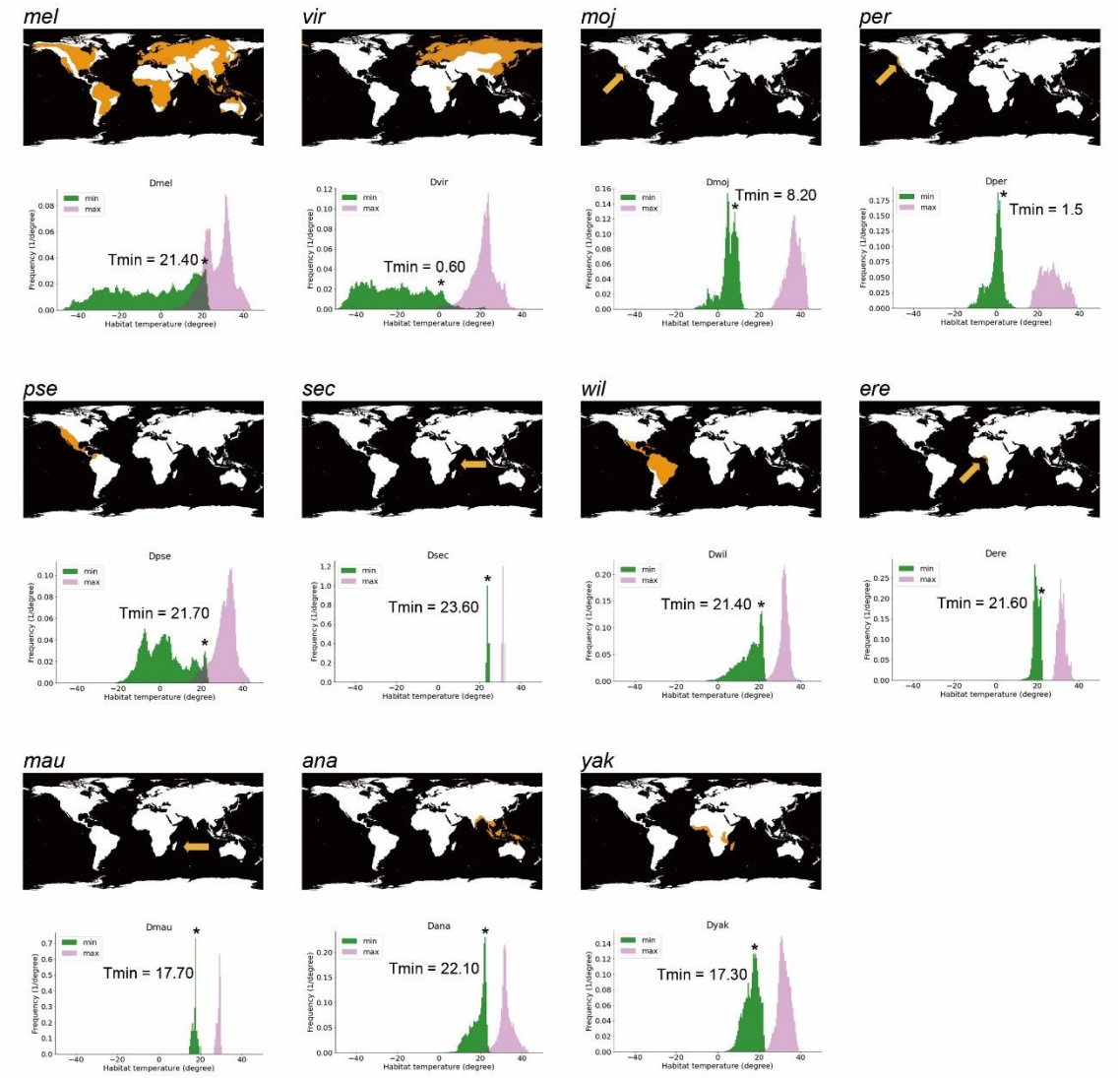
https://doi.org/10.1016/j.celrep.2019.08.030

Wertheim, B., Marchais, J., Vet, L. E. M., & Dicke, M. (2002). Allee effect in larval resource exploitation in Drosophila: An interaction among density of adults, larvae, and micro-organisms. *Ecological Entomology*, *27*(5), 608–617. https://doi.org/10.1046/j.1365-2311.2002.00449.x

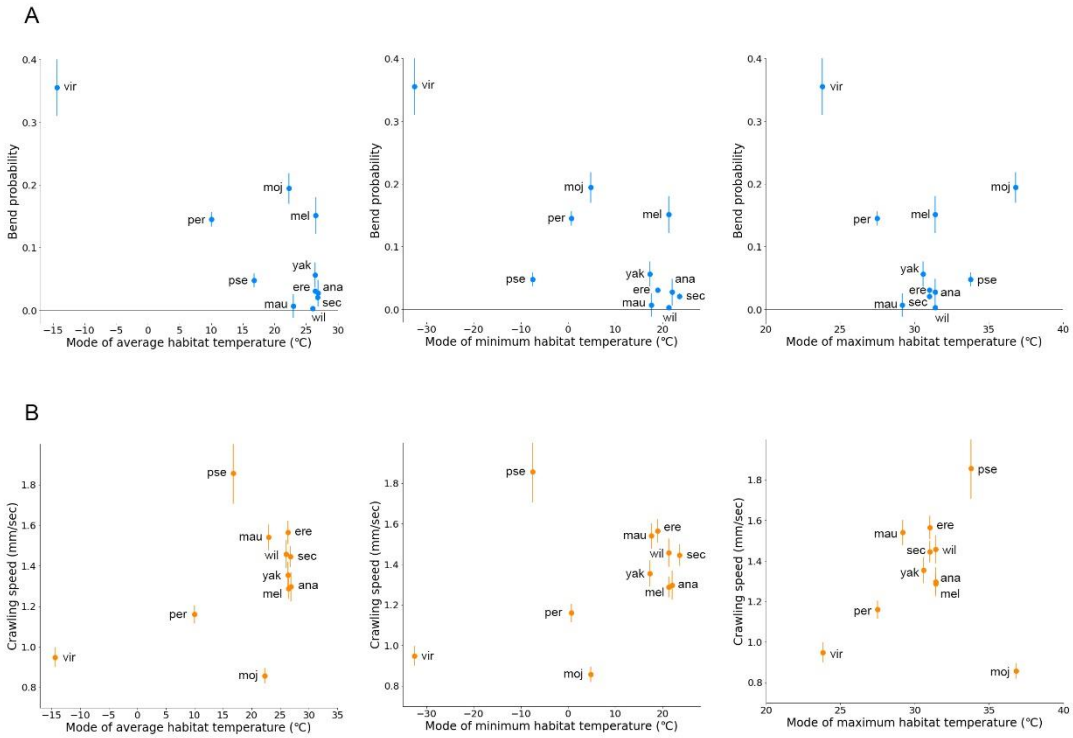
Zwart, M. F., Pulver, S. R., Truman, J. W., Fushiki, A., Fetter, R. D., Cardona, A., & Landgraf, M. (2016). Selective Inhibition Mediates the Sequential Recruitment of Motor Pools. *Neuron*, *91*(3), 615–628. https://doi.org/10.1016/j.neuron.2016.06.031



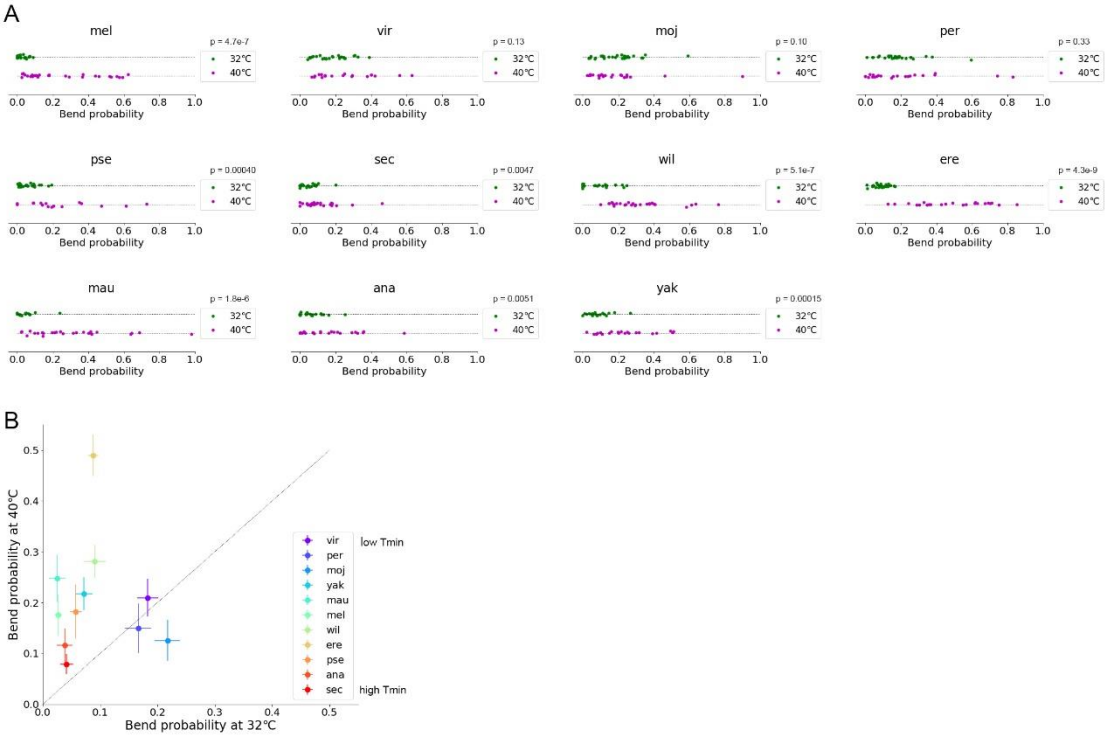
Supplementary Figure 1. Interspecies comparison between larva length and kinematics parameters. (A) Axial body length of larvae of the 11 species. Sample numbers are the following: *Dvir*: n=69; *Dmel*: n=72; *Dmoj*: n=82; *Dper*: n=76; *Dpse*: n=60; *Dsec*: n=68; *Dwil*: n=66; *Dere*: n=69; *Dmau*: n=69; *Dana*: n=68; *Dyak*: n=56. (B) Scatter plot of the crawling speed at 24°C against the larva length of the 11 species. (C) Scatter plot of the bend probability at 24°C against the larva length of the 11 species. Median ± sem are shown in B and C.



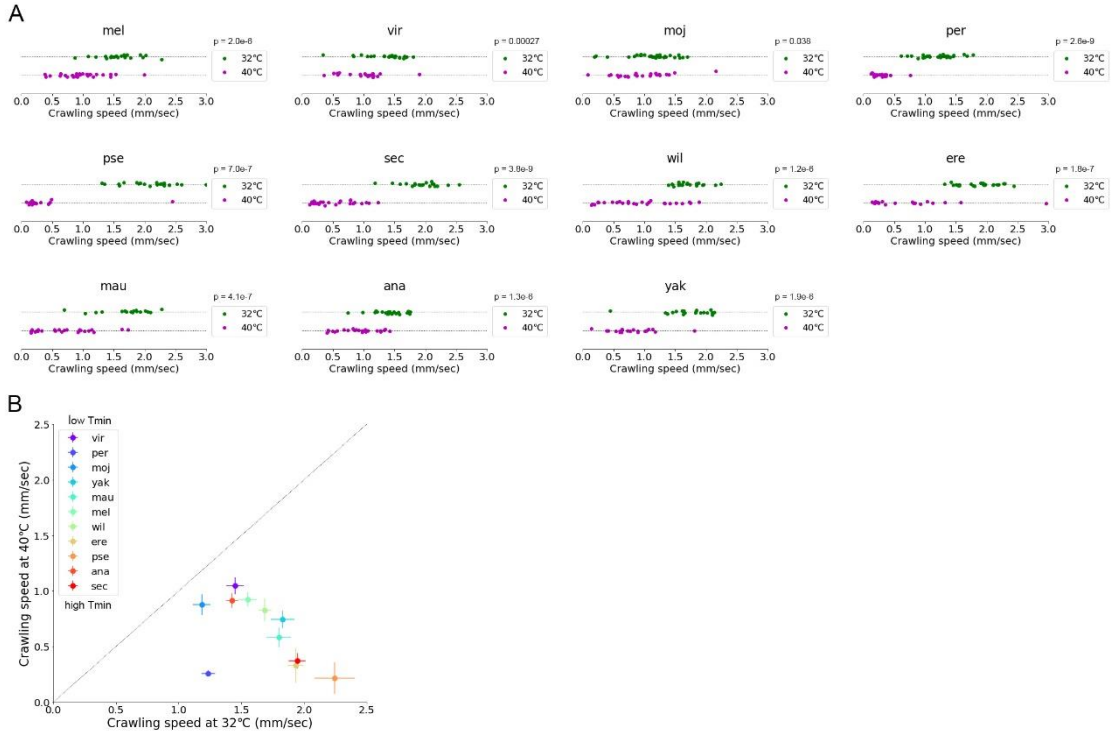
Supplementary Figure 2. World maps of histograms of habitat temperature of the *Drosophila* species. The top of each panel shows a map of the habitat of the labelled species. The bottom on each panel shows a histogram of minimum (green) and maximum (magenta) temperatures within the habitat. Asterisks denote the warmest peak in the minimum habitat temperature. The temperature at the peak is called Tmin in this study.



Supplementary Figure 3. Scatter plots of kinematics parameters and the modes of average, minimum, and maximum habitat temperatures. (A) Scatter plot of bend probability of the 11 species at 24°C against the modes of the average (left), minimum (center), and maximum (right) habitat temperatures. (B) Scatter plot of the crawling speed of the 11 species at 24°C against the modes of the average (left), minimum (center), and maximum (right) habitat temperatures. Median ± sem are shown.



Supplementary Figure 4. Bend probability in the 11 *Drosophila* species at 40°C. (A) Bend probability of the 11 species at 32°C and 40°C. The data at 32°C are the same as in Figure 4A. (B) Scatter plot of the bend probability at 40°C against at 32°C. Sample numbers at 40°C are the following: *Dvir*: n=20; *Dmel*: n=28; *Dmoj*: n=23; *Dper*: n=21; *Dpse*: n=16; *Dsec*: n=27; *Dwil*: n=27; *Dere*: n=22; *Dmau*: n=26; *Dana*: n=23; *Dyak*: n=20. Sample numbers at 32°C are described in the legend of Figure 4. Median ± sem are shown in B.



Supplementary Figure 5. Crawling speed in the 11 *Drosophila* species at 40°C. (A) Crawling speed of the 11 species at 32°C and 40°C. The data at 32°C are the same as in Figure 5A. (B) Scatter plot of the crawling speed at 40°C against at 32°C. Sample numbers at 40°C are the following: *Dvir*: n=20; *Dmel*: n=28; *Dmoj*: n=23; *Dper*: n=21; *Dpse*: n=16; *Dsec*: n=27; *Dwil*: n=27; *Dere*: n=22; *Dmau*: n=26; *Dana*: n=23; *Dyak*: n=20. Sample numbers at 32°C are described in the legend of Figure 5. Median ± sem are shown in B.