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Diurnal variation in estimated genetic parameters for locomotor activity in *Drosophila melanogaster* assessed under natural conditions

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Conflict of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Abstract

In nature organisms are exposed to variable and occasionally stressful environmental conditions. Responses to diurnal and seasonal fluctuations, such as temperature and food accessibility, involve adaptive behavioral and physiological changes. While much work has been done on understanding the genetic architecture and evolutionary potential of stress tolerance traits under constant thermal conditions, there has been less focus on the quantitative genetic background in variable environments. In this study, we use the *Drosophila* Genetic Reference Panel (DGRP) to investigate locomotor activity, a key behavioral trait, under variable natural thermal conditions during the summer in a temperate environment. Male flies from 100 DGRP lines were exposed to natural thermal and light conditions in *Drosophila* activity monitors across three experimental days. We found that activity was highly temperature- and time-dependent and varied between lines both within and between days. Further, we observed variation in genetic and environmental variance components, with low to moderate estimates of the heritability for locomotor activity, consistently peaking in the afternoons. Moreover, we showed that the estimated genetic correlations of locomotor activity between two time points decreased as the absolute differences in ambient temperature was increased. In conclusion, we find that the genetic background for locomotor activity is environment specific and we conclude that more variable and unpredictable future temperatures will likely have a strong impact on the evolutionary trajectories of behavioral traits in ectotherms.

Keywords: *Drosophila melanogaster*, DGRP, locomotor activity, environmental and genetic variation, heritability, genetic correlations, plasticity

Introduction

In their natural habitats, ectotherms are exposed to variable and sometimes stressful environmental conditions. The ability to cope with abiotic environmental variability depends on phenotypic plasticity and evolutionary adaptation (Hoffmann and Parsons, 1991; Kristensen et al., 2020). With global change, mean temperatures will increase, accompanied by more frequent and extreme climate-related events (IPCC, 2013). How ectotherms respond to altered temperature regimes will on a short-term scale depend on the existence of thermoregulatory behavior and plastic changes in physiological limits (Sunday et al., 2014; Sørensen et al., 2016; Kristensen et al., 2020). On a long-term scale, ectotherms with limited possibilities of avoiding these stressful conditions by seeking shelter in microhabitats or migrating to different latitudes or altitudes, must cope with the increasing and more variable temperatures partly through evolutionary adaptation (Chevin and Hoffmann, 2017). These changes in the genetic constitution of a population depend on adaptive genetic variation, and the speed of change is governed by the selection intensity and the heritability of relevant traits (Falconer and Mackay, 1996).

Several studies imply that evolution of the ability to survive and reproduce at high temperatures is constrained in some species, partly due to low genetic variation for these traits (Kellermann et al., 2009; Mitchell et al., 2011; Kelly et al., 2012; Hoffmann et al., 2013; Kristensen et al., 2015). However, conclusions on evolutionary trait trajectories are often based on estimates of genetic variances and heritabilities obtained from laboratory studies, even though it is known that these can be sensitive to the environment (Hoffmann and Parsons 1991). This raises the question whether deducing genetic constraints and evolutionary responses from studies in one, typically ecologically irrelevant, laboratory environment, can be extrapolated to populations in nature (Weigensberg and Roff, 1996; Sgrò and Hoffmann, 2004).

Natural environments vary spatially among habitats, latitudes, and altitudes, and temporally within and across days, seasons, and years. Consequently, organisms will encounter physiological stress or unfavorable conditions at different spatiotemporal scales (Weigensberg and Roff, 1996; Hoffmann and Merilä, 1999; Bahrndorff et al., 2021). To what extent the evolutionary potential of fitness components varies across environmental conditions, as exemplified by temperature, remains largely unexplored (Charmantier and Garant 2005; Fischer et al., 2020; Bonnet et al., 2022). However, Ørsted et al. (2018) showed that the evolvability of cold stress tolerance in adult *Drosophila melanogaster* is highly dependent on developmental temperatures experienced before testing. Thus, there is a need for more ecologically relevant studies examining the environmental dependence of the genetic parameters for behavioral, physiological, and other life history traits of ectotherms to improve our understanding of evolutionary trajectories in populations facing global climate change.

Lethal endpoints, such as the upper critical temperature, have been extensively studied under the assumption that survival at high temperatures is fundamental to a species' fitness or success. However,

numerous traits are affected by environmental variation and thermal conditions that are less extreme and more regularly encountered in nature than those causing mortality or coma (Miler et al., 2020; Braschler et al., 2021; Kjærsgaard et al., 2015). For instance, studies have shown that male *D. melanogaster* suffer from infertility at sub-lethal temperatures, which has implications for the reproductive output of the population (Jørgensen et al., 2006; Sales et al., 2018, 2021; Parratt et al., 2021; van Heerwaarden and Sgrò, 2021; Walsh et al., 2021). Other sub-lethal traits that are important fitness components include courtship, foraging, and predator avoidance. These traits are all encompassed by locomotor activity which can therefore be hypothesized to be central for the fitness of insects (Dillon et al. 2009; Munoz et al. 2022; Sunday et al. 2014; Everatt et al., 2013; Matsumura and Miyatake, 2022). Locomotor activity is a complex trait, and the genetic component is governed by many loci with small effects that are sensitive to environmental conditions, and likely their interactions (Rohde et al., 2018). Several studies have revealed abundant genetic variation for different locomotor activity measures in *Drosophila* (Burnet et al., 1988; Jordan et al., 2006, 2007; Rohde et al., 2018; Videlier et al., 2021). However, the genetic basis of such complex behavioral traits is largely uncharacterized under ecologically relevant environmental conditions, and the impact of natural varying temperatures on heritabilities and environmental and genetic variance components is unknown.

While numerous studies have investigated evolutionary aspects of lethal endpoints for arthropods, such as upper and lower temperatures, little is known about the impact of temperature variation on quantitative genetic estimates, including locomotor activity (Huey et al., 2012; Buchholz et al., 2019). Locomotion is a fundamental aspect of ectotherm behavior, playing crucial roles in survival and reproduction (Gilchrist et al., 1997), and it is vital for insects to cope with, and respond to, varying temperature conditions. Therefore, it is imperative to investigate the quantitative genetics of locomotor traits under ecologically relevant thermal conditions. Previous studies investigating the quantitative genetics of locomotor activity in *D. melanogaster* have been performed in the laboratory typically using either a negative geotaxis RING assay where the climbing ability of flies is assessed (Gargano et al., 2005) or by using *Drosophila* Activity Monitors (DAM) where activity is registered via infrared beam breaks of flies walking in polycarbonate tubes (Pfeiffenberger et al., 2010; Videlier et al., 2021). Such studies have commonly found heritabilities to be in the low to moderate range and knowledge regarding the genetic architecture explaining variation in locomotor activity between individuals is gradually emerging (e.g., Higgins et al., 2005; Jordan et al., 2006 & 2007). However, no studies have attempted to investigate genetic and environmental variance components, as well as genetic and phenotypic correlations, across natural daily temperature variation. This study seeks to address this knowledge gap.

Using the *Drosophila* Genetic Reference Panel (DGRP) (Huang et al., 2014), we here investigate the narrow-sense heritability, variance components, and phenotypic and genetic correlations of locomotor activity under variable field temperature conditions during the summer in Denmark. We argue that it is important to understand more about how environmental exposures, like temperature, impacts heritability and genetic

and environmental variance components of a key behavioral trait, locomotor activity, under natural thermal conditions, as behavioral traits can play a crucial role in the ability of ectotherms to respond to changes in temperature experienced on a daily or seasonal basis. This dataset provides unforeseen insights into the complex genetic parameters of ecologically relevant behaviors and provides strong evidence for highly temperature and time-of-day dependent genetic architecture and evolutionary potential of locomotor activity.

Materials and Methods

Drosophila stocks and maintenance

A total of 127 lines of the *Drosophila* Genetic Refence Panel (DGRP) (Huang et al., 2014) were obtained from Bloomington *Drosophila* Stock Center (NIH P40OD018537; Supplemental Table S1). The flies were maintained on a standard *Drosophila* oatmeal-sugar-yeast-agar medium (7 mL) at 20 °C, and a 12:12 h light/dark photoperiod (light on from 08:00a.m. to 08:00p.m.) for two generations before experimental start. The flies were pre-fed with additional dry yeast on the surface of the *Drosophila* medium before oviposition to generate flies used for the experiments.

Experimental setup

Experimental flies were produced from three replicate vials per line (100 DGRP lines were used, Supplementary Table S1). In each replicate vial, 15-20 randomly selected adult flies, comprising both males and females, were permitted to lay eggs for four 12-hour periods, with the flies being transferred to new vials every 12 hours. Within 48 hours of eclosion, flies were sexed under CO_2 anesthesia and males were transferred to new vials with fresh food. Given the size of the experiment, it was not feasible to include both sexes. Moreover, since we cannot be certain that our experimental animals are virgins, we decided to use males because females allocate more energy into egg production compared to males' less resource-expensive sperm production (Liker et al., 2015). Thus, including a mix of mated and unmated females in our study would introduce variation that we cannot account for. The problem is less with males and therefore we decided to use males. When the male flies were five days (± 24 h) old, 16 individuals from each line were transferred to 5 mm polycarbon tubes (TriKinetics, US) containing a droplet of *Drosophila* standard medium sealed with parafilm to prevent the media from drying out in one end and with a moist 5 mm long pipe cleaner in the other end. This was repeated on 3 experimental days. A total of 16 male flies per DGRP line were phenotypically assessed across 50 *Drosophila* Activity Monitors (DAM2, TriKinetics, US) on each of the three experimental days. The monitors quantify movement of animals over time by counting the number of times an animal crosses a laser centered at the middle of the polycarbon tube at a fixed time interval. The monitors were placed at an outdoor roofed field-site (57°00'52.6"N, 9°59'04.5"E) for 24 hours at three different experimental days in the Danish summer 2022 (May 4th, May 24th, and June 7th) where temperature and relative humidity differed markedly within and between days (Figure 1).

Processing of locomotor activity

The DAM monitors return the individual counts of infra-red laser beam breaks per 30 seconds. We refer to the count of breaks per time unit as the activity. Individuals showing no activity during field monitoring were considered dead and discarded from the analysis. The field activity was summarized for each individual by the total number of laser beam breaks per 20 minutes for the 24-hour monitoring period. In each comparison across experimental test days, we restricted subsequent statistical analyses to overlapping data points by restricting to activity data obtained between 10:00a.m. to 07:20a.m. the following morning (i.e., a total of 65 20-min bins). Besides the total activity per 20 minutes, we also computed the total activity across the entire experimental setup (the sum of activity across each 20-minute bin). For the subsequent statistical genetic analyses, the activity data was approximated to a Gaussian distribution by inverse-rank normalization (Figure S1) (McCaw et al., 2020).

Statistical genetic analyses

Initially, we investigated whether the total activity for each DGRP line varied across the three experimental test days. This was performed by comparing two linear mixed models utilizing the inverse-rank normalized total activity as response variable, activity monitor and testing day as fixed effects, and the DGRP lines as random effect, where the relationship among the DGRP lines were modeled as independent (as previously shown (Mackay et al., (2012); Huang et al., (2014)) by an identity block matrix, to a model where we included a random intercept of test day. The two models were assessed with a likelihood ratio test.

Subsequently, the data were split into each experimental test day, and analyzed separately. For the three experimental test days, two quantitative traits were analyzed: total activity across the experimental setup, and activity for 65 20-minutes periods. For these traits, we estimated the narrow-sense heritability (h^2) and genetic correlation (r_g) from variance components analysis. The narrow-sense heritability was estimated as:

$$h_g^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2}, \quad (1)$$

while the genetic correlation between activity measurements (time 1 and time 2) was computed as:

$$r_g = \frac{\sigma_{g_1 g_2}}{\sqrt{\sigma_{g_1}^2 \sigma_{g_2}^2}}. \quad (2)$$

Here we estimated variance and covariance components using a Bayesian approach with Markov chain Monte Carlo (Sørensen et al., 2015), utilizing the implementation in the R package qgg (Rohde et al., 2020; Rohde et al., 2023). For details on the model specification, please see the Supplementary Note.

In short, estimating the heritability was obtained using a univariate model, while for estimation of genetic correlation a bivariate model was applied. Utilizing multiple-trait models to estimate genetic parameters has

a long tradition in animal- and plant breeding, where repeated measurements on the same set of individuals across several days is common (Schaeffer and Jamrozik (1996); Sun et al., 2009). Thus, our statistical genetic approach is commonly used within quantitative genetics. The overall model was

$$y = Xd + Zg + e, \quad (3)$$

where y is a vector of phenotypic observations (i.e., total activity across the experimental setup, and activity during 65 20-minutes periods; note for the bivariate model y was a 2-column matrix), g was a random genomic effect capturing variation among the DGRP lines, e was a random residual effect, and d was a fixed effect capturing systematic experimental effects, here specifically accounting for that a total of 50 activity monitors were used to measure activity for all DGRP lines simultaneously. X and Z were design matrices linking the fixed effects d and random genomic effects g to the observations y.

The genomic effects (g) were defined as the sum of the effects of all markers:

$$g = \sum_{i=1}^{m} w_{ij} b_i, \quad (4)$$

where m is the total number of markers, w_{ij} is the *i*-th sequence variant for individual *j*, and b_i is the effect of sequence variant *i*. The marker effects (b_i) are assumed to follow $b \sim N(0, I\sigma_b^2)$, where the I represents an identity matrix and σ_b^2 is the prior variance of marker effects. The genomic effects (g) was assumed to follow a normal distribution $g \sim N(0, G\sigma_g^2)$, where σ_g^2 represents the estimated genomic variance component. G was the genomic relationship among the DGRP lines constructed as $G = \frac{1}{m} WW'$, where W is a centred and scaled genotype matrix. Each column vector of W is $w_i = \frac{a_i - 2p_i}{\sqrt{2p_i(1-p_i)}}$, with p_i being the allele frequency of the i-th variant, and a_i is the i-th column vector of an allele count matrix containing the genotypes encoded as 0, 1 and 2 referring to the number of the minor allele. SNP genotypes were obtained from whole-genome sequence data (available at http://dgrp2.gnets.ncsu.edu/). A total of 1,780,329 SNPs with a minor allele frequency (MAF) of ≥ 0.05 were present in the DGRP lines, distributed on the six chromosome arms; 2L, 2R, 3L, 3R, 4, and X. Similar, the residual effect (e) was assumed to follow a normal distribution $e \sim N(0, G\sigma_e^2)$, where σ_e^2 represents the estimated residual variance component. The estimated genetic and residual variance components were the mean across 100 iterations after a burn-in of 10 iterations, and the 95% credible intervals (CI) were estimated with the bayesTest R-package (Makowski et al., 2019). Increasing the number of iterations to 1,000 (and burn-in to 100) did not affect the accuracy of the estimated parameters, thus, the number of iterations were kept at 100 to minimize the computational burden.

The genetic correlation between locomotor activity assessed at two time points were estimated by fitting a bivariate model like the univariate model described above (see Supplementary Note for details). The only difference is that the estimated parameters now becomes a joint distribution, such that the marker effects are assumed to follow $[b_1\ b_2] \sim N([0\ 0], \begin{bmatrix} I\sigma_{b_1}^2 & I\sigma_{b_1 b_2} \\ I\sigma_{b_2 b_1} & I\sigma_{b_2}^2 \end{bmatrix})$, where $\sigma_{b_1 b_2}$ is the prior marker covariance effects, and the joint distribution of the genomic values becomes

$[g_1\ g_2\ ] \sim N([0\ 0\ ], [G\sigma^2_{g_1}\ G\sigma_{g_1g_2}\ G\sigma_{g_2g_1}\ G\sigma^2_{g_2}\ ])$, where $\sigma_{g_2g_1}$ is the genomic covariance between the trait measured under the two environmental conditions.

Results

Total activity

Abundant phenotypic variation was observed for total activity for the three experimental test days (Figure 2). Partitioning the genetic variance between days, compared to modeling tshe genetic variance as one combined effect, revealed a strong test day effect (P<2.2×10^{-16}, χ^2 =389.68, d.f.=5), clearly indicating, that splitting the data into experimental test day was more informative than the combined model. Generally, the DGRP lines that were most active at day 1, were also among the most active DGRP lines at test day 2 and 3 despite different environmental exposures and the correlations between total activity of the three days were positive and significant, ranging between 0.44 and 0.46 (Figure 2 and 3). The estimated heritability for total activity was $\hat{h}^2$ = 0.41-0.44 for the two first test days and dropped to $\hat{h}^2$ =0.28 on the third test day (Table 1). The credible intervals indicate that with 95% probability the true heritability estimates lie within the same interval for all three days (Table 1), however, comparing the posterior distribution of the heritability estimates using *t*-test indicated that the posterior distribution of heritability estimates was statistically different among all experimental test days (Supplementary Figure S2). The estimated genetic correlations between test day 1 and 2, 2 and 3 and 1 and 3 were $\hat{r}_g$=0.60 (CI=0.37-0.75), $\hat{r}_g$= 0.86 (CI=0.75-0.95), and $\hat{r}_g$=0.94 (CI=0.82-0.99), respectively.

Table 1. Estimated variance components for total activity (after inverse-rank normalization to approximate normal distribution [Supplementary Figure S1]). Variance components were estimated with BayesN, and the estimated genetic and residual variance components are the means across 100 iterations after a burn-in of 10 iterations. Numbers in parentheses represent the 95% credible intervals.

Test day	Genetic component	Residual component	Heritability ($\hat{h}^2$)
1	0.29 (0.22, 0.44)	0.36 (0.34, 0.39)	0.44 (0.37,0.56)
2	0.41 (0.31, 0.54)	0.58 (0.53, .61)	0.41 (0.35, .48)
3	0.27 (0.19, .40)	0.69 (0.64, 74)	0.28 (0.22, 0.37)

Activity in bins

The field activity was summarized for each line within 20-minute bins throughout the monitoring-period for each day. Among the lines we found substantial phenotypic variation in the beam-break frequency and time course of the activity throughout this period (Supplementary Figures S3-S5). The average field activity profile across the 100 DGRP lines showed a uniform distribution with a single activity peak in the afternoon and almost no activity during the night (Figure 4). The narrow-sense heritability for activity was calculated in

each 20-minute bin and showed diurnal variation in the estimated heritabilities across the day and across the test days (Figure 5). The fluctuation in heritability across the day was caused by variation in both the genetic and environmental variance components (Supplementary Figure S6). No apparent pattern was observed between estimated heritability and the average environmental temperature within each bin across the three experimental test days (Figure 6). However, within test day 1 and 2 we did see a pattern best described with a quadratic fit where intermediate temperatures resulted in the highest estimated heritabilities (Supplementary Figure S7). Time of day, across the three experimental test days, however, was a better predictor (assessed by mean variance explained across each model fit of the posterior estimated heritability and mean temperature or time of day) for heritability than environmental temperature (Supplementary Figure S7).

We then computed the genetic correlation (r_g) among all 20-minute activity bins across the three test days. The estimated genetic correlations were in the range between -1 and 1, and the closer in time two activity measurements were within the same experimental test day, the higher the estimated genetic correlation was. A similar trend was seen with ambient temperature, with an inverse relationship between estimated genetic correlation and difference in environmental temperature (Figure 7). This inverse relationship was strongest for experimental test day 1, and lower for test days 2 and 3 (Figure 7, Supplementary Figure S8). Furthermore, for experimental day 1 and 2, absolute difference in temperature was a better predictor for estimated genetic correlation than absolute difference in time of day (Supplementary Figure S8), which is opposite of what was found for the heritability.

Discussion

The main focus of the present study was to provide insight into the evolutionary potential of a key behavioral trait, namely locomotor activity, studied under variable natural thermal conditions. Knowledge on this topic is currently limited and we argue that gaining a deeper understanding of this is important for evaluating the ability to adapt to future climatic challenges through behavioral means. A previous study estimated a narrow-sense heritability of 0.26 for locomotor activity under constant laboratory conditions at 25°C, using 204 DGRP lines (Rohde et al., 2018). Another study reported narrow-sense heritabilities of 0.16 for locomotor activity in *D. melanogaster* also kept at 25°C in the laboratory (Jordan et al., 2007), whereas others found substantial variation in narrow-sense heritability between females and males, with values of 0.19 and 0.62, respectively, using similar laboratory conditions (Videlier et al., 2021). The narrow-sense heritabilities obtained in our study are seemingly in the range of these previous estimates. Interestingly, our results show that there are strong effects of natural variation in environmental conditions (e.g. temperature, light, humidity) and of time-of-day on this estimate and the genetic background for locomotor activity therefore seems to be both environment and time dependent (Figures 5 A-C, & 6). The time-of-day dependent relationship with the variation among the DGRP lines (expressed using the coefficient of variation, Supplementary Figure S9), could suggest an increased scope for the expression of genetic variation for

locomotor activity at certain time points. Similar patterns have been observed for other complex phenotypes within the DGRP system (Lin et al., 2016; Ørsted et al., 2018, Sørensen et al., 2015; Wang et al., 2017). The environmental variance is lower on day 3 compared to the two other days (Table 1). This might be linked to the lower temperature variation observed on this day and illustrates how variation in temperature can impact the evolutionary trajectory of traits. However, from our data, we cannot disentangle whether these observed patterns are because of time-of-day or because of variation in ambient temperature, although some evidence points to that time of day is a better predictor than environmental temperature (Supplementary Figure S7). Nevertheless, the estimated genetic parameters for DGRP locomotor activity vary depending on, when, and under which environmental conditions, the data is obtained (Supplementary Figure S6-S7).

Quantitative genetic theory predicts that heritabilities and genetic and environmental variance components are population- and environment-specific (Falconer and Mackay 1996). Despite this, the heritability concept is still often misunderstood, and there is a widespread misconception that the heritability of a given trait is constant across populations, trait categories (such as behavioral traits lumped together), and environments. This is, as shown in this study, not always the case, and it is important to be aware of this in models attempting to predict evolutionary responses to e.g., future climatic conditions. As shown here, we found substantial variation in heritability across the course of the three experimental days (Figure 5 A-C), suggesting that the quantitative genetic architecture of locomotor activity is environment-dependent even at the short temporal scale investigated here (Supplementary Figure S7). Other studies have found environmental dependence of the genetic background and evolutionary potential for stress resistance, life-history, and morphological traits (van Heerwaarden and Sgrò, 2011; Kristensen et al., 2015; Ørsted et al., 2019) and it has been proposed that this is caused by different sets of genes being important under different environmental conditions (Hoffmann and Merilä, 1999). We can only speculate on the causes of the day, time-of-day and/or temperature dependent variance components and heritabilities obtained in our study. Theoretically it is well described that the relative contribution of additive to non-additive (dominance and epistasis) genetic variation can be environment dependent, that variation due to environmental factors can vary across time, and that the correlation between genes and environments can also be context dependent (Falconer and Mackay 1996). It has been suggested that heritabilities depend on how harsh environments are and some hypotheses predict that the expression of genetic variation will increase in unfavorable environments, whereas others lead to the opposite prediction, and there is no consensus (Hoffmann and Merilä 1999). Thus, it will be interesting to explore the environmental-specific genetic architecture further and e.g., detect temperature-specific candidate genes of importance for explaining variation in diurnal activity patterns. Unfortunately, we did not have sufficient statistical power to perform a meaningful genome-wide association study (GWAS) in our study and have therefore refrained from that. Further support for the time-of-day / thermal dependency of the genetic architecture of locomotor activity in the investigated population comes from the fact that we found strong evidence that genetic correlations in activity were highly dependent on the difference in temperature between assessment periods (bins); the higher the temperature (or time) difference, the lower

the genetic correlations (Figure 7). Thus, our data suggests that when locomotor activity is assessed at similar temperatures / times of the day, we are investigating the same trait, to a large extent underlaid by the same genetic mechanisms (Supplementary Figure S8). In contrast, locomotion is influenced by different genes at different temperatures or times of the day. In contrast to heritabilities temperature differences between two assessments (bins) better predicted genetic correlations than time differences (Supplementary Figure S8). Ørsted et al. (2018) found a similar pattern for cold tolerance in *D. melanogaster* where genetic correlations of cold tolerance were high when flies were developmentally acclimated to temperatures close to each other and low when acclimation temperatures differed more. The evolutionary implications of these findings are complex and difficult to predict. It could be expected that more variable temperatures within and between days would lead to weaker selection for e.g. higher locomotor activity because of recurrent re-ranking of animals. On the other hand, this may lead to selection for more plastic animals if thermal environments are variable and unpredictable (see discussion and examples in Pigliucci et al. 2006; Fraebel et al. 2020).

We found that the locomotor activity in *D. melanogaster* tracked the field temperature, peaking in the afternoons between 05:00p.m.-06:00p.m. and we did not observe the morning and afternoon peaks in locomotor activity typically seen in *D. melanogaster* when investigated at constant temperatures in the laboratory (Ito and Awasaki, 2022). The observed rapid decline in activity at the start of the monitoring likely reflects disturbance of the flies during the transition from the laboratory to the semi-natural field conditions, followed by a period of cold stress before acclimating to the lower temperatures experienced in the field. Obviously, we cannot rule out that the observed activity patterns are partly shaped by exposure conditions prior to testing (maintained at a constant temperature at 20°C) and the abrupt exposure to variable temperatures in the field. However, given the isogenic nature of the individual DGRP lines, evolutionary adaptation to laboratory conditions is unlikely, and the long-term monitoring (24 hours of registrations on each day) suggests that the results do reflect patterns of locomotor behavior relevant from an ecological point of view.

Our findings affirm results from other insect studies that have shown that locomotor activity levels are partly shaped by ambient temperature (Bahrndorff et al. 2016; Ito and Awasaki, 2022; Klepsatel et al., 2013; MacLean et al., 2017; Shaw et al., 2019) and reveal that findings from laboratory studies on locomotor activity and other behavioral traits performed at constant temperatures in the laboratory can be misleading for processes in the field. Apart from temperatures, we know that other factors such as circadian rhythms, light, and interactions among animals, also have a strong impact on daily activity levels, and these interact in complex ways that we are not able to disentangle with our design (Menegazzi et al., 2012; Shaw et al., 2019). For instance, Shaw et al. (2019) demonstrated that the locomotor activity of *Drosophila suzukii* closely tracks the daily temperature during the summer. However, in the spring, the highest daily activity is observed later than the daily highest temperature. With rising future mean temperatures and more frequent heatwaves expected in the future, behavioral adjustments involving the ability of ectotherms to seek shelter and find

suitable microhabitats will be important. Exposure to higher temperatures will lead to associated increases in rates of biological processes, as illustrated here for locomotor activity, which might pose severe challenges for many species as shown recently across a broad spectrum of terrestrial and aquatic ectotherms (Jørgensen et al. 2022). Therefore, we argue that there is an urgent need to assess ecological, physiological, and genetic aspects of such traits in more detail. In forecasting species vulnerability and biogeographical shifts under future climate change, it has been argued that evolutionary adaptation should be integrated into prediction models and that current models that ignore adaptive genetic variation overestimate species vulnerabilities and future losses (Kearney et al., 2009; Bush et al., 2016). In this context, we argue that behavioral traits are important and that knowledge on the quantitative genetics of relevant traits obtained under ecologically relevant environmental conditions is rare. We believe our study is relevant in this context as the data suggest some interesting patterns: 1) there is phenotypic variation for locomotor activity across and within days, 2) the genetic background of locomotor activity is specific to the time of day / temperature at which the individuals were tested and 3) genetic correlations were temperature- and time-specific. With increasing temperature variation and more climate extremes likely to be experienced by ectotherms in the future our results suggest that this will impact on the evolution of important behavioral fitness components.

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Figure caption

Figure 1. Temperatures **(A)** and percent relative humidity **(B)** data obtained from 20 minutes bin averages from each of the three experimental days.

Figure 2. Mean of total activity within the DGRP lines for test day 1 (**A**), test day 2 (**B**), and test day 3 (**C**). Error bars represent the standard error of the mean (across the 16 biological replicates). The mean values in panel B and panel C are ordered after the DGRP line IDs in panel A.

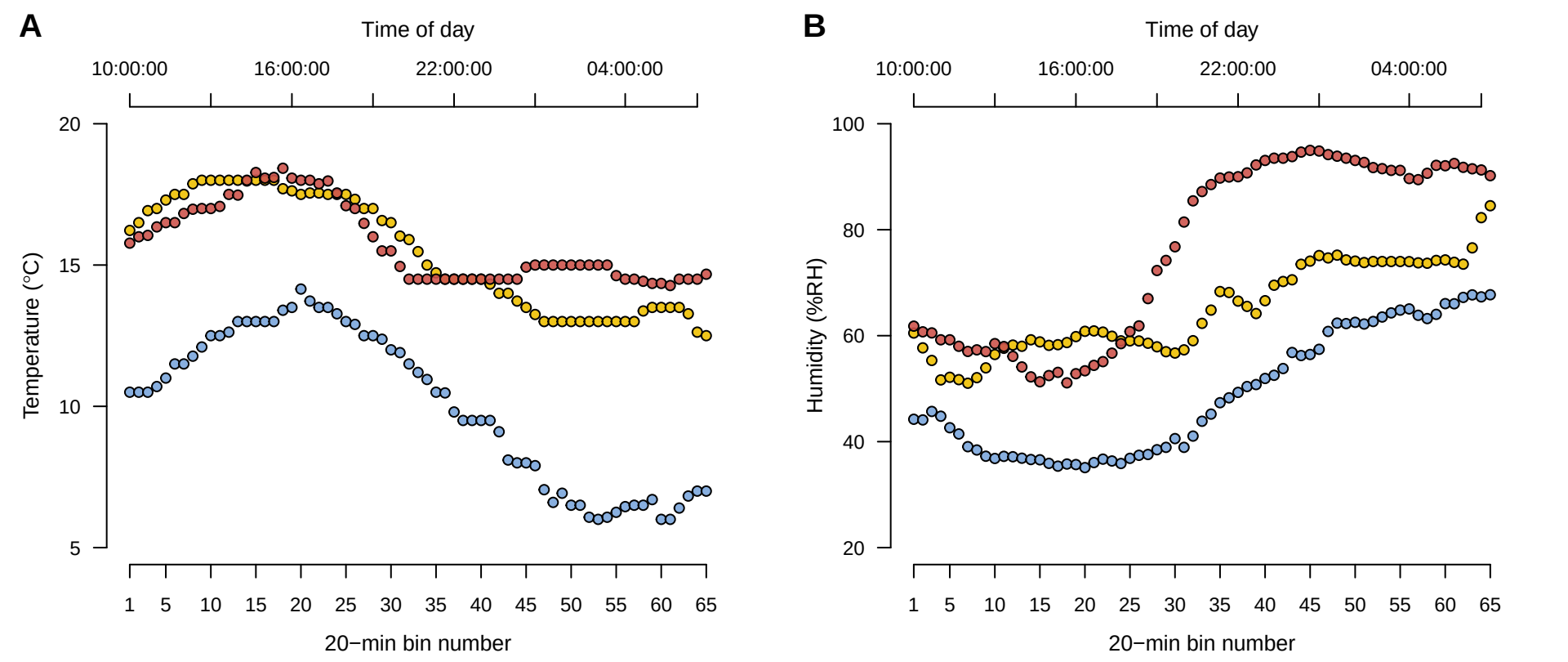
Figure 3. Phenotypic correlations of average DGRP activity between the experimental test days. Variance explained (R^2) and significance value from the best linear regression fit is indicated.

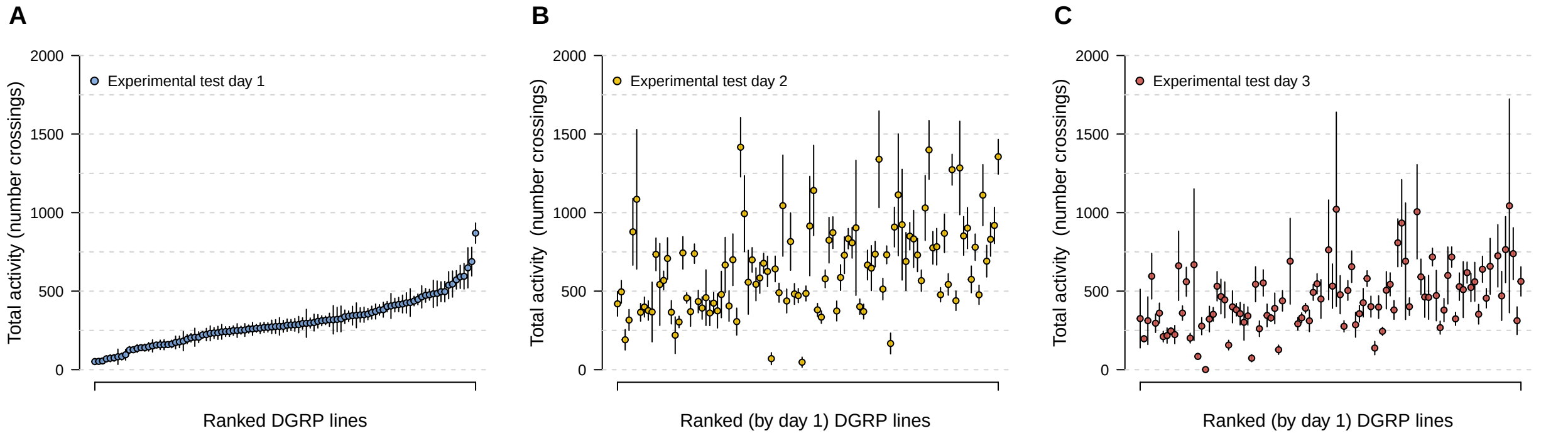
Figure 4. Mean locomotor activity across the 100 different DGRP lines summarized in 20-minute bins for the three experimental test days (panel A-C). Error bars represent the standard error of the mean. The second y-axis shows the time of day, and the shaded regions indicate the time when the sun was down.

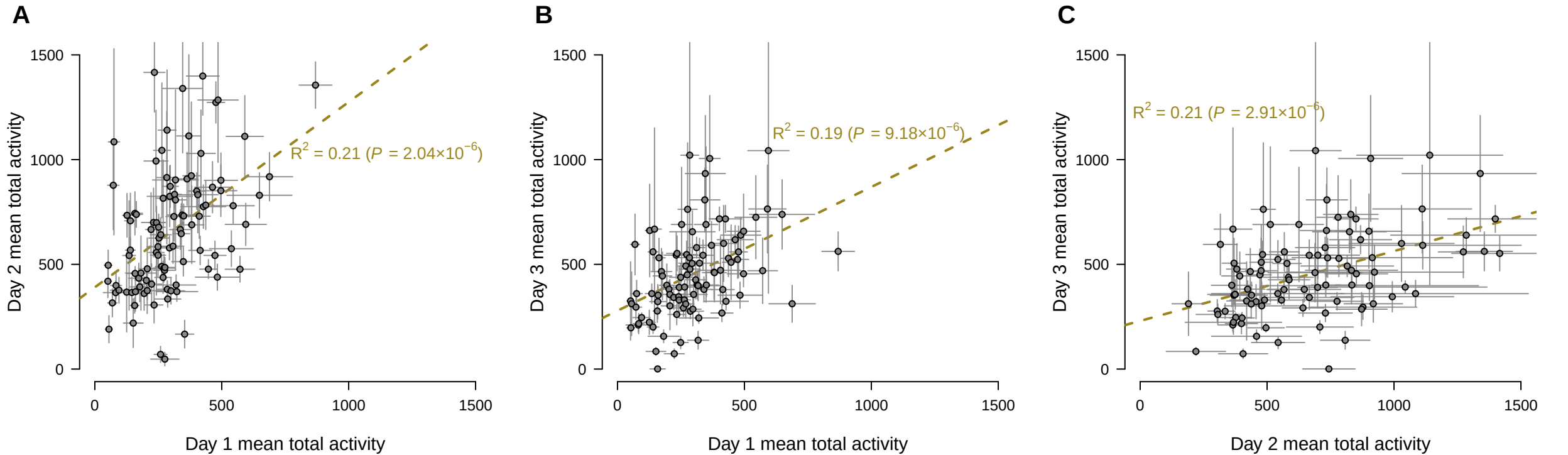
Figure 5. Estimated narrow-sense heritability (h^2) for 20-min total-activity as function of time in the field for the three experimental test days (panel A-C). Error bars represent the 95% credible interval of the estimated heritability. The second y-axis shows the time of day, and the shaded regions indicate the time when the sun was down.

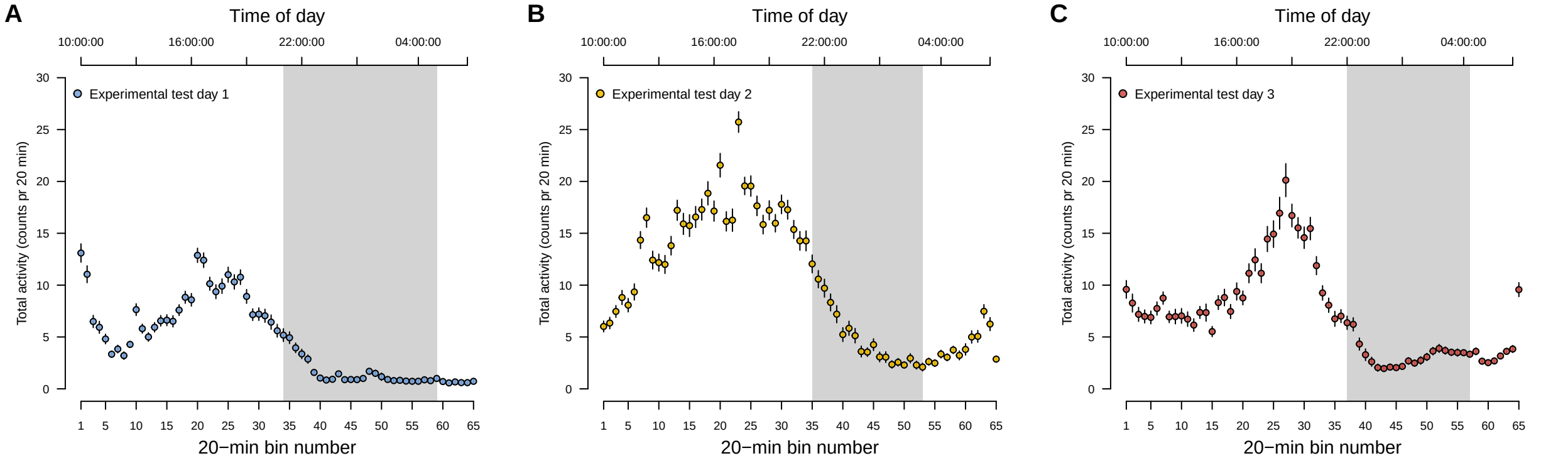
Figure 6. Estimated narrow-sense heritability (h^2) for 20-min total-activity as function of average field temperature (°C) for the three experimental test days. Vertical dashed line (and corresponding text) is the average temperature across the test day.

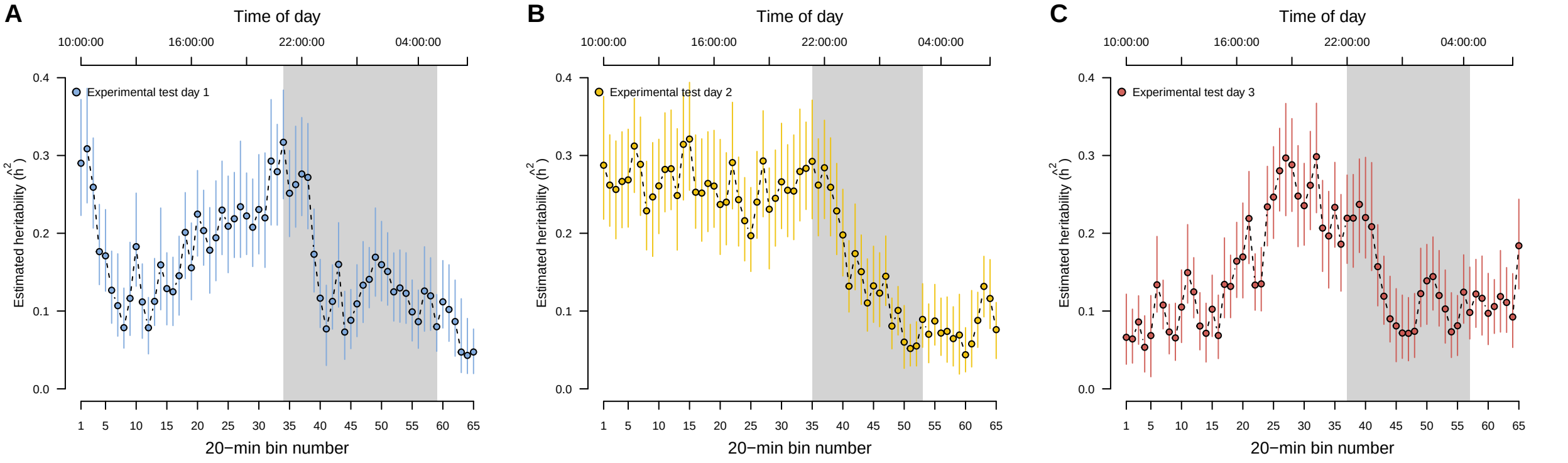
Figure 7. Comparison of genetic correlations and the absolute difference in measured temperature for the three experimental test days (panel A-C). Variance explained (R^2) and significance value from the best linear regression fit is indicated.

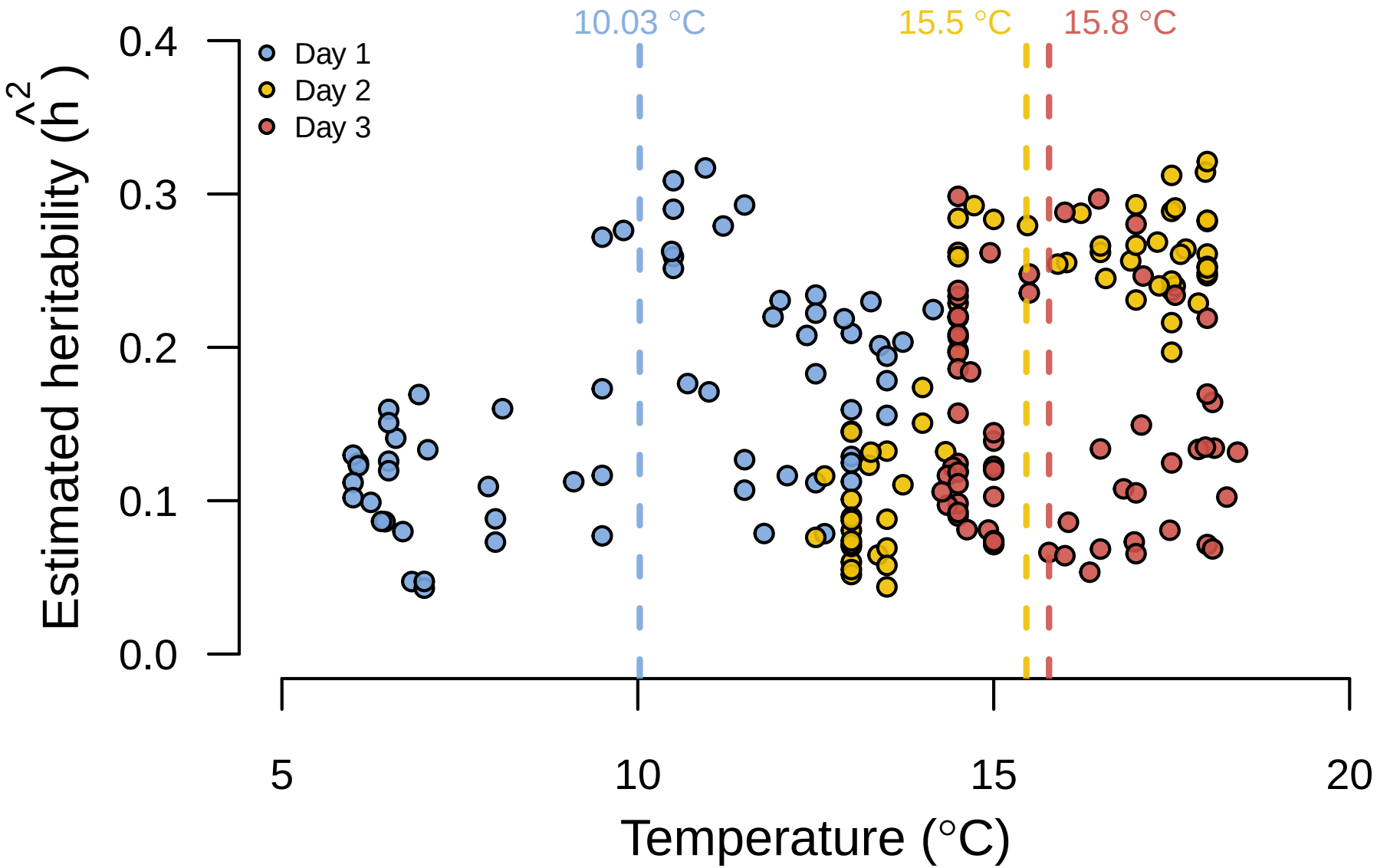

A
Time of day
10:00:00
16:00:00
22:00:00
04:00:00
Temperature (°C)
20
15
10
5
1 5 10 15 20 25 30 35 40 45 50 55 60 65
20−min bin number
B
Time of day
10:00:00
16:00:00
22:00:00
04:00:00
Humidity (%RH)
100
80
60
40
20
1 5 10 15 20 25 30 35 40 45 50 55 60 65
20−min bin number
Experimental test day 1
Experimental test day 2
Experimental test day 3

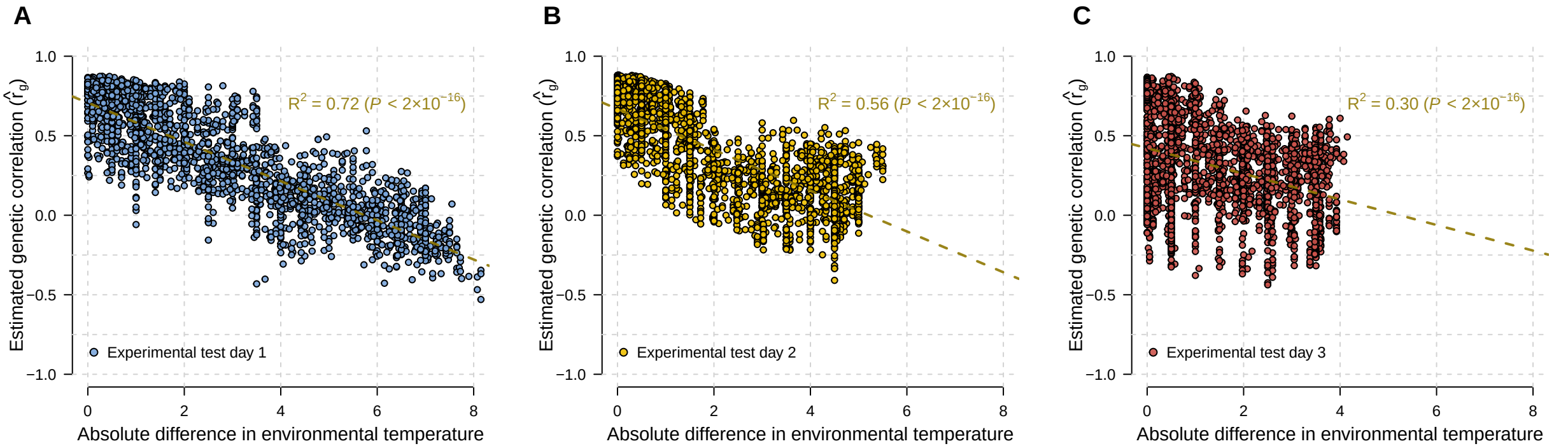

A
B
C
2000
1500
1000
500
0
Total activity (number crossings)
Experimental test day 1
Experimental test day 2
Experimental test day 3
Ranked DGRP lines
Ranked (by day 1) DGRP lines
Ranked (by day 1) DGRP lines


A
B
C
Day 2 mean total activity
Day 3 mean total activity
Day 3 mean total activity
Day 1 mean total activity
Day 1 mean total activity
Day 2 mean total activity
0
500
1000
1500
R2 = 0.21 (P = 2.04×10−6)
R2 = 0.19 (P = 9.18×10−6)
R2 = 0.21 (P = 2.91×10−6)


A
Time of day
10:00:00
16:00:00
22:00:00
04:00:00
Experimental test day 1
Total activity (counts pr 20 min)
20−min bin number
B
Time of day
10:00:00
16:00:00
22:00:00
04:00:00
Experimental test day 2
Total activity (counts pr 20 min)
20−min bin number
C
Time of day
10:00:00
16:00:00
22:00:00
04:00:00
Experimental test day 3
Total activity (counts pr 20 min)
20−min bin number


A
Time of day
10:00:00
16:00:00
22:00:00
04:00:00
Experimental test day 1
Estimated heritability (h^2)
0.0
0.1
0.2
0.3
0.4
1 5 10 15 20 25 30 35 40 45 50 55 60 65
20−min bin number
B
Time of day
10:00:00
16:00:00
22:00:00
04:00:00
Experimental test day 2
Estimated heritability (h^2)
0.0
0.1
0.2
0.3
0.4
1 5 10 15 20 25 30 35 40 45 50 55 60 65
20−min bin number
C
Time of day
10:00:00
16:00:00
22:00:00
04:00:00
Experimental test day 3
Estimated heritability (h^2)
0.0
0.1
0.2
0.3
0.4
1 5 10 15 20 25 30 35 40 45 50 55 60 65
20−min bin number


10.03 °C
15.5 °C
15.8 °C
Day 1
Day 2
Day 3
Estimated heritability (h^2)
0.4
0.3
0.2
0.1
0.0
5
10
15
20
Temperature (°C)


A
B
C
Estimated genetic correlation ($\hat{r}_g$)
1.0
0.5
0.0
−0.5
−1.0
R² = 0.72 (P < 2×10⁻¹⁶)
R² = 0.56 (P < 2×10⁻¹⁶)
R² = 0.30 (P < 2×10⁻¹⁶)
Experimental test day 1
Experimental test day 2
Experimental test day 3
0
2
4
6
8
Absolute difference in environmental temperature