

The interplay of environmental and social factors influences balancing selection: 6Pgdh in bulb mites

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Abstract

Balancing selection, the process by which multiple alleles are maintained in a population, can occur through genotype by environment interactions. The polymorphism of 6Pgdh in bulb mites involves two alleles, S and F, which have opposing effect on male reproductive success. While S-bearing males have a reproductive advantage and the S allele fixes in the laboratory populations, polymorphism is regularly observed in natural populations. We hypothesise that 6Pgdh is under balancing selection in bulb mites, driven by fluctuations of temperature and sexual selection intensity. This study uses experimental evolution to track allele frequency changes over 13 generations under different temperatures (12 °C and 18 °C) and sexual selection intensities (equal and female-biased sex ratios). The results show a significant increase in F allele frequency under higher sexual selection intensity at 12 °C. This suggests that individuals with the F allele gain a reproductive advantage at lower temperatures, and 6Pgdh polymorphism is maintained through a three-way interaction genotype x abiotic (temperature) x social environment. However, in another experiment we did not find significant associations between male genotype and reproductive success across different temperatures. The complex interplay between environmental factors and social dynamics in maintaining genetic polymorphism highlights that multiple factors can interact in driving balancing selection.

Introduction

Genetic variation, which can be considered the fundamental raw material for natural selection, is a crucial factor for evolutionary processes to manifest. An increase in genetic variation within a population increases the potential for evolutionary change, as postulated by Fisher, “the rate of increase in the average fitness of a population is equal to the genetic variance of fitness of that population” (Fisher 1941). Such genetic variation, which results in diverse phenotypes, allows populations to adapt to environmental challenges (Barrett and Schluter 2008). Nevertheless, directional selection tends to diminish genetic variation by selecting for particular variants, thereby increasing their frequency within a population (Bürger and Lynch 1995). This often results in the eventual fixation of the favoured variant (Wright 1932; Endler 1986). Therefore, the persistence of genetic polymorphism, despite the expectation of decreased variation under directional selection, presents a paradox (Lewontin 1974; Radwan et al. 2016; Hallsson and Björklund 2012).

Selection processes that result in the maintenance of polymorphisms fall under a common umbrella term, balancing selection. However, a number of different mechanisms of balancing selection have been proposed and their relative importance is still under debate (Stamp and Hadfield 2020; Fijarczyk and Babik 2015). In some populations, heterozygote advantage can be observed, as in the case of the X-linked glucose-6-phosphate dehydrogenase gene in humans, where heterozygous individuals are protected from severe malaria (Manjurano et al. 2015). This increases the survival and reproductive success of heterozygous individuals, ensuring that both alleles remain present in the population (Manjurano et al. 2015). The maintenance of polymorphism can also be attributed to negative frequency-dependent selection, in which fitness of alleles changes depending on their frequency, with rare variants having an advantage over more common ones. Fitzpatrick et al (2007) demonstrated this mechanism for *Drosophila melanogaster* foraging (*for*) gene, which is responsible for larval foraging behaviour, where the highest fitness always goes to individuals with the rare allele and the polymorphism of the gene is maintained. Antagonistic pleiotropy is another balancing selection mechanism, where the trade-off between different fitness traits leads to the maintenance of polymorphism (Connallon and Clark 2013). For example, in the case of the *Mpi* and *Idh-2* enzyme loci in red deer (*Cervus elaphus*), each with two different alleles associated with juvenile survival, genotypes associated with low survival in the first two years of development were found to have higher female reproductive fitness, resulting in the maintenance of gene polymorphism (Pemberton et al. 1991). An analogous mechanism is sexually antagonistic pleiotropy, where the trade-off is between the fitness of the sexes. Because trait fitness varies between the sexes, polymorphism is maintained within a population. Nuzhdin et al (1997) showed that the genes within five autosomal quantitative trait loci (QTL) associated with lifespan and viability in *D. melanogaster* showed strong sex-dependent effects, maintaining genetic variation in lifespan.

The significance of heterogeneous environments in maintaining genetic variation has been well documented (Hedrick et al. 1976; Slatkin 1987), particularly from early theoretical modelling studies (Via and Lande 1987; Gillespie and Turelli 1989; Levins 1968). The phenomenon termed environment-dependent balancing selection occurs when the fitness of alleles depends on the environment (there is a crossover genotype-by-environment interaction for fitness), and environmental factors vary in space or time (Kokko and Heubel 2008). Given the expected variability of the environment, such environment-dependent selection can potentially be a powerful driver of balancing selection. Early studies on this topic used simple models focusing on simple genetic systems, often considering only a single environmental factor and assuming constant selection pressure over time (Levins 1968; Hedrick et al. 1976). These models suggested that the parameter space in which polymorphism could be maintained was relatively narrow, implying that the maintenance of genetic variation required rather specific conditions that may rarely occur in nature. In contrast, more recent studies have adopted more complex and realistic models that consider polygenic traits, multi-allelic genes, and the simultaneous effects of multiple environmental factors (Carley et al. 2021; Abdul Rahman et al. 2021). These newer models consider the dynamic nature of natural environments, where multiple factors can change in unpredictable ways and interact with genetic systems. As a result, these newer studies have shown that the conditions for maintaining polymorphism are less

restrictive than previously thought. The broader and more inclusive parameter space found in these models suggests that genetic variation can be maintained under a wider range of environmental conditions, highlighting the importance of considering complex interactions between genetics and environment to reflect the nature of real-world scenarios. The findings of the newer models have been corroborated by empirical studies, thereby demonstrating their robustness (Brook 2009; van Dongen et al. 2016; Zan and Carlborg 2024).

As mentioned above, the role of environmental heterogeneity in driving balancing selection in natural systems can be quite complex. The potential of a given environmental factor to shape genotype-by-environment interactions may depend on another environmental variable. For example, several studies have demonstrated genotype-by-environment interactions for sexual fitness, including male reproductive success (Patlar and Ramm 2020; Greenfield and Rodríguez 2004; Ingleby et al. 2010). The potential of such interactions to drive selection should vary with conditions that influence the strength of sexual selection, such as population density or sex ratio. Consequently, one might observe a three-way interaction between genotype, abiotic and biotic (social) environment.

In the case of the 6-phosphogluconate dehydrogenase (*6Pgdh*) polymorphism in bulb mite *Rhizoglyphus robini*, a three-way interaction may be expected as the mechanism of polymorphism maintenance, where one of the alleles (the S allele) is associated with increased male reproductive success (Konior 2006; Skwierzyńska and Plesnar-Bielak 2018), which should lead to its rapid fixation and loss of the polymorphism. However, while the alternative F allele is indeed rapidly lost in the laboratory, both alleles are often maintained in natural populations (Unnikrishnan et al. 2024). A previous experimental evolutionary study showed that the selective advantage of the S allele is reduced (but not lost) in populations evolving at a reduced temperature of 18 °C compared to the standard laboratory temperature of 24 °C (Plesnar-Bielak et al. 2020). Similarly, the study also showed that S allele frequency increased less rapidly in populations where sex ratio manipulation relaxed the intensity of sexual selection (Plesnar-Bielak et al. 2020). It has been speculated that these two factors, if applied simultaneously, could have led to a reversal of fitness rank and a selective advantage for the F allele. In such a case, the interaction of temperature and social environment and their heterogeneity could explain the maintenance of the *6Pgdh* polymorphism in the wild.

Here we use experimental evolution to test how the interaction of temperature and sexual selection intensity shapes *6Pgdh* allele frequencies. We track changes in *6Pgdh* frequencies in replicate populations for 13 generations under two levels of temperature (18 °C and 12 °C) interacting with two levels of selection intensity (equal sex ratio and female-biased population with relaxed sexual selection). We predict that there is a cost for the S allele at lower temperature. If this cost is associated with life history fitness (as opposed to reproductive fitness), we predict an increase in F-allele frequency under lower temperature and relaxed sexual selection. This is because under weak sexual selection intensity, the reproductive advantage of the S-allele should be negligible, hence it may be outweighed by the life history cost of the S-allele at lower temperature. Alternatively, if the cost is associated with reproductive fitness, the reproductive advantage of the S allele itself might be temperature dependent. Under such a scenario, the F allele might gain reproductive advantage at low temperature, and we would expect an increase in its frequency under low temperature and intense sexual selection.

Materials and Methods

Study system

6-Phosphogluconate dehydrogenase (*6Pgdh*) plays a pivotal role in the pentose phosphate pathway, which, in conjunction with glycolysis, represents a major glucose catabolism pathway. The pentose phosphate pathway generates ribose-5-phosphate sugars and NADPH molecules. The pathway exerts a significant influence on redox homeostasis and the biosynthesis of ribonucleotides and represents a principal source of NADPH (Stincone et al. 2015).

A single nonsynonymous nucleotide polymorphism in *6Pgdh* is associated with male-fitness differences in bulb mites, representing a case of sexual selection. The two alleles of *6Pgdh*, designated S and F, differ in one amino acid substitution (arginine to methionine). Under standard laboratory conditions, males carrying the S allele exhibit increased reproductive fitness, as evidenced by higher mating frequency and sperm production (Skwierzyńska and Plesnar-Bielak 2018) compared to F-bearing males. Heterozygotes appear to exhibit an intermediate phenotype (Łukasik et al. 2010; Skwierzyńska and Plesnar-Bielak 2018). The *6Pgdh* genotype does not appear to affect female reproductive fitness. However, females mated with SS males exhibit a decreased fitness compared to those mated with FF males, indicating the presence of sexual conflict. Once more, mating with heterozygous males result in an intermediate level of fitness reduction (Skwierzyńska and Plesnar-Bielak 2018). As predicted, given its advantage in male-male competition, the S-allele rapidly fixes in laboratory populations. However, *6Pgdh*-polymorphic populations are common in natural populations, with the level of polymorphism varying across locations and through time (Unnikrishnan et al. 2024).

Source population

Experimental evolution populations were established from a *6Pgdh* polymorphic population (source population), in which the frequency of the F allele had been increased before the start of the experiment. It was done in order to start experimental evolution from approximately

equal frequencies of both alleles. To do it, we used a field collected population originating from >100 individuals found on onions collected in Łazany (50.0206 N, 19.8924 E), near Kraków in July 2020. This field collected population was kept at 8 °C, except for the first week after establishment, when they were kept at 24 °C to let juvenile individuals develop so that the population would expand.

The source population was created in spring 2021. We randomly paired virgin females and males from the field-collected population, in which the F allele frequency was about 0.23. After the pairs mated (almost 300 pairs) and females laid eggs, both parents were genotyped. We then looked for pairs with at least 2 copies of the F allele (either both parents FS, or one FF and one SS, or one FF and one SF, or both parents FF). The offspring from such pairs were used to establish the source population. The population was allowed to expand freely for ca. 2 months at 24 °C (which corresponds to 3-4 mite generations), before it was moved to 12 °C to extend generation time, to prevent adaptation to 24 °C and the loss of the F allele. This population was used to start the experimental evolution populations within one month after transferring to 12 °C (less than one mite generation at that temperature). The F-allele frequency in the source population was 0.6 when the experimental evolution started.

Culturing conditions

All populations (source and experimental) were maintained in plastic containers (approximately 2.5 cm in diameter) with a plaster of Paris base soaked with water. They were maintained at >90% humidity, constant darkness, with powdered yeast provided *ad libitum* as a food source. Individually isolated mites were maintained in glass tubes with diameter of approximately 1 cm.

Experimental evolution

Experimental evolution was carried out with two levels of temperature (18 °C and 12 °C) crossed with two levels of sexual selection intensity achieved by manipulating the sex ratio (equal sex-ratio and female biased sex-ratio treatments with strong and weak sexual selection intensity respectively). The lowest temperature employed in the preceding study (Plesnar-Bielak et al. 2020) was selected as the high temperature level of 18°C, whereas the lower temperature level of 12 °C is pertinent to the average annual temperature in Poland. In order to achieve a weaker selection intensity, the proportion of males in the weak sexual selection intensity level was reduced to 0.2, in comparison to the previous study where this proportion was 0.3 (Plesnar-Bielak et al. 2020). Five replicate populations were used for each treatment, resulting in a total of 20 populations.

For each generation, individuals were isolated at the final nymphal stage (tritonymphs) into separate vials. The individuals were then allowed to develop individually into adults and were sexed (still in individual vials). These virgin mites were then put in common containers (one container per replicate) controlling for the sex ratio (64 females + 16 males in female-biased populations and 40 females + 40 males in equal sex-ratio populations) and allowed to interact for 6 days, during which the mites could mate freely, and females could lay eggs. After this time, adult individuals were removed, and eggs were left for further development. Allele frequencies of *6Pgdh* were measured from a random sample of around 40 individuals from every two consecutive generations followed by a five-generation break (Fig 1a).

Reproductive success

We measured the success of the males with *6Pgdh* different genotypes in reproductive competition with SS-homozygous males from the stock laboratory population. The reproductive success was assessed using the sterile male technique, a well-established method in bulb mite research (Parker 1970; Radwan 1991; Radwan and Siva-Jothy 1996). This technique involves using an experimental male and a tester male and allowing them to compete for the female. The tester males are irradiated with 20 kRad (200 Gy) of gamma radiation from Co⁶⁰. Following irradiation with such a dose, males are still able to fertilize eggs, but the eggs do not hatch (Radwan and Siva-Jothy 1996). Therefore, since bulb mite females do not lay unfertilized eggs, an experimental male's reproductive success can be calculated as a fraction of the eggs that hatched. The fraction of eggs that are fertilized by normal, non-irradiated males and still do not hatch is negligible. The experimental males were taken from three populations that had evolved under 12 °C and equal sex-ratio, since that particular treatment showed F-allele frequency increase. Only three populations from this treatment were included because of logistic constraints, and these were chosen because they had the highest F-frequency within the treatment. The females and tester males originated from the base population maintained under 24 °C, since this population is fixed for S allele.

Firstly, 50 females were randomly selected from both the experimental and tester populations and kept in separate containers for one week to lay eggs at 12 °C. After this period, the females were removed and the containers with the eggs were transferred to the respective temperatures. The experiment was conducted at three temperatures: 24 °C (standard laboratory temperature), 12 °C (average yearly ground temperature at 5 cm depth in Poland) and 8 °C (low temperature relevant to colder periods in Poland). When the eggs hatched and the individuals reached the tritonymph stage, approximately 600 tritonymphs from the control population were isolated in individual vials in order to obtain virgin females for the experiment. Following the emergence of the adults in the populations, the experimental and tester (irradiated) males were isolated for a period of three days prior to the commencement of the experiment. The tester males were provided with food

containing blue dye, which enabled the distinction between experimental and tester males. The experimental setup comprised one experimental male, one tester male (identifiable due to the blue dye) and the virgin female from the base population. After three days, the tester males were removed, and the experimental males were genotyped. The females were kept for two more days (or four days when there were insufficient number of eggs) and then removed. From each vial, 10 to 20 eggs (depending on the number of eggs available) were then transferred to a different vial as a sample, and the fraction of the eggs hatched was recorded and used for analysis. The experiment was conducted in two separate blocks, each containing experimental males from all three populations, with the populations that were the source for the experimental males differing by two generations between the two blocks (Fig 1b).

DNA extraction and 6Pgdh genotyping

For DNA extraction, individual mites were placed in 1% chelex solution (40µl) and were crushed. After adding 3µl of proteinase-K (EurX, Poland), such a mixture was incubated in a thermocycler (Thermofisher Scientific, Poland) (10 min 94°C, 15 min 75°C). Such prepared samples were stored in a fridge (ca. 6°C) until the genotyping assay.

Real-Time PCR was used for *6Pgdh* genotyping. Fluorogenic TaqMan probes (Thermofisher Scientific) specific for the missense single nucleotide polymorphism were used to determine the F and S alleles (see Unnikrishnan et al. 2024). The Bio-Rad CFX96 Real-Time PCR detection system was used for the genotyping. TaqMan Genotyping Master Mix (Thermofisher Scientific) and Custom Genotyping Assay (Thermofisher Scientific) that included allele-specific primers and fluorescent probes were mixed in 10:1 ratio. 5.5µl of such a mix and 4.5µl of chelex with suspended DNA were put in a 96 well plate for genotyping. PCR was performed in 41 cycles (15 sec 95 °C, 1 min 60 °C).

Statistical analysis

To test how *6Pgdh* allele frequencies were changing over generations depending on the selection treatment, we used Generalized Estimating Equations (GEE) approach using the `geeglm` function (`geepack`, version 1.3.10). Compared to repeated measures ANOVA, which is usually used to handle generational data, GEE is better suited here because it accommodates various correlation structures and non-Gaussian distributions, providing robust estimates even with complex data. The GEE model here, used a vector containing the S and F alleles counts (per population obtained from each generation tested) as the response variable, with temperature and sexual selection intensity (along with their interaction) as the predictor variables. To incorporate the repeated measure nature of the data in the model, we used population ID as the vector for the clusters (`id=Population`) and the generation number specifying the order of the repeated measures in each unit (`waves=Generation`). We used a binomial model with an AR1 (autoregressive) correlation structure.

We have also checked for significant increase/decrease in F-allele frequencies within the experimental evolution period. To do it, we calculated differences in F-allele frequencies between generation 13 and generation 0 for each population. For each selection treatment, these differences were compared to 0, using two-tailed one sample t-test (`stats` package, version 4.3.0).

For the reproductive success data, we used Generalized Linear Mixed-Effects Model (GLMM) using `glmmTMB` function (`glmmTMB`, version 1.1.9) with a vector containing the number of eggs that hatched (successes) and the number of unhatched eggs (failures) as the response variable, with temperature and genotype of males (with their interaction) and block id as fixed factors and population id of the experimental males as a random factor. The model was fitted assuming a betabinomial distribution to account for the overdispersion.

Data visualizations were created using the `ggplot2` package in R (`ggplot2`, version 3.4.4). All statistical analyses were conducted using R version 4.3.0 (2023-04-21 ucrt).

Results

Experimental evolution

We found that sexual selection intensity and temperature affected *6Pgdh* allele frequency changes. Notably, the interaction between sexual selection intensity and temperature had a significant effect (GEE, $\chi^2 = 4.58$, $p = 0.03$) on the allele frequency of the experimental populations after 13 generations. This indicates that the impact of sexual selection intensity on allele frequency varied with temperature (individual effects: - sexual selection intensity: $\chi^2 = 9.56$, $p = 0.0019$; temperature: $\chi^2 = 1.51$, $p = 0.22$).

To further understand this interaction, we analyzed the effects of sexual selection intensity on allele frequencies at each temperature separately. The results show that sexual selection intensity had a significant effect at 12 °C ($\chi^2 = 24.58$, $p < 0.001$) (Fig 2) resulting in an increase in F-allele frequency. This result was confirmed by the t-test, showing an increase in F allele frequency in populations with higher sexual selection intensity at 12 °C ($t = 5.06$, $p = 0.007$), but not in populations with lower sexual selection intensity ($t = -0.47$, $p = 0.66$). At 18 °C, sexual selection intensity did not significantly affect allele frequencies ($\chi^2 = 2.96$, $p = 0.085$), as evidenced by the lack of significant

changes in F allele frequency for populations with low sexual selection intensity ($t = 1.97$, $p = 0.12$) and those with high sexual selection intensity ($t = -0.83$, $p = 0.45$) (Fig 3).

Reproductive success

We did not find any significant associations between reproductive success of the experimental males and the predictors tested (Fig 4). Specifically, the interaction between temperature and male genotype was not significant ($\chi^2 = 2.86$, $p = 0.58$), suggesting that the reproductive success of males with different genotypes did not differ in different temperatures. Neither temperature ($\chi^2 = 2.16$, $p = 0.34$) nor male genotype ($\chi^2 = 3.60$, $p = 0.17$) were significant individually. The only predictor variable which was significant was block id ($\chi^2 = 6.4323$, $p = 0.011$).

To further investigate the significant effect observed for block id, we tested the model at each of the blocks separately. There were no significant effects for the interaction between temperature and male genotype on the reproductive success at either of the blocks separately (block 1:- temperature X male genotype: $\chi^2 = 6.03$, $p = 0.20$; block 2:- temperature X male genotype: $\chi^2 = 4.87$, $p = 0.30$).

Discussion

The process of balancing selection maintains multiple alleles within a population. Several mechanisms have been proposed to explain this phenomenon, including negative frequency-dependent selection, heterozygote advantage, and environment-dependent selection (Charlesworth 2006; Huang et al. 2023). In systems involving environment-dependent balancing selection, there may be cases where interactions between biotic, abiotic and social environments contribute to the maintenance of polymorphism (Hedrick et al. 1976; Ferrer-Admetlla et al. 2008). Such a situation may occur in the bulb mite metabolic gene *6Pgdh*. Although males with the S allele show higher reproductive fitness in laboratory populations, polymorphism persists in natural populations (Unnikrishnan et al. 2024). The aim of this study was to investigate whether environmental factors - temperature and intensity of sexual selection - act alone or in combination to drive this balancing selection.

The results of our study provide evidence for the existence of environment-dependent balancing selection on the gene *6Pgdh* in bulb mites. In particular, a significant increase in the frequency of the F allele at low temperature and high sexual selection intensity highlights that balancing selection in the bulb mite *6Pgdh* gene is driven by a complex interplay between genotype, environmental factors and social dynamics. Furthermore, although there have been several other studies of the factors contributing to balancing selection, many of these have focused on the separate effects of individual factors (Carley et al. 2021; Smith et al. 2021; Lonn et al. 2017). A multifaceted approach to the study of balancing selection has rarely been used. A recent study on seed predation in an African savannah small mammal community system (Schoepf and Pillay 2023) is a notable exception. It shows that multiple factors such as seed size, seed type, predation risk, habitat type, seasonality and their interactions drive seed variation, with the possibility that these factors are likely to be hierarchically ordered. Similarly, a study by Joswig et al (2022) showed how the interaction of climate and soil fertility influences global plant variation. Our study adds to these by highlighting the role of social dynamics in considering such interactions within species.

In particular, our results suggest that at lower temperatures, males carrying the F allele gain an advantage in the competition for mates. The observed increase in F allele frequency under low temperature and intense sexual selection supports the notion that the reproductive advantage of the S allele is not absolute but varies with temperature. However, reproductive advantage of the F allele at low temperature was not confirmed by the reproductive success assay using direct male-male competition at different temperatures. It is possible that the conditions of the experiment were not optimal for the reproductive advantage of the F-males to be expressed. It is possible that the reproductive advantage of the F-males could have been observed under conditions with more competitors or if the duration of the competition had been longer. In addition, the design of the experiment introduces some additional variation due to factors such as differences in egg-laying ability of the females (less than 10 eggs from some females to more than 200 eggs from some females) or due to the non-uniform nature of the irradiation effects on the males. This high level of variation indicates that the current experiment lacks sufficient statistical power to detect moderate but still biologically relevant, differences. On the other hand, this method has been used previously in this (Radwan and Siva-Jothy 1996; Plesnar-Bielak et al. 2018; Skwierzyńska and Plesnar-Bielak 2018) and other systems (Sirot et al. 2007; Mazza et al. 2015; Magris et al; 2015) and allowed the detection of significant results.

Different factors interacting with each other have been shown to influence adaptation rates, which may be linked to the impact of these factors on genetic variation and polymorphisms in important genes. The importance of considering heterogeneous environments and social structure to study genetic variance in the design and execution of experimental evolution has been recognized and highlighted (Van den Bergh et al. 2018). An experimental evolution study of the bruchid beetle (*Callosobruchus maculatus*) investigated the effect of varying natural (food type) and sexual (mating system) selection intensities on the rate of adaptation (Fricke and Arnqvist 2007). The study showed that mating system treatment (social factor) influenced the rate of adaptation, depending on food type (abiotic factor). In *Drosophila serrata*,

an experimental evolutionary study showed that the effect of natural selection on the rate of adaptation to novel environments was greater in a monogamous mating system (Rundle et al. 2006). A similar result was observed in an experimental evolutionary study of *Rhizoglyphus robini*, where polygamous populations adapted rapidly to changes in thermal environments, whereas monogamous populations suffered fitness declines and were more likely to go extinct in novel environments (Plesnar-Bielak et al. 2012). These studies, together with our results, emphasize the need to consider methods to incorporate multi-factorial interactions (abiotic, biotic and social factors) in laboratory studies such as experimental evolution in order to obtain meaningful results and ultimately unlock the answers to many evolutionary processes. Furthermore, although such experimental systems and designs could become considerably more complex due to the addition of multiple factors and their interactions, inference could be refined by focusing on individual and key metabolic genes, as demonstrated in our study.

By focusing on a key metabolic gene, the insights gained are fundamental to evolutionary biology, and understanding the mechanisms of balancing selection has practical implications for conservation biology (Hoffman et al. 2017; Muirhead 2001). Maintaining genetic diversity is crucial for the adaptability and resilience of populations, particularly in the case of key metabolic genes, as variation in these genes has the potential to significantly affect the fitness and chances of survival of the organism (Koshiba et al. 2020; Whitt et al. 2002). By identifying the mechanisms that maintain specific genetic variation, we can better predict which populations might be especially vulnerable to environmental change and develop targeted strategies to conserve biodiversity. Our results suggest that monitoring multiple factors, such as environmental conditions and social structure, may be a key to predicting the fate of many populations under conservation (Parrett and Knell 2018; Peniston et al. 2020).

While our study provides knowledge and insight into the system, it is important to recognize and address its limitations. Although populations were maintained at constant census size (N) and sex ratio was controlled, the effective population size (N_e) may have varied between the populations with female-biased and equal sex-ratio. The potential differences in effective population size (N_e) between treatments might slightly influence allele frequency changes, but these effects are expected to be minor. This discrepancy in N_e has been identified and discussed as a potential limitation in previous experimental evolutionary studies that manipulate sex ratio (LaMunyon et al. 2007; Wigby and Chapman 2004). However, experimental evolution studies in other species have suggested that such differences in N_e between experimental populations should not considerably bias the results (Snook et al. 2009; Edward et al. 2010; Rice and Holland 2005). In addition, recent results on *R. robini* suggest that N_e does not differ significantly between populations with much higher differences in sex ratio than the ones used here (Chmielewski et al. unpublished), making our comparison between our experimental populations valid. Furthermore, although our results suggest that the F allele is further stabilized in the long term in the low temperature/high selection intensity treatments, evolutionary timescales are much longer compared to a 13-generation span. Hence, it would be interesting to experimentally assess the stability of these interactions over longer timescales. Finally, although we have gained insight into the maintenance of polymorphism, we have yet to determine the precise advantage gained by individuals with the F allele at lower temperatures and the associated trade-off in this system. Addressing these limitations in future research will enhance our understanding of the intricate interplay between genetic, environmental, and social factors in shaping population dynamics.

Overall, our study provides strong evidence for a three-way environment-by-genotype interaction as a mechanism of balancing selection in *6Pgdh* in bulb mites. We found that it is the interplay between the abiotic environmental factor (temperature) and the social environment (intensity of sexual selection) that likely drives the maintenance of polymorphism in such a system, although we were unable to detect differences in reproductive fitness between genotypes at different temperatures. The results of this study highlight the importance of taking a comprehensive approach to the study of balancing selection, where we consider the combined effects of different factors. This broader perspective not only deepens our understanding of evolutionary dynamics, but also paves the way for future research into the complex interplay between genotype, environment, and selective pressures.

Declarations

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Author contributions

PU, APB and WB were involved in the planning and design of the experiments. PU, APB, AS and MT did the lab work. PU analyzed the data. PU, APB and WB wrote the manuscript. All authors read and approved the final draft.

Author declarations

The authors declare no competing interests.

Data archiving

The dataset from this study will be uploaded to Dryad repository (yet to be uploaded).

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Figures

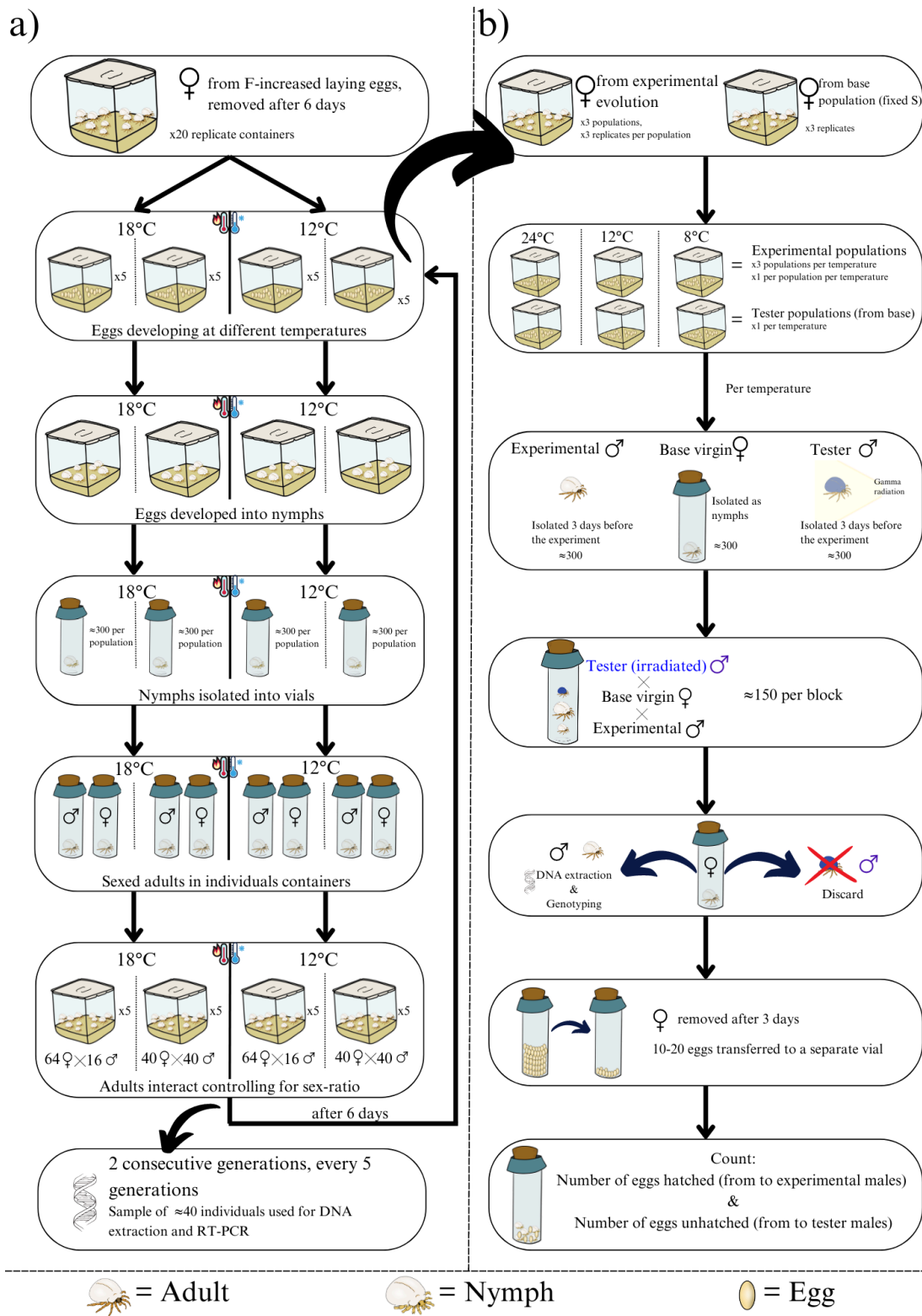


Figure 1

Steps involved in experimental evolution (a) and reproductive success assay (b).

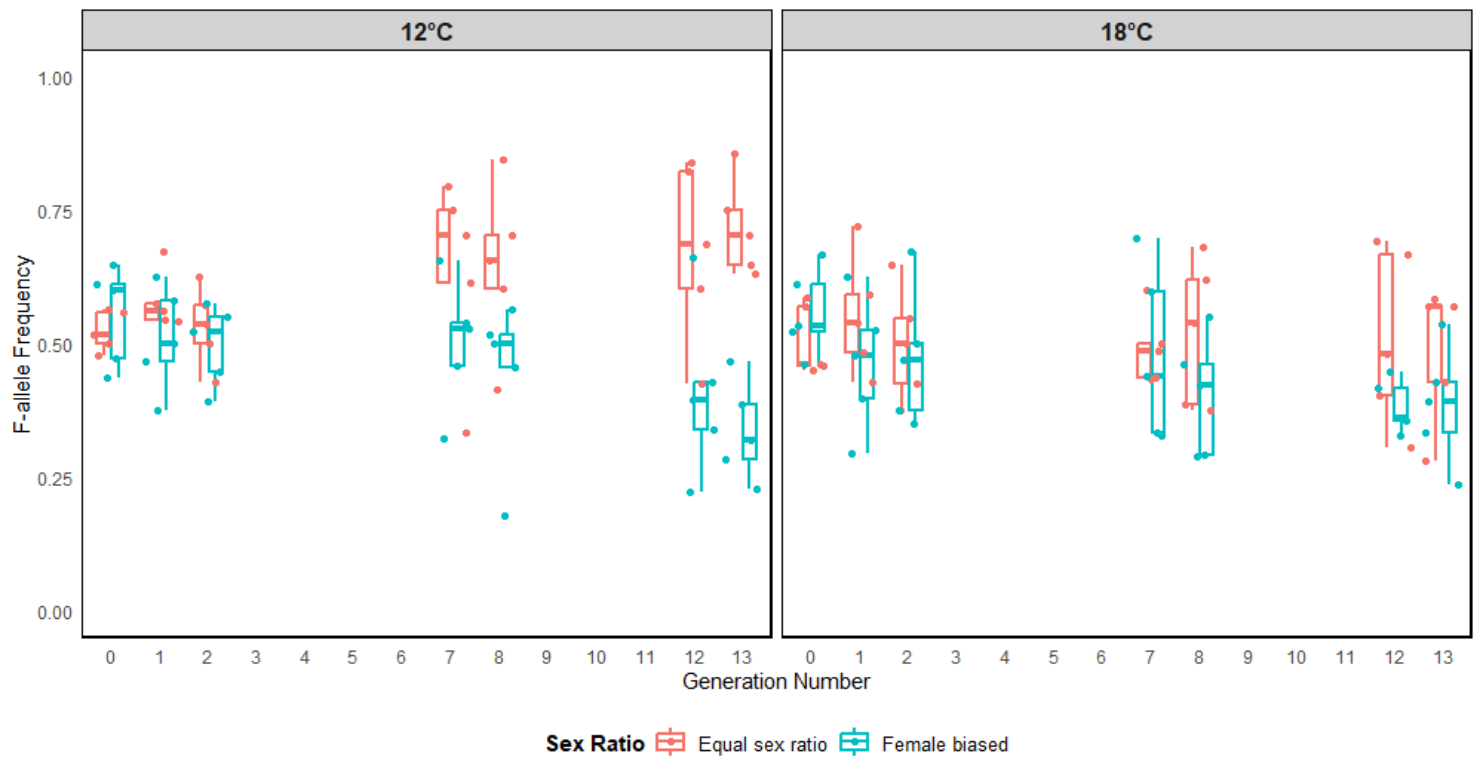


Figure 2

Evolution of F-allele frequency of the populations over 13 generations. The colours represent the different sex-ratio levels (equal vs female-biased) corresponding to the difference in sexual selection intensity (strong vs weak). The boxplots show the IQL of F-allele frequencies of each treatment group in that generation with the center line representing the median.

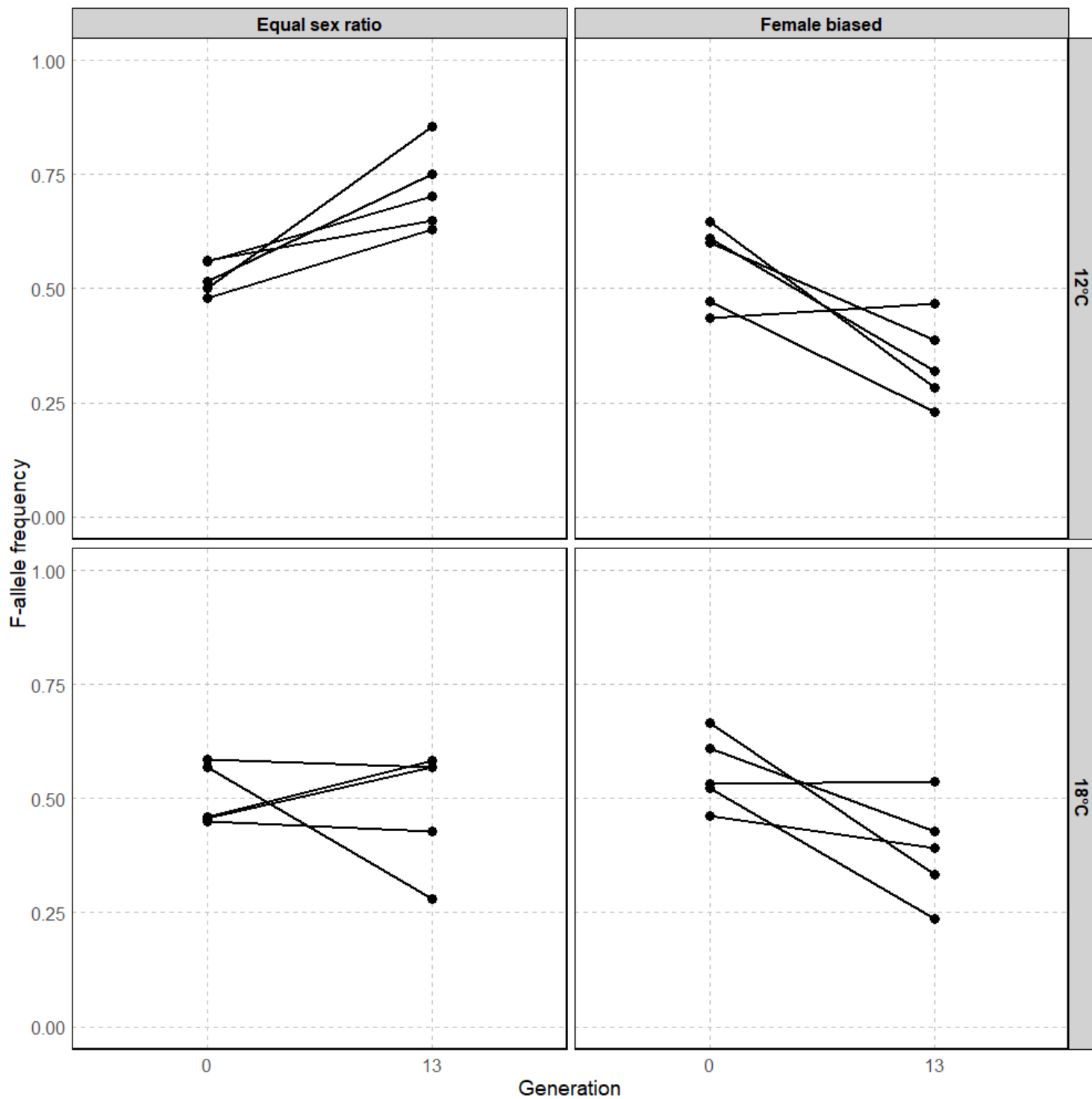


Figure 3

Comparison of generation 0 and generation 13 of each treatment. Only the populations from the treatment with equal sex-ratio (strong sexual selection) and 12 °C (lower temperature) show an increase in F-allele frequency. Each point represents the F-allele frequency of an individual population, and the lines connect the same replicate populations.

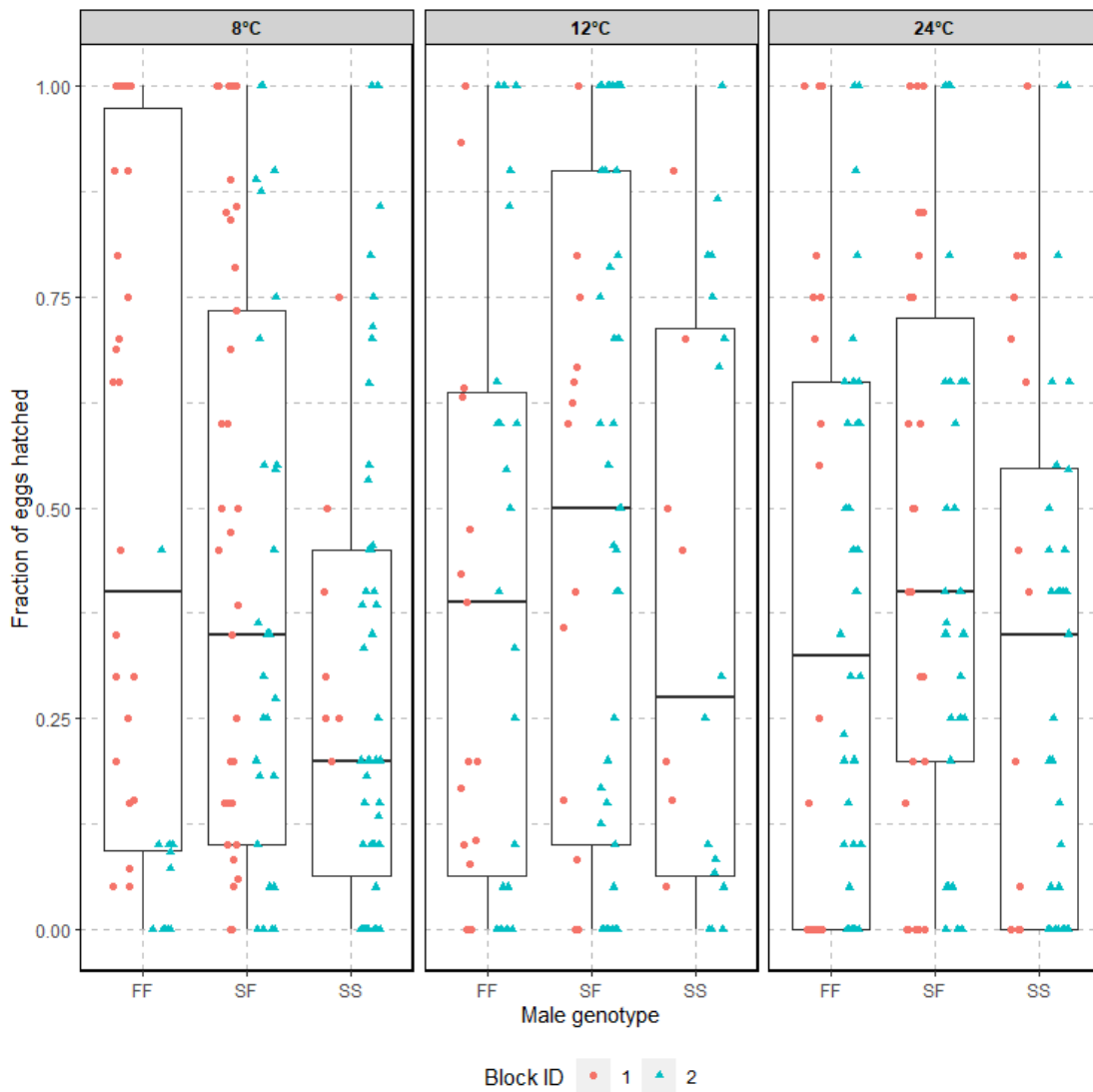


Figure 4

Comparison of reproductive success of different genotypes in different temperatures, the y-axis shows the fractions of eggs that hatched which is the representation of the level of reproductive success. The boxplots represent the IQL of the fraction of eggs hatched (reproductive success) with the center line being the median, and the points represent the success of individual males. The two different blocks are represented by the shape and colour of the individual points.