The effect of temperature on sex determination

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CHAPTER 4

Temperature and fitness of houseflies with different sex determining factors

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Abstract

Multiple sex determining mechanisms persist in natural populations of the housefly, *Musca domestica*. Their geographical distribution follows geographical clines, with the standard XY system present mainly at higher latitudes and altitudes and autosomal sex determining factors prevalent at low latitudes and altitudes. Previous studies showed a positive correlation between temperature and frequency of autosomal factors in natural populations, suggesting that they have a fitness advantage over the XY system at higher temperatures. In this study, we experimentally investigated the relative fitness of flies with autosomal sex determining factors versus standard flies under different temperature conditions. We determined whether autosomal *M* factors could invade the standard XY populations. We obtained different results for different *M* factors: the *M* factor on autosome II replaced the Y, but *M* on autosome III did not increase in frequency. However, we did not find an effect of temperature on the outcome. We also compared fitness of females with and without F^D. We found great variation between populations, but no effect of temperature on the fitness of *F* and F^D females. We discuss our results in the context of natural variation in housefly sex determining factors. We conclude that the role of temperature on the spread and distribution of different sex determining mechanism in the housefly still remains unclear. Future experiments should also include interaction of different sex determining factors under different temperatures.

Introduction

Multiple sex determining factors co-exist in many populations of the housefly, *Musca domestica* (Dübendorfer *et al.,* 2002; Table 4.1). The distribution of these factors follows geographical clines. The "standard" system, with a male-determining factor, *M*, located on the Y chromosome prevails at higher latitudes and altitudes. At lower latitudes and altitudes *M* factors have also been found on any of the five autosomes. Such populations often also harbour a dominant autosomal factor, F^D, which induces female development even in the presence of several *M* factors (Çakir & Kence, 1996; Franco *et al.,* 1982; Hamm *et al.,* 2005; Tomita & Wada, 1989b; Kozielska *et al.,* 2008; Feldmeyer *et al.,* 2008). It has been proposed that this distribution is governed to a great extent by temperature (for details see Kozielska *et al.,* 2008; Feldmeyer *et al.,* 2008). Support for this hypothesis comes from the correlation between the frequencies of autosomal sex determining (SD) factors and the ambient temperature in natural populations of houseflies (Kozielska *et al.,* 2008; Feldmeyer *et al.,* 2008). The prevalence of autosomal SD factors in warmer localities and their lack in colder ones suggests that autosomal SD factors have a fitness advantage over the XY system at higher temperatures and a disadvantage at lower temperatures. However, it has never been shown experimentally that this is indeed the case.

Numerous studies have been performed to measure different fitness components at different temperatures of houseflies collected in various localities (e.g. Bryant, 1980; Chapman & Goulson, 2000; Elvin & Krafsur, 1984; Fletcher *et al.,* 1990; Lysyk, 1991; West, 1951), often with contrasting results (see Lysyk, 1991; West, 1951), but virtually none of them took the sex determining mechanism of the investigated flies into account. To our knowledge, only one study intended to compare the competitive abilities of houseflies from autosomal and standard populations (Çakir & Kence, 1999). Çakir and Kence found that the frequency of XX males increased in most of the treatments, but they did not know the exact frequencies of different SD factors, neither *M* nor F^D. They also did not control for the genetic background of different factors, which makes the interpretation of their results difficult.

The objective of the present study was to more directly compare the fitness of flies with different SD factors under different temperatures. For *M* we measured the invasion success of two different autosomal *M* factors into a standard XY population at two different temperatures. This approach reflects presumed ancestral conditions when autosomal *M* factors emerged in XY populations (Franco *et al.,* 1982). A similar approach was impossible for comparing the fitness of standard *F* females with F^D females (see below). Therefore we decided to measure lifetime reproductive success of females with and without F^D from different populations at two different temperatures.

Because we used two different approaches, we will present our experiments in two separate sections. Part I contains the methods, results and a short discussion of the experiment on invasion of autosomal *M* factors. Part II includes methods, results and discussion of the experiment measuring fitness of *F* and F^D females. At the end of the chapter, we present a general discussion on the effect of temperature on different SD factors in the housefly.

Part I: Invasion of autosomal *M* factors

Material and methods

Housefly strains

We used several strains with *M* located on different chromosomes.

1) Marker XY strain – a lab marker strain homozygous for five recessive visible mutations: *ac* (*ali curve* – tips of the wings are curved upwards), *ar* (*aristopedia* – aristae of antennae are substituted by tarsal segments), *bwb* (*brown body*), *ye* (*yellow eyes*) and *snp* (*snip wings* – part of the wing is missing) on autosome I, II, III, IV and V, respectively. This strain has the standard XY sex determining system.

2) SFE-M^{II} autosomal strain – a lab strain created by a number of generations of backcrosses of one wild type XX male with an *M* factor located on autosome II with the marker-strain females (described in Table 4.1). A wild type male used for generation of this strain came from the strain collected in Santa Fe, Spain, in 2004. All females in this strain are homozygous for all five autosomal markers similar to females from the marker strain; males are homozygous for the mutations on all the autosomes except II. They are heterozygous for

autosome II: one autosome comes from the marker strain and the other one is the wild type autosome II with *M*. Since in male houseflies there is almost no recombination the *M* factor is always linked to the wild type *ar*+ allele and males always develop normal antennae.

3) CAM - M^{III} autosomal strain - a lab strain created by a number of generations of backcrosses of one wild type XX male with an *M* factor located on autosome III with the marker-strain females (Table 4.1). A wild type male used for generation of this strain came from the strain CAM collected in Camargue, France, in 2004. Similar to the SFE-M^{II} strain, all females are homozygous for all markers. Males are homozygous for the mutations on all the autosomes except III. They are heterozygous for autosome III: one autosome comes from the marker strain (with *bwb* allele) and the other one is the wild type autosome III with *M*. Males and therefore black, since the *M* factor is linked with the wild type *bwb*+ allele.

Since there are no visible mutations on the X or Y chromosome it is possible that both an X chromosome from the XY marker strain and an X chromosome from the original wild type males is present in both autosomal strains. However, since there have been no structural genes described so far on the X or Y chromosome (see Dübendorfer *et al.,* 2002), we do not expect much effect of sex chromosomes from different strains. Both autosomal strains were created approximately one year (approximately 12 generations) before the start of the experiment in July 2005.

Usage of the strains described above allows us to compare the performance of males with a Y chromosome (strain 1) or autosomal *M* factor (strains 2 and 3) in the same genetic background (except for the genes located on the autosome with the *M* factor). Additionally, the presence of visible markers linked with autosomal *M* factors allows us to precisely score the frequencies of different *M* factors each generation. This is particularly important since there are no molecular markers to distinguish between *M* factors on different autosomes. So far *M* location can be checked only after a tedious procedure involving two generations of backcrosses to marker strains (see Kozielska *et al.,* 2008), making analysis of frequencies of different *M* factors from a large number of males difficult. However, a potential drawback is that in our autosomal strains *M* is linked with the wild type phenotype, which may confer an increase in

fitness, compared to XY marker-strain males which are homozygous for all mutant alleles. Therefore, we created control males to assess the effect of the wild type marker by separating it from the effect of the *M* factor.

4) C-III - control males were created by single pair backcrosses of XY males from the same wild type CAM strain from which CAM-M^{III} males were derived, to virgin marker-strain females (Table 4.2). Males whose F2 offspring did not show a sex limited inheritance of visible markers possessed the Y chromosome and no autosomal *M* factor (see e.g. Denholm *et al.,* 1983). These male offspring were used in one more generation of backcrosses to marker-strain females from which male offspring with all visible mutations except for brown body were used as a control to CAM-M^{III} males, since they were also homozygous for the four mutant alleles, but heterozygous for wild type autosome III, but without the *M* factor. They possessed a Y chromosome (to assure maleness), in contrast to autosomal *M* males, which were XX (Table 4.1 and 4.2). This should not influence the results considerably, since both the X and the Y chromosome seem to be equivalent with respect to viability and fertility (Dübendorfer *et al.,* 2002; Franco *et al.,* 1982). Construction of the control males for the SFE-M^{II} strain was impossible, since in the original wild type strain all males were homozygous for M^{II}, therefore there was no autosome II without an *M* factor present in that population.

Table 4.1. Schematic representation of the crosses performed to create the CAM-M^{III} strain. *ac*, *ar*, *bwb*, *ye* and *snp* represent recessive visible mutations on each of the autosomes (autosome III in bold); + represents a wild type allele of any of the mutations and *M* is always linked with the wild type allele of *bwb*, since there is no recombination in males. In the first generation, wildtype males are crossed with marker-strain females, resulting in heterozygous progeny with a wildtype phenotype. Male offspring is then crossed again with marker-strain females yielding a variety of phenotypes among the F2 (four examples represented here). All females are homozygous for *bwb* and show the brown body phenotype, all males are heterozygous and show the wildtype phenotype (black body). Males homozygous for all visible mutations, except *bwb,* (framed) were again crossed with marker-strain females to establish the CAM-M^{III} strain. The SFE-M^{II} strain was obtained in a similar way, but there the *M* was linked with the *ar*+ allele.

Marker-strain ♀ Wildtype ♂

X *ac ar bwb ye snp* / X *ac ar bwb ye snp* X X + + + + + / X + + *M* + +

offspring

♀ X *ac ar bwb ye snp* ♂ X *ac ar bwb ye snp*

2nd cross:
Marker-strain ♀

X *ac ar bwb ye snp* / X *ac ar bwb ye snp* X ♂ X *ac ar bwb ye snp*

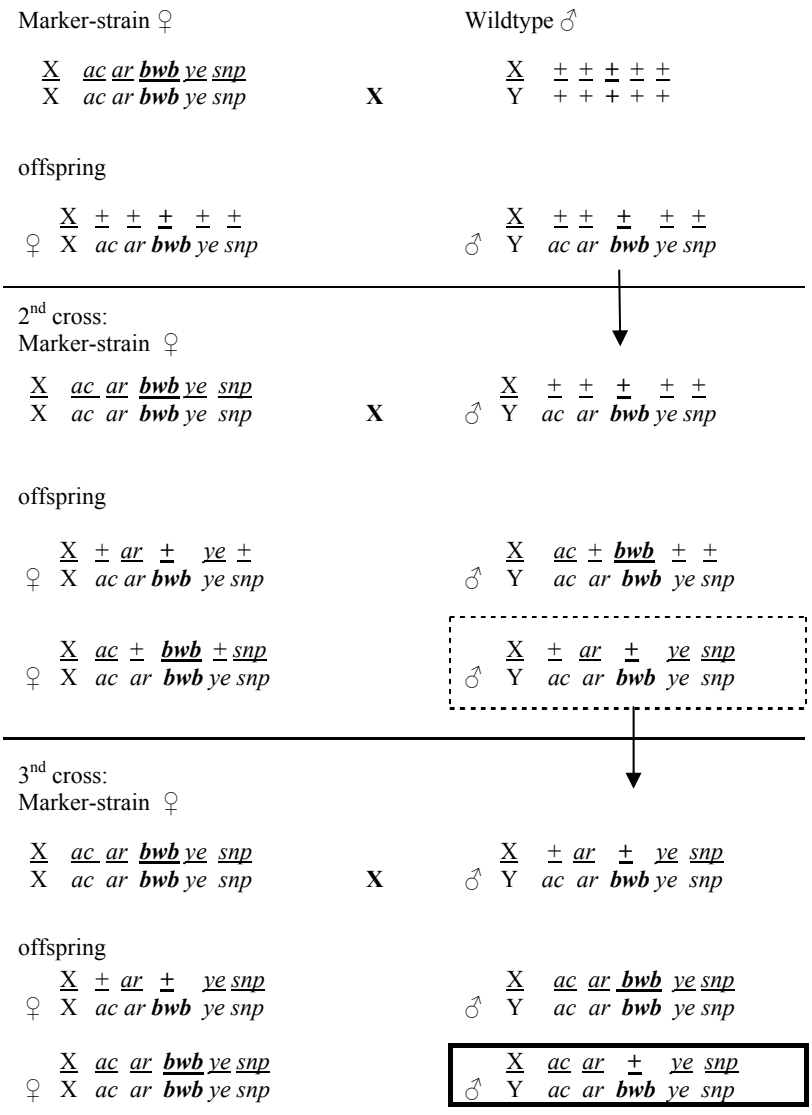
offspring

♀ X *ac ar bwb ye* + ♂ X *ac ar bwb ye snp*

♀ X *ac* + *bwb* + *snp* ♂ X *ac ar bwb ye snp*

♂ X *ac ar M ye snp* / X *ac ar bwb ye snp*

Table 4.2. Schematic representation of the crosses performed to create C-III control males. The procedure is similar to the one present in Table 4.1, but now the *M* is located on the Y chromosome and all visible mutations segregate randomly in both sexes (some examples of offspring genotypes are shown). Male offspring from the 2nd cross heterozygous for *bwb* (dashed frame) were crossed with marker-strain females, and F3 males homozygous for all visible mutations except *bwb* (solid frame) were used as C-III males in the control experiment.



Experimental setup

We set up population cages to measure the fitness of males with different *M* factors. Each experimental cage started (generation 0) with 150 females and 120 males from the marker strain and 30 males from one of the autosomal *M* strains or C-III control males. We kept populations at a temperature of either 20°C or 25°C and replicated each treatment five times (five cages per strain per temperature). We used this narrow range of temperatures, since under laboratory conditions there is a very high mortality of larvae below 20°C and a high mortality of adults above 25°C (personal observation).

We kept the adult flies in population cages (13×13×22cm) and provided them with constant access to water, sugar water and milk powder (as food). When flies were about 5 (in 25°C) or 7 (in 20°C) days old, females reached full maturity and were most prone to lay eggs (personal observation) and film boxes with standard egg laying medium (see Hilfiker-Kleiner *et al.,* 1994) were placed in the cages. After one day at 25°C or two days at 20°C they were replaced by a second set of boxes of egg laying medium and eggs were transferred to bigger boxes where larvae could develop. The second egg laying medium was collected again after two or one day(s) (in 20/25°C) and eggs were transferred to new larval boxes, leading to two larval boxes per population. This protocol for egg collection yields many eggs and at the same time prevents large age differences between offspring. Larvae were fed *at libidum* with the same medium which was used for egg collection. When larvae from a box pupated, 150 random pupae per box were collected and placed in a new population cage while 150 other random pupae were collected in separate boxes and later used to calculate the frequencies of different *M* factors (see below). Since the pupal emergence rate is almost 100% (personal observation), each population cage contained approximately 300 flies each generation. These rearing conditions reflect the standard fly-keeping procedure used in our lab, except that temperature is usually 20°C and adult population density is approximately 500 flies. The experiment lasted for 8 generations under the same protocol and rearing conditions.

In experiments with the invasion of M^{II} and M^{III} males, every generation we calculated the frequency of males with a Y chromosome (all five mutations present) and males with an autosomal *M* factor (only four mutations; see Table

4.1). For the control we scored the number of black and brown males and females. *bwb* is a recessive mutation and we estimated the frequencies of the wild type *bwb*+ allele assuming a Hardy-Weinberg equilibrium. In the first generation we tried to score the phenotypes of adult flies after the new generation had been started and adults had been killed by freezing in -20°C. However, antennae get damaged very easily after death and scoring the *ar* mutation after freezing was impossible. Therefore, from the 2nd generation onwards, we phenotyped adults from a different, but representative batch of pupae, that was not used for further culturing (see above).

Statistical analysis

For the statistical analysis we used the proportion of M^{II} males, the proportion of M^{III} males or the proportion of the wild type *bwb*+ allele in the last generation of the experiment. We analyzed each of these proportions separately with a generalized linear model with binomial errors in R (R Development Core Team, 2006). We used a likelihood-ratio approach to judge the significance of the effect of temperature, using an *F* test to correct for overdispersion. We compared the final frequencies of M^{II} and M^{III} males and the frequency of the *bwb*+ allele with their initial frequencies using a binomial test.

Results and discussion

The average frequencies of the *M* factor located on autosome II increased significantly during the course of the experiment at both temperatures (Table 4.4, Figure 4.1A and B). The average proportion of autosomal *M* males after eight generations was one or close to one in most populations and did not differ between temperatures (Table 4.3), although at higher temperature M^{II} seems to reach fixation faster (Figure 4.1). This suggests that males with the autosomal *M* factor on the second chromosome have a selective advantage over males with *M* located on the Y chromosome in the temperature range we used.

Unfortunately, we cannot exclude the possibility that other genes linked with an autosomal *M* factor, in particular a wild type *ar*+ allele, have an effect on the fitness of autosomal *M* males. As described above, we were not able to set up a control experiment to test whether a wildtype autosome II without an *M* factor

Table 4.3. Absence of a temperature effect on the frequency of autosomal males and *bwb+* allele. Results from a generalized linear model analysis of the frequencies of males with the *M* factor located on autosome II (A), autosome III (B) and frequencies of the *bwb+* allele (C) in the last generation of the invasion experiment. There is no effect of temperature on the frequency of any of the genetic factors studied.

Model	DF	Deviance	*F*	P
A. M^{II}				
Temperature	7	391.38		
Null model*	8	392.44	0.016	>0.5
B. M^{III}				
Temperature	8	622.97		
Null model*	9	631.20	0.132	>0.5
C. Control				
Temperature	8	392.36		
Null model*	9	449.10	1.370	>0.2

would invade as well. We have some evidence that flies which are homozygous for the *ar* mutation do not have decreased egg to adult viability comparing to heterozygous *ar*/*ar+* males (not shown), but mal-developed antennae might have a detrimental effect in the adult stage.

Table 4.4. Changes in the frequencies of males with M^{II} and M^{III}, and the frequency of *bwb+* allele in the control. The initial and final frequencies (in generation 8) are given, together with the P value from the binomial test comparing them. For each experiment the results from the two temperatures were pooled together, since there is no difference between them. The frequency of males with M^{II} and *bwb+* allele in control increased significantly during the experiment.

Experiment	Initial frequency	Final frequency	P
Males with M^{II}	0.20	0.85	<0.001
Males with M^{III}	0.20	0.38	>0.05
Control – *bwb+* allele	0.05	0.11	<0.01

The frequency of the *M* factor located on autosome III was not affected by the temperature and it did not significantly increase during the experiment (P>0.5 in binomial test for both temperatures pooled together; Table 4.4; Figure 4.1C and D). Although on average the frequencies of autosomal *M* males did slightly increase when compared to the initial frequencies, they were relatively stable between generations. Therefore, males with an M^{III} factor do not seem to have a noticeable fitness advantage over XY males. This result is puzzling, since the M^{III} factor is the most common among autosomal *M* factors in most of the studied populations worldwide (Denholm *et al.,* 1990; Franco *et al.,* 1982; Hamm *et al.,* 2005; Tomita & Wada, 1989b; Kozielska *et al.,* 2008).

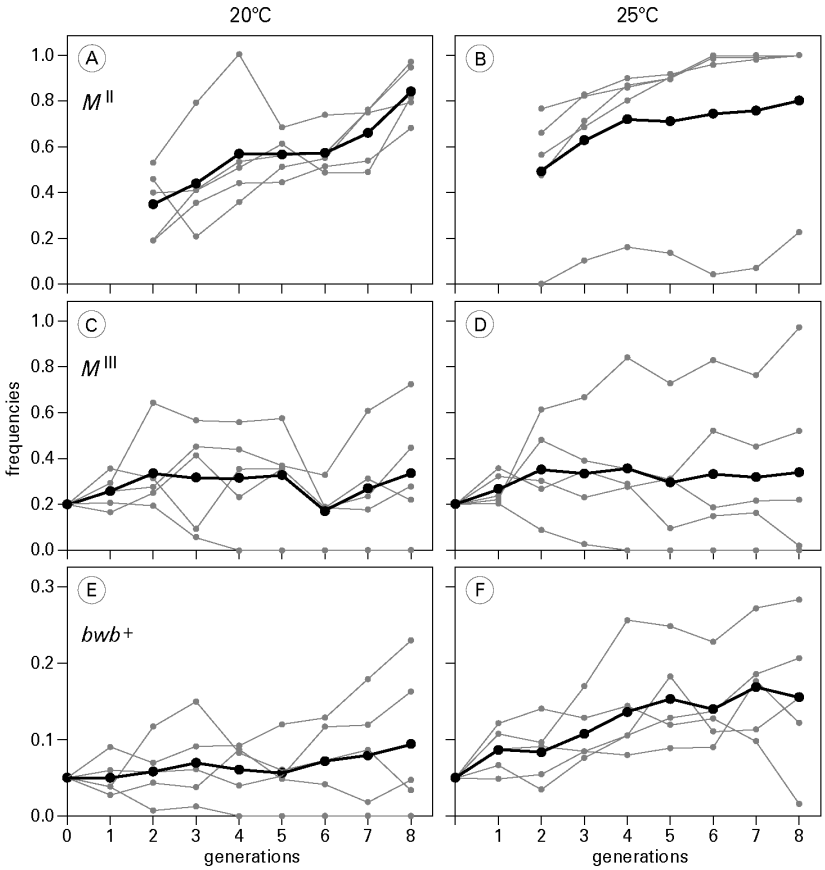


Figure 4.1: Frequencies of males with the M^{II} factor (A and B), males with the M^{III} factor (C and D) and the frequency of *bwb+* allele in control (E and F) at two different temperatures (20ºC and 25ºC) during the invasion experiment. Grey lines represent five different replicates and the black line their average. Data for generation 1 for the invasion of M^{II} is lacking (see Material and Methods).

Moreover, in contrast to the M^{III} factor, the average frequency of the *bwb*+ allele in the control experiment increased significantly during the experiment ($P<0.01$ for both temperatures pooled together; Figure 4.1), suggesting that the *M* on autosome III actually confers a fitness disadvantage to its bearer (Sokal & Sullivan, 1963; Sullivan & Sokal, 1965). However, we cannot exclude the alternative explanation for this pattern, that some genes on the wild type autosome III are incompatible with the marker-strain background. When linked with the *M* factor, they could not be removed from the population by recombination, since they were present only in males and crossing-over does rarely occur in males (see Franco *et al.,* 1982). In contrast, in the control experiment the wild type autosome could also be present in females in which recombination could have removed initial linkage of incompatible wildtype alleles with the *bwb*+ allele. Future experiments measuring the invasion success of autosomal *M* factors under variable genetic backgrounds may be able to minimize the effect of genetic incompatibility on the spread of autosomal *M* factors.

Although the invasion experiments allow a more realistic assessment of competitive abilities associated with different SD factors than individual fitness essays, they still may not be able to include all fitness aspects. For example, if the presence of an autosomal factor confers a fitness advantage mainly in the later lifetime period, our experiment would not have measured it, since for logistic reasons we only allowed females to lay eggs for a relatively short period. Therefore, only early life time fitness was taken into account in our experiment. Also, all males and females emerged within a relatively short time period, which may increase competition between males above levels seen in nature. Alternatively, if males with M^{III} have a slightly longer developmental time than marker-strain males (Sokal & Sullivan, 1963), they may miss most of the mating possibilities, since female houseflies usually mate only once before laying eggs (Andres & Arnqvist, 2001; Hicks *et al.,* 2004; Riemann *et al.,* 1967).

Part II: Relative fitness of females with the F^D factor

Material and Methods

Housefly strains

Introduction of F^D factor into different genetic background is very slow and labour-intensive, since usually multiple *M* factors segregate in different lines and both types of females (with and without F^D) are produced. Therefore, instead of performing an invasion experiment, we decided to assess life time fitness (and some of its components) of individual F^D and *F* females. We used females from three different wild type strains:

1) CAM – a wild type strain where the frequency of F^D females is around one quarter. This strain possesses *M* factors located on the Y chromosome and autosome III. This strain was established from flies collected in Camargue, France, in 2004. It is the same wild type strain from which the CAM-M^{III} strain used in the *M* factor invasion experiment was established. It was maintained at a population size of approximately 500 flies prior to the experiment (as were all the other strains).

2) FVG – a wild type strain in which the frequency of F^D is around 0.5. The *M* factor has been found on autosome II, but since fewer than 5 males were checked, it can be present also on other chromosomes. This strain was established from flies caught in Faverges, France, in 2004

3) UML – a wild type strain in which the frequency of F^D females is around 0.5, *M* factors are located on autosomes I, II, III and V. It was established from flies caught in South Africa in 2005 (Feldmeyer *et al.,* 2008).

Experimental procedure

F and F^D females cannot be distinguished phenotypically. Therefore we measured several fitness components of 50 randomly chosen females from each population. After death the genotype of those females was determined using the molecular technique described in Kozielska *et al.* (2008). The experiment started in February 2006.

Since the temperature sensitive period of development starts already during oogenesis (Schmidt *et al.,* 1997a), we placed mothers of focal females at the

experimental temperatures just after emergence. 50 females and 50 males were placed in population cages at two different temperatures: 20°C and 27°C. We used a slightly wider temperature range than for the *M* invasion experiments to increase the chance of detecting an effect of temperature. This increase of temperature was possible because higher temperatures do not seem to affect adult flies as negatively when they are kept in single pairs, compared to larger numbers of flies in population cages. A further increase in temperature would largely exceed conditions found in nature (see below). A lower temperature than 20°C would have yielded very low offspring numbers, especially from single-female egg batches (personal observation). The rearing conditions were the same as in the invasion experiment unless mentioned otherwise.

When the females reached maturity they were allowed to lay eggs which later developed at the same temperature as experienced by the mothers. After pupation around 1000 pupae from each population and temperature were collected and when the flies started to emerge in large numbers, 50 females from each temperature treatment of each population were collected within 24 hours after emergence and weighed individually on an electronic laboratory scale. All 50 females used in the experiment emerged within one day or sometimes two days. Each female was placed individually with two males from the same population and of the same age in 180 ml transparent containers and provided with sugar water and milk powder. At the same time we collected around 50 additional males and placed them together with an equal number of females. These males were used to replace dead males in containers with experimental females. We used two males per female to reduce the chance that a female would not produce eggs should her mate be infertile. After 7/5 days (in 20/27°C) females were provided with egg-laying medium. Every 5/3 days the egg-laying medium together with eggs or larvae was transferred to bigger boxes where the larvae developed at the same temperature as the mothers and new egg-laying medium was provided to females. Every day we checked for dead females, which were frozen for later molecular analysis. We let all the offspring develop till the adult stage and we counted all emerging flies.

Statistical analysis

Many females did not have any offspring, leading to a strongly skewed distribution of offspring number with an excess of zeros. Therefore, the lifetime

offspring production was modelled with a hurdle model in R, using the hurdle function from the pscl package (Zeileis *et al.,* 2007). It is a two-component model: a truncated count component is employed for positive counts and a hurdle component models zero vs. larger counts. For the latter a binomial distribution was used. Females' lifespan was modelled with Generalized Linear Models with gamma errors in R (R Development Core Team, 2007).

In both models, for lifespan and offspring production, weight was used as a continuous explanatory variable and temperature, population and SD factor (F^D vs. F) as discrete variables. We started with a full model (including all interactions between discrete variables) and used backward selection to find a minimum adequate model. Significance of the models was assessed with a likelihood-ratio approach.

Results and discussion

Average weight, lifespan and lifetime offspring production of females with F and F^D from different populations and under different temperature conditions are presented in Figure 4.2. Neither the SD factor (F/F^D) nor the interaction of the SD factor with temperature had a significant effect on female fitness. Female fitness is differentially affected by temperature in different populations, as shown by a significant effect of interaction between temperature and population on the females' lifespan and on the lifetime offspring production (Table 4.5). Offspring production seems not to be governed only by differences in lifespan, since at higher temperature lifespan was always shorter (as expected: Fletcher *et al.,* 1990; Lysyk 1991), but higher temperatures had a positive effect on lifetime reproductive success in two populations (CAM and UML) and a negative effect in one (FVG).

The different effects of temperature on the lifetime fitness of females from different populations could stem from adaptation to the local conditions of the original population. In the field the FVG population probably only rarely experienced temperatures above 20°C, whereas the average temperatures experienced by CAM are about 5°C higher, and average maximum daily temperatures exceed 22°C throughout the year in the location from which the UML population originated (temperature data from http://www.worldclim.org; Hijmans *et al.*, 2005; not shown). The low fitness of CAM females can be an

indicator of their general low genetic quality and may also explain the low fitness of males from this population (see invasion experiment above).

We did not find any evidence that F^D females have higher fitness at higher temperatures and F females under lower temperatures, or any other effect of SD factor (F vs. F^D) on female fitness. Theoretically, it is possible that under the temperatures we studied F and F^D are neutral and only higher temperatures are favourable for the F^D factor, but temperature data from natural populations would contradict this hypothesis (see General Discussion). A more plausible explanation is that the fitness differences between F^D and F females are visible only under more competitive conditions than experienced by the females and their offspring in this experiment.

Table 4.5. Factors affecting female fitness. Results of statistical analysis of the lifespan and lifetime offspring production of females. Only statistically significant effects are listed. Δ DF represents the difference in degrees of freedom between the final model and the model without the listed variable.

Model	Δ DF	χ^2	P
Lifespan[1]			
Population×Temperature	2	5.529	0.006
Lifetime offspring production[2]			
Weight	2	10.925	0.004
Population×Temperature	4	26.901	<0.001

[1] – Final model (Population + Temperature + Population×Temperature) has residual DF = 278 and deviance 85.115

[2] – Final model (Weight + Population + Temperature + Population×Temperature) has DF = 15 and log-likelihood = -958.45

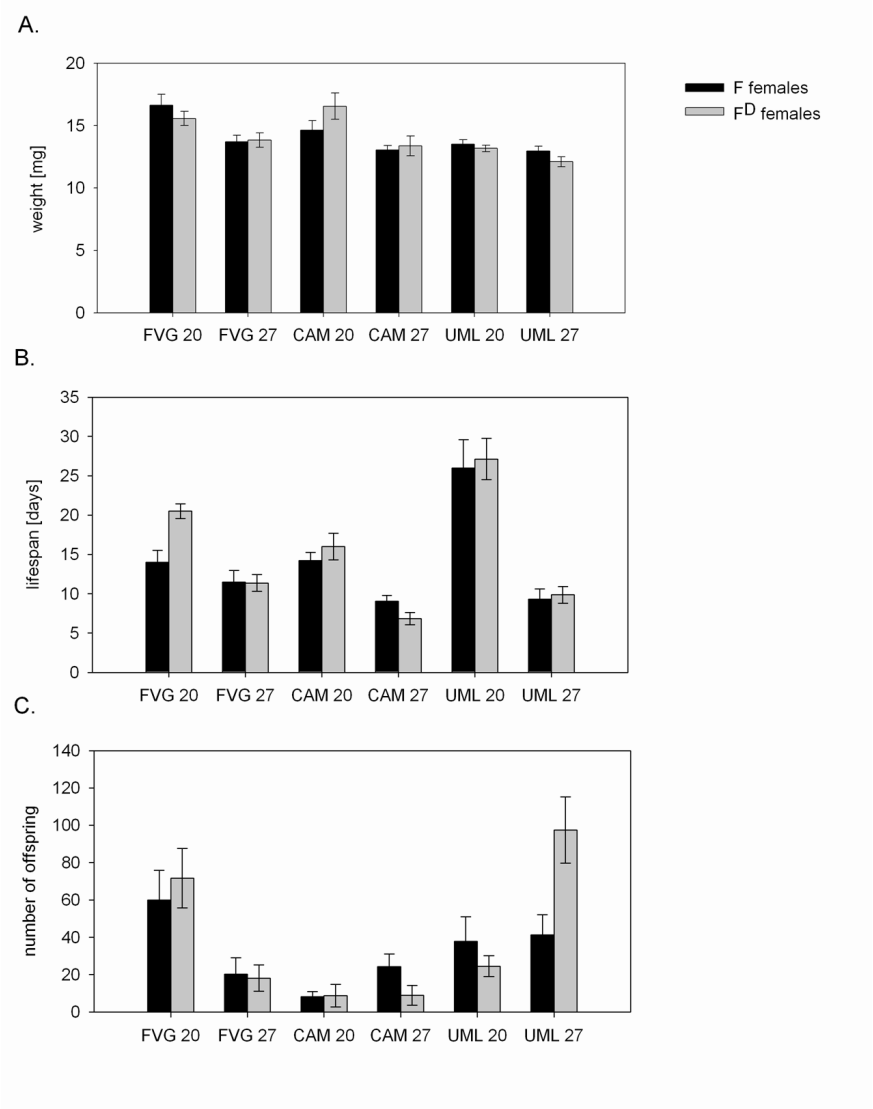


Figure 4.2: Average weight (A), lifespan (B) and lifetime offspring production (C) of females with F and FD from different populations at 20 and 27°C (as listed after the strain name). Error bars represent standard errors.

General discussion

We did not find a clear general effect of temperature neither on the fitness of autosomal *M* males nor on the fitness of females with or without an F^D factor. One might argue that the temperature range we used was too narrow and not representative of the temperatures experienced by the houseflies in nature. Although there may be some truth to this explanation, it does not fully explain our results.

Since M^{II} spread quickly in both temperatures, it may be that the temperatures we used were too high to detect a fitness advantage of XY males that presumably exists under low temperatures in nature (see Feldmeyer *et al.,* 2008). Indeed, average yearly ambient temperatures of 25°C or even 20°C are rare (at least in Europe; temperature data from http://www.worldclim.org; Hijmans *et al.,* 2005) and high frequencies of autosomal *M* factors already occur at lower temperatures (Kozielska *et al.,* 2008; Feldmeyer *et al.,* 2008). In contrast to M^{II}, M^{III} did not increase in frequency during the experiment, suggesting one obvious explanation that the range of temperature studied was too low for the M^{III} factor to show its fitness advantage. However, as discussed above, this is rather improbable. Similarly, female fitness at different temperatures was not affected by the presence or absence of the F^D factor, suggesting that the *F* and F^D factors are neutral at the used temperatures. As before, this explanation is improbable, since high frequencies of F^D females were also found in populations in which even in summer months the maximum daily temperature is below 20°C (e. g. in most of Italy, Kozielska *et al.,* 2008; temperature data not shown).

Under natural conditions ambient temperatures are much more variable than in our experiments and although average yearly temperatures correlate with the frequencies of autosomal SD factors (Feldmeyer *et al.,* 2008), the effect of temperature may be a complex phenomenon (see Feldmeyer *et al.,* 2008). Seasonal or daily temperature extremes or temperature fluctuations may be more important for the long term fitness of different SD factors than average temperatures *per se*. In the wild, flies can also actively seek temperatures that are optimal for them, which may be different at different developmental stages (West, 1951). Another possibility is that other climatic factors, e.g. humidity,

interact with temperature, creating the geographical distribution of SD factors seen today (see Kozielska *et al.,* 2008).

Different SD factors may need to be studied together, since they can affect each other dynamics. For example, the F^D factor may not by itself be affected by temperature, but if at higher temperatures autosomal *M* factors confer higher fitness to both sexes, then F^D females would indirectly gain fitness since they, in contrast to standard *F* females, can possess autosomal *M* factors. The fact that only females from UML seem to follow the expected pattern of higher fitness of F^D females under higher temperatures could support this hypothesis, since in this population all males, and presumably all F^D females, possess at least one autosomal *M* factor (Feldmeyer *et al.,* 2008). In the CAM population, on the other hand, the frequency of XY males was around 65% (4 months prior to the experiment, results not shown). Therefore, the frequency of autosomal *M* factors in F^D females is probably relatively low. We do not know the exact frequencies of different *M* factors in the FVG population. Indirect fitness gain of F^D females through possessing autosomal *M* could explain why F^D has been found mainly in populations in which autosomal *M* factors were present (Denholm *et al.,* 1990; Franco *et al.,* 1982; Tomita & Wada, 1989b; Kozielska *et al.,* 2008; Feldmeyer *et al.,* 2008). Also, even if autosomal *M* factors were beneficial only to males, that would lead to male biased sex ratios and consequently could facilitate spread of F^D to assure even sex ratios. Future experiments controlling for the presence of *M* factors in F^D females are necessary to determine any fitness effect of the presence of *M* factors in females.

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