The effect of temperature on sex determination

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CHAPTER 5

Does temperature affect the sex ratio and frequency of intersexes in the housefly, *Musca domestica*?

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Abstract

Sex determining factors in the housefly follow a clinal distribution and there is evidence that temperature is the driving force leading to the observed pattern. It suggests that the fitness of individual houseflies varies depending on the environment and the composition of sex determining factors. It has also been observed that the number of intersexes increases in winter, suggesting that a gene involved in the sex determining cascade is influenced by temperature leading to an unbalanced sex determination. Here we test experimentally whether different temperature regimes lead to a change in the proportion of intersexes produced from standard XY as well as autosomal *M* populations with different frequencies of the dominant female determining factor F^D. We do not find an effect of temperature on the number of intersexes produced at any of three temperature treatments, but do observe that the sex ratio is affected by temperature. We can however not rule out that reduced survival of one of the sexes is the cause for the sex ratio bias.

Introduction

The housefly, *Musca domestica*, is a cosmopolitan species that is especially interesting because of its variable sex determination (Dübendorfer *et al.,* 2002). Within a single population male heterogamety, female heterogamety and a mixture of both can occur. In northern populations on several continents the "standard" XX/XY sex determining system is found, where females possess two X chromosomes, both sexes are homozygous for the female determining factor *F* on chromosome IV, which in males is overridden by the male determining factor *M* on the Y chromosome (Hediger *et al.,* 1998a; Dübendorfer *et al.,* 2002). In more southern and low altitude populations however, male determining factors can be found on any of the five autosomes and some proportion of females carry a dominant female determining factor (F^D), which induces female development even in the presence of *M* (Franco *et al.,* 1982; Tomita & Wada, 1989b; Çakir & Kence, 1996; Dübendorfer *et al.,* 2002; Hamm *et al.,* 2005; Kozielska *et al.,* 2008; Feldmeyer *et al.,* 2008). Towards more southern regions, males are homozygous for autosomal *M* factors and the frequency of F^D increases.

Several authors have proposed that the observed cline in sex determining factors might be due to linkage of the *M* factor to insecticide resistance genes (Kerr, 1970; Franco *et al.,* 1982; Tomita & Wada, 1989b), however a recent study shows that this is not the case (Hamm *et al.,* 2005). An alternative hypothesis is that temperature causes the observed cline (Franco *et al.,* 1982; Çakir & Kence, 1996). Recently we were able to show that seasonality, which was measured as yearly temperature range, influences the distribution of autosomal *M* factors whereas temperature in interaction with humidity might be causing the observed distribution of the F^D factor (Feldmeyer *et al.,* 2008). The gradual distribution of sex determining factors along a temperature gradient with autosomal *M* factors and F^D occurring at higher temperatures, suggests that autosomal *M* and F^D have a fitness advantage at higher temperatures over the XY system and a disadvantage at lower temperatures. Previous experiments measuring housefly fitness components at different temperatures did not take the sex determining system of the investigated populations into account (Fletcher *et al.,* 1990; Lysyk, 1991; Chapman & Goulson, 2000). Bryant (1980) who compared populations from different localities neither did so, but could

show faster mating speeds in populations from high latitudes compared to flies from low latitudes.

In Chapter 4 we tested whether high temperatures have a positive fitness effect on males with autosomal *M* and lead to the invasion of autosomal *M* males in a population of otherwise M^Y males, but we did not find a clear effect. We also looked at different fitness measures comparing *F* and F^D females at different temperatures, but again did not find a clear result. Here, we focus on one specific fitness parameter; the proportion of intersexes. Intersexes (individuals possessing both male and female characteristics) which can be recognized by deformed genitalia and an aberration in interocular distance, have been reported from the housefly by several authors (Sullivan, 1961; Milani, 1967; Vanossi Este & Rovati, 1982; Schmidt *et al.,* 1997a; Hediger *et al.,* 1998a). Milani (1967) reports that the number of intersexes in houseflies increases in winter months. This finding implies that temperature affects sex determination in the housefly, but it is not clear whether these observations come from autosomal *M* or XY populations. In the housefly two laboratory strains are known in which sex determination is affected by temperature (Vanossi Este & Rovati, 1982; Schmidt *et al.,* 1997a). F^{man} is a loss of function mutation of *F* and results in male offspring when females are homozygous for this mutation. At higher temperature F^{man}/F^{+} offspring of F^{man}/F^{+} mothers develop more often into intersexes or even fertile males than under lower temperatures (Schmidt *et al.,* 1997a). The second mutation, *Ag*, is probably a weak form of *M* on autosome I (M^I) which is too weak for a zygotic male determining effect but strong enough to interfere with maternal *F* activity (Vanossi Este & Rovati, 1982). *Ag* exerts its activity during oogenesis and becomes weaker at higher temperatures which results in mostly female development, but more males and intersexes at lower temperatures (Schmidt *et al.,* 1997b). These two mutations show that the female as well as the male determining factor can be affected by temperature.

Comparable to *Drosophila*, *doublesex* is the switch gene at the bottom of the sex determining cascade in the housefly *Mddsx*, (Hediger *et al.,* 2004). Through alternative splicing a male- or female specific protein variant is produced leading to either male or female development. Sex specific splicing of *Mddsx* is regulated by the *F* gene, which if present leads to female development, if absent

to male development (Hediger *et al.,* 2004). Recently it has been found that *F* corresponds to transformer (*Mdtra*) and is homologous to the *Drosophila* as well as *Ceratitis capitata* transformer (D. Bopp University Zürich, personal communication). *F* is activated in the early zygote by maternal *F* product (Dübendorfer & Hediger, 1998). For the auto-regulation of *F* and female splicing of *Mddsx* constant expression of *transformer2* (*Mdtra2*) is necessary, which is expressed equally in both sexes (Burghardt *et al.,* 2005). At any time between early embryogenesis and metamorphosis *M* activity can interrupt the self-regulatory loop of *F*, and lead to male development (Hilfiker-Kleiner *et al.,* 1993). Generally enzyme function, like enzyme-substrate or enzyme-modulator interaction (Somero, 1968), as well as gene expression levels, both up and down regulation (Maurelli & Sansonetti, 1988; Howarth & Ougham, 1993; Smoot *et al.,* 2001; Carroll *et al.,* 2003), but also mRNA and protein stability (Podrabsky & Somero, 2004) have been shown to be affected by temperature. It is conceivable, that temperature leads to an altered expression of one of the sex determining factors which then can not be properly regulated by the other, or through underexpression can not fulfill its function. Alternatively if one of the factors is a protein or based on secondary DNA structure then its' conformation might be altered by temperature which in turn can alter its functionality (Podrabsky & Somero, 2004). In principle any gene or gene-product in the sex determining cascade could be affected by temperature.

In this study we investigate experimentally whether (1) temperature has an effect on the number of intersexes produced, (2) XY and autosomal *M* populations differ in their response to temperature in the number of intersexes produced, and (3) whether temperature affects progeny sex ratio. For each of the sex determining factors we can generate sex ratio predictions depending on whether a certain temperature leads to a decrease or increase in strength of the factor; (a) *M* becomes stronger: since *M* is able to override *F*, stronger *M* can only be determined through a male biased sex ratio in crosses with F/F^D females since *M* in *F/F* individuals is the "standard setting" leading to male development. (b) *M* becomes weaker: *M* is not able to suppress *F* anymore which results in female instead of male development leading to female biased sex ratios in crosses with *F* and F^D females. (c) *F* becomes stronger: *F* is not/less affected by *M* leading to a female biased sex ratio as *F/F* individuals carrying *M* develop into females instead of males (d) *F* becomes weaker: similar

to the F^{man} mutation, *F* is not strong enough to start the auto-regulatory loop which leads to male development, therefore a male biased sex ratio is expected. (e) F^D becomes stronger: no noticeable effect as F^D is insensible to *M* and leads to female development even in the presence of *M* (f) F^D becomes weaker: *M* is able to interrupt the self-regulatory loop leading to a male biased sex ratio.

In order to keep the effect of temperature on maternal *F* product apart from sex factor interactions in the zygote we set up three different experiments. In the first experiment all adults were reared and kept under one temperature regime and only shifted to experimental temperatures for egg laying and offspring development. In the second experiment adults and offspring experienced the same temperature throughout all developmental stages. Additionally, we set up a third experiment in which we conducted reciprocal crosses between two strains with *M* on autosome II (M^{II}) and either *F/F* or *F/* F^D females.

Material and Methods

To test whether intersex frequencies vary at different temperatures for different sex determining factors, we used two types of populations, two strains each. Two strains were of the M^Y - type (from Germany, GR1 and GR2), the other two strains contained M^{II} (from France, Carmarque (CAM) and Africa, Bagamoyo (BAG)). Originally we thought we had two comparable autosomal populations, however later they appeared to vary in the frequency of M^{II} and differed in the presence/absence of the dominant female determining factor F^D. To determine the frequency of homozygous *M* males, we crossed 20 males from the CAM and BAG population to mutant *F/F* females with homozygous, recessive mutations on each autosome (for more details on the crosses refer to Chapter 4). Offspring resulting from homozygous *M* males are always male, whereas heterozygous males produce mixed broods. We also backcrossed five of the resulting F1 males to mutant females in order to double check the location of *M*. To determine whether females were carrying the dominant female determiner F^D we tested 20 females with molecular markers (for details see Chapter 4). The CAM population mainly consisted of males that were heterozygous for M^{II} but some males contained M^{II} plus M^Y; all females were homozygous for *F*. The BAG population consisted of males that were homozygous for M^{II}, males that were heterozygous for M^{II} and males that contained M^{II} plus M^Y; all females contained F^D. The variation in frequency of

sex determining factors leads to differences in expected sex ratios of the different populations (Table 5.1).

The setup of the following three experiments is identical in that adult flies in population cages (13x13x22cm) were subjected to three different temperatures, 18°C, 22°C, and 26°C, but the developmental stage of the flies that was exposed

Table 5.1: Frequencies of different sex determining factors per population and resulting expected sex ratios (proportion males).

Cross	♂ genotype	♀ genotype	Genotype frequency (%)	Genotype specific sex ratio	Expected overall sex ratio
GR1 x GR1	M^Y/X	F/F	100	0.5	0.5
CAM x CAM	$M^{II}/+$	F/F	83	0.5	0.54
	$M^{II}/+$, M^Y/X	F/F	17	0.75	
BAG x BAG	$M^{II}/+$	F/F^D	31	0.25	0.38
	M^{II}/M^{II}	F/F^D	31	0.5	
	$M^{II}/+$, M^Y/X	F/F^D	38	0.375	
CAM♀ x BAG♂	$M^{II}/+$	F/F	31	0.5	0.75
	M^{II}/M^{II}	F/F	31	1.0	
	$M^{II}/+$, M^Y/X	F/F	38	0.75	
BAG♀ x CAM♂	$M^{II}/+$	F/F^D	83	0.25	0.27
	$M^{II}/+$, M^Y/X	F/F^D	17	0.375	

to the temperature treatment differed (see below for details). Thirty females and 30 males per population were put in a population cage to mate. The adult flies were given constant access to water, sugar water and milk powder. When the flies were about 10 days old, they were provided with film boxes filled with standard egg laying medium. Eggs were collected from each of the population cages four times with a three day interval. The eggs were transferred to bigger boxes where larvae could develop while adult flies in the cages were provided with new egg laying medium. Larvae were fed every second to third day until pupation. For each of the 12 subpopulations (4 strains x 3 temperatures) 1000 emerging flies were sexed and intersexes counted. In cases where fewer flies emerged we counted as many as available. Intersexes were determined by closely examining the genitalia of all flies for abnormalities.

Three experiments were set up to find out whether temperature has an effect on sex determination in the housefly, which was measured as the number of intersexes produced as well as progeny sex ratio aberrations from the expected (Table 5.1). In addition, the developmental stage at which temperature affects sex determining factors, as adult or zygote respectively, was tested.

Experiment 1

This experiment served to find out whether temperature affects sex determining factors of the zygote. Therefore all adults were reared and kept at 20°C to keep any possible effect of temperature acting in adults, especially in adult females, constant. When the adult flies reached an age of about 10 days they were allocated over the population cages and transferred to one of the three temperatures. The rest of the procedure followed the protocol described above.

Experiment 2

In this experiment temperature could effect any developmental stage. Offspring from the previous experiment were used as parental population and thus produced and kept at the according temperature throughout their life and for reproduction. Thus adults as well as offspring experienced the same temperature regime.

Experiment 3

Reciprocal crosses were set up to break apart possible co-adaptations between autosomal *M* and *F* or F^D masking a possible temperature effect. Additionally, we aimed to disentangle which of the sex determining factors was affected by temperature and whether the response was positive or negative. We therefore compared intra-strain crosses of the CAM and BAG populations with crosses of 30 CAM females x 30 BAG males and 30 BAG females x 30 CAM males.

Statistical Analysis

Statistical analysis was performed using R (R Development Core Team, 2006). By means of contingencies a "Fisher's exact test" was performed to test whether the number of intersexes differed between the three temperature treatments. A χ^2 - test was performed to test whether the number of males and females (sex

ratio) differed between temperature treatments, as well as to test whether the number of males and females deviated significantly from the expected sex ratio.

Results

An overview of the results is presented in Table 5.2.

Frequency of intersexes

In the first experiment where adults were reared at 20°C before putting them at the three experimental temperatures, the number of intersexes ranged between one to eight (N=1000 tested) but no effect of temperature was detected in any of the crosses. The same holds true for the second experiment where adults developed at the according temperature. Here the number of intersexes in some cases is slightly higher than in experiment 1 ranging between zero to 13 intersexes but no effect of temperature is detected. In the third experiment, there are two crosses in which the number of intersexes decreases significantly with increasing temperature. In the intra-strain BAG cross the number of intersexes is highest at 18°C with nine intersexes and decreases down to two and three at 22°C and 26°C respectively. In the CAM females x BAG males cross the number of intersexes is also highest at 18°C with 35 intersexes, decreasing to seven at 26°C. The reciprocal cross BAG females x CAM males does not result in different numbers of intersexes between the different temperatures.

Sex ratios

In the first experiment temperature has no effect on the sex ratio of all four populations. In experiment 2 the sex ratios are affected significantly by temperature and increase in all crosses with exception of the CAM intra-strain cross where the sex ratio decreases significantly with increasing temperature. In experiment 3 only the sex ratios of the CAM females x BAG males cross decrease significantly with increasing temperature, while all other sex ratios are not affected by temperature. Even though sex ratios seem to increase significantly with temperature in the two XY populations (GR1 and GR2) in experiment 2, none of the sex ratios of a single temperature treatment deviates significantly from 50:50, except GR1 at 26°C. In the CAM intra-strain cross the sex ratio is significantly more male biased than the expected 0.54 (Table 5.1) at 22°C in experiment 1 and 18°C in experiment 2. In contrast in the BAG intra-

Table 5.2: Number of males (♂), females (♀), intersexes (♂/♀) and sex ratio (SR) under three different temperature treatments for the three different experiments. Experiment (1) adults reared at 20°C, egg to adult development at one of the three temperatures; (2) adult and offspring development continuously at one of the three temperatures; (3) fly treatment according to first experiment, however only autosomal *M* populations and reciprocal crosses were investigated. Fisher's exact test was used to test for different numbers of intersexes between temperature treatments. χ^2- test was performed to test whether sex ratios differed significantly between temperatures and from the expected sex ratio (Table 1). Asterisks indicate the significance level of the sex ratio deviation from the expected value. *-p<0.5; **-p<0.01; ***-p<0.001.

Experiment		**1**					**2**					**3**					
Cross	**Sex**	**Temperature (°C)**			χ^2	**p**	**Temperature (°C)**			χ^2	**p**	**Cross**	**Sex**	**Temperature (°C)**			χ^2	**p**
		18	**22**	**26**			**18**	**22**	**26**					**18**	**22**	**26**		
GR1 x GR1												CAM♀ x BAG♂						
	♂	536	521	537			490	513	561				♂	523	668	687		
	♀	462	473	458			506	479	427				♀	127	300	306		
	♂/♀	2	6	5		0.467	4	8	12		0.146		♂/♀	35	11	7		**<0.001**
	SR	0.54	0.52	0.54	0.55	0.758	0.49	0.52	0.57*	11.86	**0.003**		SR	0.8*	0.69**	0.69**	31.23	**<0.001**
GR2 x GR2												BAG♀ x CAM♂						
	♂	481	507	501			466	487	526				♂	400	425	374		
	♀	513	485	503			528	512	474				♀	589	572	619		
	♂/♀	6	4	4		0.841	6	1	0		0.135		♂/♀	11	3	7		0.110
	SR	0.48	0.51	0.5	1.47	0.479	0.47	0.49	0.53	6.79	**0.034**		SR	0.4***	0.43***	0.38***	5.12	0.077
CAM x CAM												CAM x CAM						
	♂	560	593	551			438	579	530				♂	354	477	350		
	♀	436	406	441			285	418	465				♀	256	336	303		
	♂/♀	4	1	8		0.061	2	3	5		0.784		♂/♀	0	1	3		0.197
	SR	0.56	0.59*	0.56	3.37	0.186	0.61*	0.58	0.53	9.91	**0.007**		SR	0.58	0.59	0.54	4.26	0.119
BAG x BAG												BAG x BAG						
	♂	434	467	416			402	443	464				♂	293	470	425		
	♀	563	529	577			594	546	523				♀	373	528	572		
	♂/♀	3	4	7		0.497	4	11	13		0.080		♂/♀	9	2	3		**0.007**
	SR	0.44*	0.47***	0.42	5.23	0.073	0.4	0.45**	0.47***	9.23	**0.010**		SR	0.44*	0.47**	0.43*	4.18	0.124

strain cross the sex ratio is more male biased than expected in all three experiments at most of the temperature treatments. The cross between CAM females x BAG males shows significant change in sex ratio bias with temperature. At 18°C the sex ratio is more male biased than expected whereas at the intermediate and high temperature the sex ratio is female biased. In the reciprocal cross with BAG females x CAM males there is no effect of temperature on the sex ratios, however all sex ratios are significantly less female biased than expected. Thus sex ratios vary between different temperature treatments, however the observed trends are inconsistent even among the same type of cross (e.g. BAG intra-strain cross).

Discussion

In this study we wanted to test experimentally whether temperature has an effect on sex determination in the housefly by investigating the number of intersexes produced at different temperature regimes. As sex determination in the housefly is not only dependent on the sex determining factors present in the zygote but also on maternal product which is put in the zygote by the mother to induce the self-regulatory loop of *F* production, we investigated the effect of temperature on adult females and zygotes respectively. No clear effect of temperature on the frequency of intersexes is detected. For the BAG crosses, the results of intersex numbers between different temperature treatments are inconsistent as in experiments 1 and 2 the number of intersexes increases with increasing temperature, although not significantly, whereas in experiment 3 it significantly decreases with increasing temperature. The only other cross where an effect of temperature on the number of intersexes is observed is the CAM females x BAG males cross in the third experiment where at 18°C the highest number of intersexes is observed. Since the number of individuals at that particular temperature is low, we can not be sure that other effects like genetical incompatibility or chance might play a role instead of altered expression or functionality of a sex determining factor.

Although there is no clear effect of temperature on the number of intersexes, several crosses, mostly of the second experiment, resulted in biased sex ratios that differed significantly between temperature treatments, and most sex ratios deviated significantly from the expected value (Tables 5.1 and 5.2). This might be an indication that temperature acts on the production of the *F* and/or F^D

product in the adult female. When looking at the pattern of significant sex ratio deviations from the expected value we find two crosses with CAM females that result in more male biased sex ratios then expected at low temperature, which could be interpreted as reduced *F* function. In most of the within strain BAG crosses sex ratios are significantly higher than expected. This can be explained in two ways. First, we only checked 20 males for being hetero- or homozygous for *M* and out of these only five males were tested for the exact sex determining factor composition, which means that there is a large error margin in our estimate of the predicted sex ratio. Second, in comparison with our earlier expectations this could mean that either *M* increases in strength, which can only be recognized in crosses with F^D females (under normal *F*, individuals with *M* become males in any case), or the strength of F^D decreases at low temperatures as more males are produced. Unfortunately these two possibilities are not distinguishable since both effects are only visible under F^D, thus in crosses with BAG females. However, since the pattern of sex ratio deviation is not consistent over the different experiments it is likely that the effects are due to the error margin in predicted sex ratios.

Our results should be seen as a preliminary cue to a possible effect of temperature on sex determining factors in the housefly. We note that first, our experiments lack replica to strengthen our findings, and second, possible sampling errors determining the population composition in respect to sex determining factors make predicted sex ratios less precise. In the future, molecular techniques like RT-PCR or protein analysis may be used to investigate the question whether sex determining factors differ in their expression level or protein function. However, up to date only the gene and function of *F* and F^D is known, the molecular nature of the *M* factor is still unrevealed. In summary, we conclude that under our experimental regime the number of intersexes is not affected by temperature. Nevertheless, sex ratios seem to be somewhat influenced by temperature but additional research is needed to determine the extent to which sex determination in the housefly is temperature dependent.