# The Effect of Temperature on Sex Determination

Robert Bosch Stiftung

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# The Effect of Temperature on Sex Determination

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# Contents

# Preface

This PhD thesis is a result of a program consisting of three PhD projects funded by the Robert Bosch Stiftung. The goal of these projects is to better understand the effects of temperature at different organismal levels, from genes via individuals to populations and ecosystems. Each project covers one or more of these levels which will later be integrated to obtain a broader insight into the effects of temperature in nature at different levels of organization. The top-level project covering ecosystem to population level deals with climatic effects on termite distribution and function in a savanna ecosystem. The second project comprises population and individual levels by investigating the effect of temperature on several plant traits with an emphasis on the metabolic rate in response to acclimation and adaptation. The third and present project covers the genetic to population level and investigates the effect of temperature on sex determination.

# CHAPTER 1

## General introduction and thesis overview

Currently temperature is a hot topic, not only in biology. We all hear and read in the daily news about global warming and possible effects of temperature changes. On a global level temperatures are rising, the Arctic and glaciers are melting, and extreme weather events seem to become more common. This is not only affecting us humans who start encountering water shortage, harvest losses and floods but this also affects all other living organisms. For example population distributions of many animal and plant species start changing and migratory birds shift their departure and arrival dates (for review see Archaux, 2003; Mills, 2005; Beaumont *et al.,* 2006; Parmesan, 2006). These and other effects of global warming illustrate that temperature has an impact on a large scale. What is less obvious is that temperature has an effect also on single individuals. Virtually everything that an organism does is influenced by and dependent on its thermal conditions (Johnston & Bennett, 1996). In the following parts of the introduction I will first discuss the effect of temperature on organisms and clinal distributions of traits that seem to be caused by temperature. Second, I will give an overview of different sex determining systems and variations within and between the different systems, some of which are affected by temperature. Third, I will present some knowledge on the genetic basis of temperature dependent sex determination. Fourth, I will consider the different sex determining systems from an evolutionary point of view, discussing theory and the adaptive significance of the different systems, and lastly give an overview of the thesis.

## Effect of temperature on organisms at multiple levels

Life on earth seems to follow clinal patterns coinciding with temperature clines in many respects. On a global scale biodiversity hotspots are found in the tropics and species numbers decline towards the poles (Gaston, 2000; Bromham & Cardillo, 2003). Body size of animals tends to be larger in colder compared to warmer regions in most endotherms but also in many ectotherms. This phenomenon is known as Bergmann's rule and seems to be effected by temperature per se (for review see Blanckenhorn & Demont, 2004).

Other life history traits like litter size, offspring mass, flowering time, diapause response, and activity patterns are also affected by temperature and have been shown to follow clinal distributions (Vernberg, 1962; Bradford &

Roff, 1995; Caicedo *et al.,* 2004; Jin & Liu, 2007; Simunovic & Jaenike, 2006; Demont & Blanckenhorn, 2008).

Extreme temperatures lead to range limitations in some species or to special adaptations in others. At high temperatures animals have to prevent overheating, protein damage and aquatic organisms have to deal with a mismatch of oxygen demand and the capacity of oxygen supply to tissues (Moseley, 1997; Melzner *et al.,* 2007; Pörtner & Knust, 2007). To prevent overheating of the body, heat transfer to the skin is increased and the sweating threshold decreased (Moseley, 1997). To ensure e.g. protein assembly and protein folding at high temperatures the base level of heat shock proteins is increased (Moseley, 1997). On the other extreme at cold temperatures it is crucial for an organism to escape starvation during longer cold periods, cell damage by ice crystals, and of course freezing. Organisms developed a number of physiological adaptations to help them deal with cold conditions. For example, some fish are known to possess antifreeze proteins which bind to ice-crystals preventing them from growth (Chen *et al.,* 1997). Seals and whales make use of a process called counter-current heat exchange, by which they hardly lose any body heat to the cold water (Scholander & Schevill, 1955). Organisms that do not live in constant cold climates but e.g. in regions with seasons survive winter by hibernating (Carey *et al.,* 2003; Ultsch, 2006). The metabolism and body temperature are decreased drastically to reduce energy expenditure during this period. In reptiles, a trait that is mostly found in cold climates is viviparity. The transition from oviparity to viviparity has taken place at least 100 times independently within reptiles and mainly in cold climates (Shine, 2005; Blackburn, 2006; Ji *et al.,* 2007). The idea is that eggs laid in cool climate will develop slowly or not at all, whereas eggs retained in a sun-basking female will experience warmer conditions and thus develop faster.

On the genetic level a well-known example of a clinal distribution on several continents is the geographical variation in allele frequencies of the *Adh* gene in Drosophila (Berry & Kreitman, 1993; Sezgin *et al.,* 2004). It has been suggested that the distribution of the *Adh* alleles follows a temperature cline (Oudman *et al.,* 1991; Oudman *et al.,* 1992; Parkash *et al.,* 1990). Recently it has been shown in Australian drosophilids that the genetic constitution of the

*Adh* locus has shifted in accordance with increasing temperatures due to climate change (Umina *et al.,* 2005).

The most important traits influenced by temperature in the context of this thesis and also showing clinal distributions in some species are sex determining systems. In the Atlantic silverside, *Menidia menidia*, northern populations have genetic sex determination (GSD) whereas southern populations exhibit environmental sex determination (ESD) (Conover, 1984). Conover *et al.* (1992) showed experimentally that low temperature selected for GSD whereas at high temperature ESD was retained in the population. In the frog *Rana rugosa* different GSD systems are found in Japan (Eggert, 2004; Ogata *et al.,* 2008). In north-west populations differentiated sex chromosomes are found with females being heterogametic (ZW), whereas the other populations have male heterogametic sex determination (XY). The housefly, *Musca domestica*, is a third example and will be discussed in more detail in Part II of this thesis (see also Box 1.1). In the housefly, northern populations on several continents exhibit the standard XY system whereas in populations towards the south some males carry the male determining factor on one or multiple autosomes and in some populations females evolved a dominant female determining factor thus resembling a female heterogametic system (Figure 1.1) (Dübendorfer *et al.,* 2002). The clinal variation in sex determining systems makes the housefly an interesting study organism to investigate the effect of temperature on sex determination (SD). The question is how did these clines come about? In principle the effect of temperature on SD can be divided into two categories. First, temperature as a proximate mechanism with a direct effect on organisms themselves, e.g. with temperature as a cue for SD. In many species with temperature dependent sex determination (TSD), temperature directly affects the development of an embryo to either male or female.

Temperature can also influence the sex ratio in species with genetic SD, e.g. leading to female development in otherwise XY individuals (sex reversal) at extreme temperatures. There are several examples of temperature dependent sex reversal in amphibians, but also in the bearded dragon, *Pogona vitticeps*, the cichlid fish *Oreochromis niloticus*, and the goldfish *Carassius auratus* (Dournon *et al.,* 1990; Eggert, 2004; Goto-Kazeto *et al.,* 2006; Quinn *et al.,* 2007; Baroiller *et al.,* 1995). In the second category temperature acts as ultimate

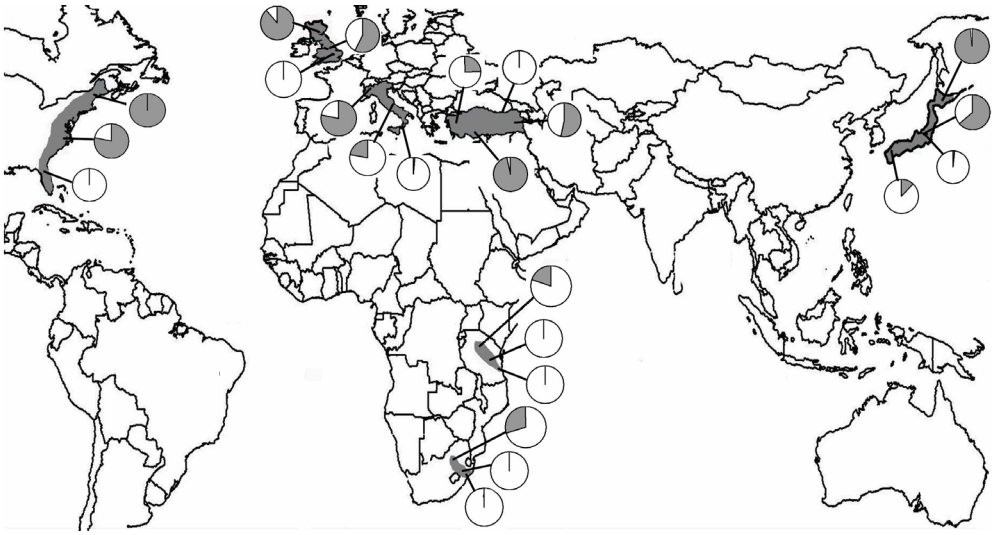

**Figure 1.1:** Global distribution of sex determining factors in the housefly. Relative frequency of *M* factors located on sex chromosomes (grey) or on autosomes (white). (See Chapters 6 and 7 for details.)

trigger, setting the selective environment for the evolution of and transition between different sex determining systems, thereby also affecting the geographical distribution of sex determining mechanisms in nature.

If the fitness of individuals with certain sex determining systems varies in a temperature dependent manner, selection will lead to a distribution of sex determining systems that reflects environmental differences in temperature. In this context not only temperatures per se, but also temperature fluctuations and seasonality have to be considered and can influence the distribution.

The main emphasis of this thesis will be on the second aspect, on temperature as ultimate trigger, especially to investigate to what extent temperature can act as selective force leading to transitions between different sex determining mechanisms.

## Overview of sex determining mechanisms

Before discussing selective forces, and transitions between different sex determining systems, I will first give an overview of existing sex determining systems and underlying mechanisms.

As sex determination is one of the most fundamental processes in the development of an organism, impeccable development into either sex is crucial for an organism in order to be reproductively viable. Therefore, one would expect sex determination to be a conserved process without much genetic and developmental variation. However, in recent years it has become increasingly clear that this is not the case. Not only has it been shown that much variation in sex determining systems exist within the animal and plant kingdoms, but also between closely related species and even within single species (Marin & Baker, 1998; Organ & Janes, 2008) (Figure 1.2).

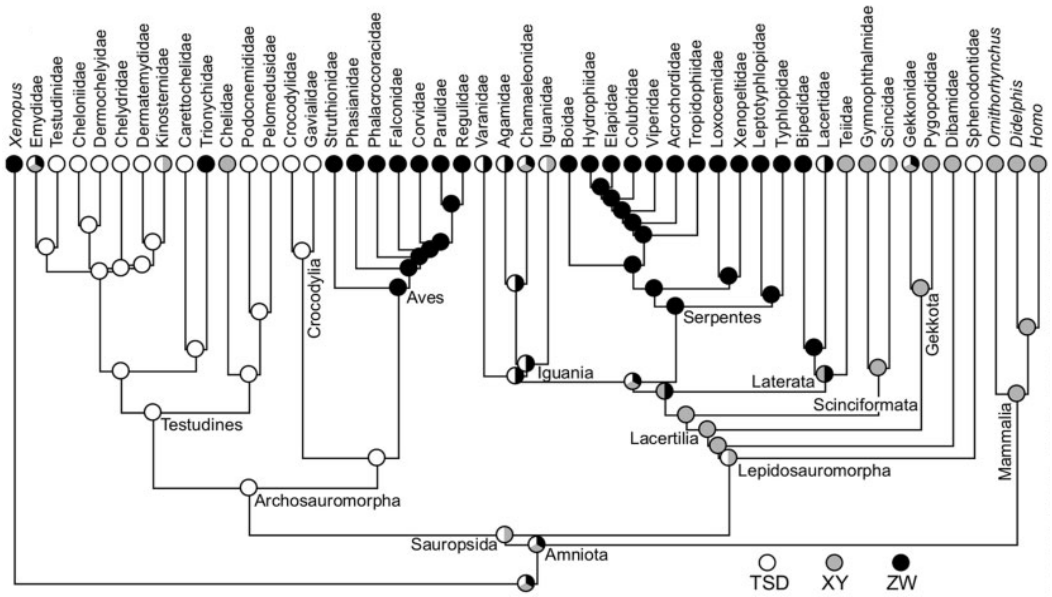


**Figure 1.2:** Phylogeny of sex determination systems focused mainly on Sauropsidae on a family level (three mammal and one amphibian species are added as outgroup). Three of the main sex determining systems, ZW, XY, and TSD can be found in Sauropsida with many transitions between the different systems. Adapted from Organ & Janes, 2008.

## Genetic sex determination (GSD)

In organisms with GSD an individual's sex is determined by its genotype. Several different forms of GSD are known at the chromosomal and gene level.

Here I will briefly summarize different GSD systems to give an idea on the existing variation within the GSD system.

### *Heterogamety/Single-factor system*

Heterogamety comprises two different systems. In male heterogamety, males are heterozygous for the sex chromosomes and are annotated XY, while females are homozygous for the X chromosome, thus XX. This is the most common system and can be found mainly in mammals but also in reptiles, and some invertebrates. In mammals sex determination depends on the presence/absence of the male-determining Y chromosome which carries the testis determining factor *SRY*. In *Drosophila melanogaster* and *Caenorhabdis elegans* for example, sex is determined by the X:A ratio, thus the ratio of feminizing X chromosomes to masculinizing autosomes (Cline & Meyer, 1996).

The second system is female heterogamety with females being the heterogametic sex and are denoted ZW, whereas males are homozygous ZZ. This system is best known from birds, snakes and butterflies but can also be found in many other animal groups, e.g. some fish (Devlin & Nagahama, 2002; Traut *et al.,* 2007). The exact mechanisms underlying SD in the ZW system, thus whether a female determining gene or a gene ratio is responsible for sex determination is still unresolved yet (Sechman, 2005).

### *Haplo-diploidy*

In haplo-diploid species females develop from diploid, fertilized eggs which carry the father's and half of the mother's genome, whereas males develop from unfertilized, haploid eggs, which only contain maternal genes. The underlying mechanism varies between species, however the most common system seems to be complementary sex determination (CSD) (Cook, 1993; Beukeboom, 1995). Under single-locus CSD, sex is determined by multiple alleles at a single locus, in contrast to multi-locus CSD, where two or more loci contribute to SD. Haplo-diploidy is mainly found in hymenopteran insects but also in mites and some beetles (Cook, 1993; Normark *et al.,* 1999; Mori *et al.,* 2005).

*Multifactorial system*

The combined effect of multiple factors results in the development of a certain sex. For example, several loci on multiple chromosomes segregate as sex factors which together act as signal to develop to either male or female (Bull, 1983). Multifactorial systems can be found in several fish species and is commonly observed in Diptera like the midge *Chironomus tentans*, mosquito *Culex tritaeniorhynchus*, and the phorid *Measelia scalaris*.

## Environmental sex determination (ESD)

In ESD sex is determined during embryogenesis in response to the local environment, with some environments producing males and others producing females (Bull, 1983). In ESD (unlike GSD) there is little if any genetic difference between the sexes, thus sex cannot be predicted by zygotic genotype (Bull, 1983; Valenzuela *et al.,* 2003).

Environmental factors that have been found to serve as cues for ESD, are for example pH, nutrient availability, day length, and temperature. In case of temperature dependent sex determination (TSD) the sex of the offspring is determined by incubation temperature during the so-called temperature sensitive period. TSD is found in many reptiles (Bull, 1983; Ciofi & Swingland, 1997), some fish (Conover & Heins, 1987; Strussmann *et al.,* 1997; Ospina-Ãlvarez & Piferrer, 2008), and some nematodes (Pires-daSilva, 2007). The relationship between incubation temperature and the probability of developing into a certain sex varies between species and is depicted in Figure 1.3. In many turtles and fish low temperatures lead to male development whereas high temperatures lead to female development (Figure 1.3A). In several lizards the opposite pattern is observed; females develop at low and males develop at high temperatures (Figure 1.3B). In crocodilians, some turtle and some lizard species males develop at intermediate temperatures while females develop at low and high temperatures (Figure 1.3C). One example of a less commonly found sex ratio-temperature pattern is shown here for the bearded dragon, *Pogona vitticeps*. In this species intermediate temperatures lead to an even sex ratio, high temperatures lead to female development, and at cold temperatures offspring are unviable (Figure 1.3D). It seems conceivable that scenarios 1.3A and 1.3B might be special cases of 1.3C. By taking scenario 1.3C and shifting the response curve to left or to the right, and/or decrease the strength of the sex ratio

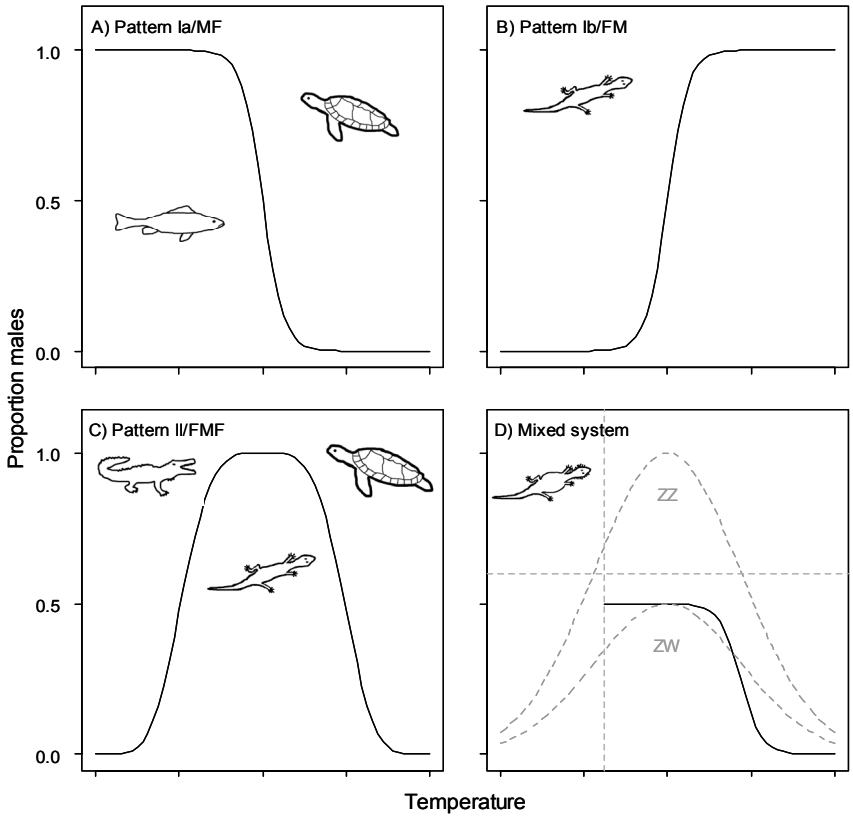


**Figure 1.3:** Temperature dependent sex ratio patterns in reptiles and fish. A) TSD pattern referred to as Ia or MF (male-female), which can be found in many turtles and some fish species. B) TSD pattern Ib or FM which can be found in some lizard species. C) TSD pattern II or FMF, which is found in crocodilians, some turtle and some lizard species. D) Mixture of GSD and TSD. Example of sex ratio response to temperature for the bearded dragon, *Pogona vitticeps* (Quinn *et al.,* 2007). The black line indicates the sex ratio response to temperature. The dashed, grey vertical line indicates the lower viable range for this species, below this point offspring are not viable. The dashed, grey curves indicate the amount of gene product produced by individuals with ZZ or ZW chromosomes respectively, which depends on temperature. The dashed, grey horizontal line indicates the gene product threshold that has to be reached in order to become male.

bias somewhere along the temperature range, one can end up at many different sex ratio response scenarios, e.g. also scenario 1.3D. Thus by modifying scenario 1.3C one can illustrate most of the diversity of temperature dependent sex ratio patterns within reptilians and amphibians, which is by far much more diverse than the four depicted examples (see Valenzuela & Lance, 2004 and authors therein).

## Mixed systems

Even though TSD and GSD are most often discussed as the two main sex determining systems it becomes more and more clear that both systems are the ends of a continuum with a wide variety of intermediate forms in between (Valenzuela *et al.,* 2003; Sarre *et al.,* 2004; Uller *et al.,* 2007). One of these intermediate stages is genotypic sex determination where certain temperatures can induce sex reversal. The best known examples for this kind of system are found among Amphibians (Dournon *et al.,* 1990; Eggert, 2004). In *Rana sylvatica* it has been shown that high rearing temperature of the larvae leads to male biased sex ratios, whereas in *Pleurodeles poireti* high temperatures lead to feminization (reviewed in Eggert, 2004). However, it has been argued that in most studies only temperatures that are irrelevant in nature have been investigated (Hayes, 1998).

Recently, some reptile species with presumed GSD systems have been found to show an effect of temperature on sex ratios, and some species have been discovered where both GSD and TSD seem to co-exist (Shine *et al.,* 2002; Quinn *et al.,* 2007). Shine *et al.* (2002) found heteromorphic sex chromosomes in the montane lizard, *Bassina duppereyi*, but observed 70% males under cold incubation regimes. In the bearded dragon, *Pogona vitticeps,* offspring are not viable at low temperatures, intermediate temperatures lead to a balanced sex ratio and high temperatures lead to female biased sex ratios (Figure 1.3D) (Quinn *et al.,* 2007). Sex ratio patterns in the snow skink, *Niveoscincus ocellatus*, are even more complicated and interesting. In a warm low-altitude population a TSD-like sex ratio response has been observed, whereas in a cold high-altitude population the sex ratio consistently stays at 1:1, thus GSD-like (Wapstra *et al.,* 2004; Wapstra *et al.,* 2009). Even though the sex ratio of the lowland population seems to be temperature dependent, average sex ratios only range between 0.3-0.7 (proportion males). This pattern suggests that sex

determination in the lowlands is based on a GSD system which is affected by temperature. The occurrence of two seemingly different sex determining systems in two populations of the same species, which also differ in the experienced environmental conditions and life-history traits, render *N. ocellatus* an interesting study organism, which we will investigate in this thesis. Within this species the effect of environmental as well as life-history effects on transitions from GSD to TSD as well as a mixture of the two systems can be investigated.

## Unified sex determining mechanism on the molecular level?

After discussing the different sex determining systems I will show here that the basic structure of sex determining pathways follows unified patterns, however the number of underlying genes and their function within a pathway can be quite diverse, and small changes can be enough to render a new sex determining system.

One current hypothesis on the evolution of sex determining systems is that they evolve from bottom up (Wilkins, 1995). This means that the bottom gene of the sex determining cascade, leading to either female or male development, is most conserved and can be found in many closely, but also less close related species. Further up the cascade alterations on regulatory genes are easier to achieve, and are mainly manifested in two ways (Marin & Baker, 1998; Pomiankowski *et al.,* 2004). Either a gene takes control of the expression of the top gene in the cascade (Marin & Baker, 1998), or a mutation in a downstream gene renders it insensitive to the gene further up the hierarchy (Nöthiger & Steinmann-Zwicky, 1985). Thus at higher levels of the cascade most of the variation can be found. This has also been shown recently by molecular studies, which revealed that the variation in sex determining systems is not only due to the presence of a female or male determining gene, but there is also variation in the genetic makeup of the underlying sex determining cascades, or the function of genes within a cascade (Schütt & Nöthiger, 2000; Hodgkin, 2002; Valenzuela *et al.,* 2006; Valenzuela, 2008a) (Figure 1.4). In *Caenorhabditis elegans* Hodgkin (2002) showed that new sex determining systems can easily be generated by single mutations or changes in one section of the sex determining cascade. He created 18 *C. elegans* strains that exhibited GSD or TSD. Temperature sensitive mutations were obtained for most of the major sex

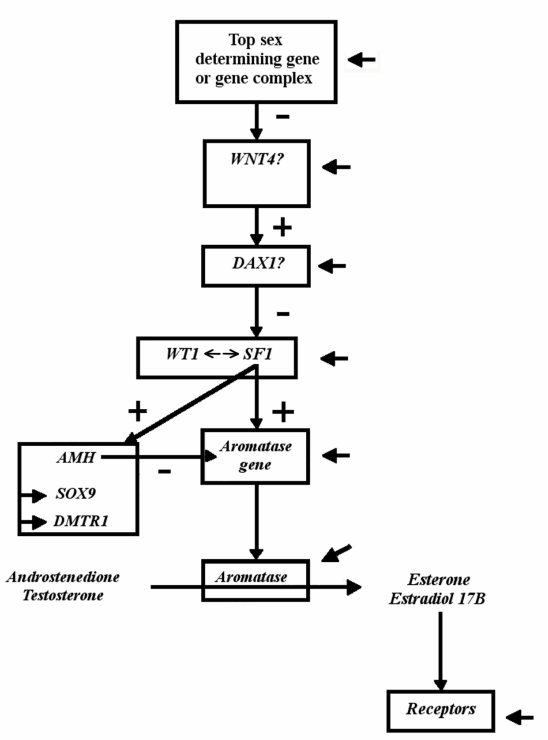


**Figure 1.4:** Proposed pathway for the sex determining cascade in GSD reptiles. Short arrows point to potentially temperature-sensitive genes in species with TSD (for more details see text) (adapted from Sarre *et al.,* 2004).

determining genes, which are scattered across the genome, and several autosomes were turned into de facto sex chromosomes.

In reptiles the sex determining cascade for GSD species is known to start at the top of the cascade with a sex determining gene, which at the end of the cascade, at the bottom gene, will lead to the production of either female or male determining hormone (Figure 1.4). The cascade in TSD species is assumed to be similar, however the temperature sensitive gene, which would have the function of the top sex determining gene, is still unknown and possible focal points for temperature to act are plenty (Figure 1.4). Recent work on the genetics of TSD species brought new insights, and again revealed significant variation (overview in Valenzuela, 2008a). The bottom gene of the cascade, the

*aromatase* gene, is differentially expressed in males and females in some turtles and some crocodilians, but there are other species where no differential expression was detected (Valenzuela & Shikano, 2007). The upstream gene in the cascade, *Sf1*, shows temperature sensitive expression in the turtles *Chrysemys picta* and *Trachemys scripta*, but their expression patterns differ (Fleming & Crews, 2001; Valenzuela *et al.,* 2006). One of the genes upstream of *Sf1* and responsible for its regulation is *Wt1*. The expression level of *Wt1* has been found to be significantly higher at male producing temperatures than at female producing temperatures in both *C. picta* as well as *T. scripta*, whereas again the expression pattern between the two species differed (Spotila *et al.,* 1998; Valenzuela, 2008b). For *C. picta* the second gene upstream of *Sf1* was also investigated and did not show temperature sensitivity which lead the authors to the conclusion that *Wt1* possibly together with *Sf1* could be the thermal master switch gene in *C. picta* (Valenzuela, 2008a). Interestingly Valenzuela (2008a; 2008b) also showed that in *Apalone mutica*, a GSD species closely related to *C. picta, Dax1* as well as *Wt1* both show thermal sensitivity. However, *Sf1* downstream of these two genes probably is insensitive to the differential signal leading to unbiased sex ratios throughout the temperature range.

This section showed that SD on the molecular level can be extremely diverse and evolve rapidly. The fast evolution is possible as small changes along the cascade, even a single mutation, can result in a new sex determining system. This fact will be important for the theoretical part of this thesis where we model transitions between different sex determining systems. With complicated mechanisms underlying transitions between different systems the models would become difficult to implement and probably unrealistic.

## Transitions between sex determining systems in theory

ESD is a sex determining mechanism whose existence still puzzles many scientists. Since in ESD- species SD solely depends on environmental conditions during development, species with this sex determining system are prone to experience biased sex ratios. If the bias goes towards extremes, population extinction might be the consequence. Alternatively one could also pose the question why SD in most species is realized through a rigid GSD system and not by a flexible system which uses environmental information,

such as ESD? In the following I will list and briefly discuss some hypotheses specifically established for the evolution and persistence of ESD, as well as factors that promote the transition from ESD to GSD. Box 1.2 provides more information on additional selective forces and factors that might lead to transitions between sex determining systems in general.

## Factors that may lead to the evolution or persistence of TSD

Hypotheses on the evolution and persistence of TSD can be grouped in two categories, the non-adaptive and adaptive hypotheses.

### *Non-adaptive hypotheses*

The two non-adaptive hypotheses are the (1) *phylogenetic inertia hypothesis* which states that TSD persists because TSD species are genetically not variable enough to evolve other sex determining systems (Ewert & Nelson, 1991). (2) The *quasi neutrality hypothesis*, *w*hich assumes that sex ratio fluctuations in TSD species have a slightly negative effect. Through overlapping generations sex ratio biases even out over time, and render TSD quasi neutral (Girondot & Pieau, 1996). Both of these hypotheses assume TSD to be the ancestral sex determining system whereas currently GSD is considered to be the ancestral sex determining system from which TSD evolved (Janzen & Krenz, 2004; Organ & Janes, 2008). Additionally it has been shown that TSD evolved several times independently even between closely related species, and TSD is found not only in long-lived but also in short-lived species, which contradicts the "quasi neutrality" hypothesis.

### *Adaptive hypotheses*

The sib-avoidance hypothesis assumes that single sexed clutches from natural nests reduce deleterious effects of inbreeding (Ewert & Nelson, 1991). This hypothesis would only be applicable in species where mating takes place directly after hatching between offspring of unrelated nests, however, in many crocodilians and turtles offspring from many annual cohorts interbreed (Burke, 1993). Second, the group adaptation hypothesis states that group fitness increases due to skewed sex ratios, possibly caused by geographically structured population dynamics (Ewert & Nelson, 1991). The assumption here is that female biased populations have higher productivity compared to populations

with even sex ratios, however, male biases are also regularly observed in natural populations (Ewert & Nelson, 1991). In line with the group adaptation theory Freedberg and Taylor (2007) suggested that ESD might be favored over GSD since it allows for female biased sex ratios, which is widely observed in TSD species. The idea here is that female biased populations have a higher reproductive output which decreases extinction risk, and at the same time more offspring also lead to a colonization advantage in new environments. However, they also find that even though ESD is favorable for colonization, ESD populations are susceptible to subsequent colonization attempts by GSD populations at a later stage. This would rather imply that ESD is only advantageous during a transitional stage, but not on the long run.

The best known and also empirically supported hypothesis, is the *differential fitness hypothesis* (Charnov & Bull, 1977). Here it is assumed that male and female fitness is differentially affected by certain environmental conditions. Offspring maximize their fitness by developing either into male or female, depending on the experienced environmental circumstances during development. For example, this theory has been shown to hold true in some fish and some reptile species (Conover, 1984; Ewert & Nelson, 1991; O'Steen, 1998; Harlow & Taylor, 2000; Freedberg & Taylor, 2007; Warner & Shine, 2008). In reptiles however, there are also numerous counterexamples where no differential fitness between the sexes was detected (Rhen & Lang, 1995; St. Clair, 1998; O'Steen & Janzen, 1999). In line with the differential fitness hypothesis comes the *nest-site philopatry hypothesis* (Reinhold, 1998). The idea here is that the fitness of daughters and sons is differentially affected by nest-site quality. Daughters stay at their natal place and therefore inherit high quality sites, which result in higher survival and lead to female development, while males are mainly produced at low quality sites. Empirical data is equivocal with pro (Reinhold, 1998) and contra (Valenzuela & Janzen, 2001) evidence. Another problem with this hypothesis is that, according to theory, female philopatry should select for male biased sex ratios, not for female bias as observed in the populations studied by Reinhold (1998) (Clark, 1978).

#### *Factors selecting against ESD*

The two commonly named factors negatively affecting the evolution and persistence of ESD are environmental fluctuations and short lifespan which are

closely linked. Environmental fluctuations over space and time can lead to strong sex ratio fluctuations, which might lead to population extinction and therefore lead to selection against ESD (Bulmer & Bull, 1982; Leimar *et al.,* 2004). In long-lived species sex ratio biases caused by environmental fluctuations will even out over generations. However, in short lived species or species with a small number of reproductive cycles, strong sex ratio fluctuations can cause population extinction and thus lead to selection for GSD (Bull & Bulmer, 1989).

This overview shows that theory on the evolution and persistence of ESD is plenty. However, it also makes clear that for many species, the evolutionary significance of TSD is still unresolved. Several hypotheses have either not been tested empirically yet, did not find empirical support, and most hypotheses have not exceeded the state of verbal argumentation. This shows the necessity for more thorough theoretical and empirical investigations and the scope for new additional ideas and hypotheses.

## Scope and outline of the thesis:

Recent studies challenge the classical dichotomous view between genetic sex determination (GSD) and temperature dependent sex determination (TSD). Several species have been discovered where both genes and temperature affect sexual development, however not much is known when and why such mixed systems evolve. The main goal of this thesis is to investigate which selective forces lead to transitions between GSD, TSD, and intermediate forms, with the main emphasis on the effect of temperature. This will be accomplished by a combination of theoretical and experimental approaches.

The evolution of TSD from GSD and intermediate forms was modeled on two levels. First from a general point of view, to get insights in the dynamics of transitions between different sex determining systems. And second, transitions between the different sex determining systems were investigated in one specific example with detailed life-history and climatic data of the snow skink, *N. ocellatus*. In both theoretical approaches existing hypotheses on factors that might affect the evolution of TSD were included in the models and their influence studied. To investigate the effect of temperature on the evolution of different GSD systems, the housefly was chosen as experimental model

organism. In the housefly genetic sex determining systems follow clinal patterns, suggesting an effect of temperature on their distribution. This hypothesis was tested by means of fitness assays as well as the collection of additional data on clines in the southern hemisphere which made it possible to determine the effect of temperature on the distribution of housefly sex determining factors.

The following seven chapters of this thesis are arranged in two parts. Part I (Chapters 2 and 3) concerns theoretical models for evolutionary transitions between GSD and ESD; Part II (Chapters 4 - 8) includes field studies, lab experimental and molecular data on the diversity and evolution of housefly sex determining mechanisms.

## Part I: Theoretical approach

**Chapter 2**: Many hypotheses on the evolution and persistence of ESD exist. However, only few have found empirical support. By means of individual based simulations it was investigated whether sex ratio selection under local mate and local resource competition can lead to the evolution of ESD, the more flexible system in terms of (unfacultative) sex ratio bias. In addition, factors that supposedly influence the evolution and persistence of ESD like lifespan and temperature fluctuation were investigated.

**Chapter 3:** The aim of this chapter is to model the evolution of the sex determining system in the snow skink, *Niveoscincus ocellatus*. This species is interesting since the sex ratio of some populations seems to be affected by temperature whereas other populations show a 1:1 GSD-like sex ratio response independent of temperature. As the exact sex determining mechanism in this species is not known, we chose to assume a previously published system for alligators and a lizard species (Deeming & Ferguson, 1988; Quinn *et al.,* 2007). By means of individual based simulations we investigate whether observed life history traits and environmental conditions can lead to the observed pattern, and whether the differential fitness hypothesis (Charnov & Bull, 1977) is applicable in this species.

## Part II: Empirical approach

**Chapter 4:** The clinal distribution of sex determining factors in the housefly might be explained by fitness differences between autosomal *M*, $F^D$ and the standard XY system. Autosomal *M* factors and/or $F^D$ might have a fitness advantage over the standard XY system at high temperature and a disadvantage at low temperature. To test this hypothesis, we performed temperature-controlled laboratory experiments that allowed us to quantify the effects of temperature on the fitness of flies with different sex determining factors. Experiments were performed to see whether males with autosomal *M* factors can invade a population with a standard XY system and whether the invasion prospects were temperature dependent. In females, we investigated whether individuals with *F* or $F^D$ differed in lifespan and lifetime reproductive success, under various temperatures.

**Chapter 5:** As motivated in Chapter 4, different sex determining factors might influence fitness traits under certain temperature regimes. It has been reported previously, that the frequency of intersexes (individuals with mixed female and male features) in houseflies increases in winter (Milani, 1967). This suggests that temperature might directly affect SD in the housefly. We reared flies with different sex determining factor compositions at different temperatures and examined the offspring for the occurrence of intersexes.

**Chapter 6:** Based on the fact that autosomal *M* factors appeared only around 1960, and the spread of autosomal *M* and $F^D$ factors in Japan, it has often been argued that autosomal *M* and $F^D$ are spreading north. To test whether the distribution of sex determining factors in the housefly is a dynamic and still ongoing process or a rather stable distribution, we re-sampled populations in Europe that have been investigated by Franco *et al.* (1982).

**Chapter 7**: In this chapter we investigate the clinal distribution of housefly sex determining systems more closely and try to answer the question whether temperature, as proposed by several authors, and/or additional climatic factors influence the distribution of male and female sex determining factors. Since the clinal distribution of housefly sex determining factors has so far only been shown for the northern hemisphere we collected houseflies from several locations in the southern hemisphere (Africa) to study their distribution. For the

statistical meta-analysis we also included data from previously published studies of the northern hemisphere.

**Chapter 8**: Experimental work on housefly sex determining factors often relies on crosses with mutant flies. If one is assessing fitness traits, differences in genetic make-up of the mutants can lead to distorted results. The variation in sex determining systems in housefly and the fact that different housefly populations harbor autosomal *M* factors on different autosomes, render the housefly a good model organism to study the evolution of sex chromosomes. As part of an attempt to locate *M*, new microsatellite markers were developed, and in addition with previously published microsatellite markers a linkage map was constructed.

**Box 1.1 Sex determination in the housefly**

The purpose of this Box is to give more detailed information on the sex determining system of the housefly on a molecular basis, the distribution of different housefly sex determining systems, and some information on two laboratory populations of the housefly that show temperature sensitivity in sex determination.

*Molecular basis*

Sex determination in the housefly works as follows. In females the auto-regulatory loop of *F* is activated in the early zygote by maternal *F* product and leads to female specific splicing of the switch gene *Mddsx* at the end of the cascade (Dübendorfer & Hediger, 1998; Hediger *et al.,* 2004) (Figure B1). The *F* factor corresponds to the "transformer" gene (*Mdtra*) and is homologous to the *Drosophila* as well as *Ceratitis capitata* transformer (D. Bopp, personal communication). Comparable to *Drosophila*, the constant expression of an additional factor, *transformer2* (*Mdtra2*) is necessary for female splicing of *Mddsx* and also the auto-regulation of *F* (Burghardt *et al.,* 2005). In males however, the male determining factor *M*, interrupts the self-regulatory loop of *F*, and thus leads to male specific splicing of *Mddsx* (Hilfiker-Kleiner *et al.,* 1993). The molecular nature of *M* is unknown. It is conceivable that *M* on different autosomes might actually represent different genes or chromosome regions that all express the same function, namely inactivation of the key gene *F*, which in principle is possible any time between early embryogenesis and metamorphosis.

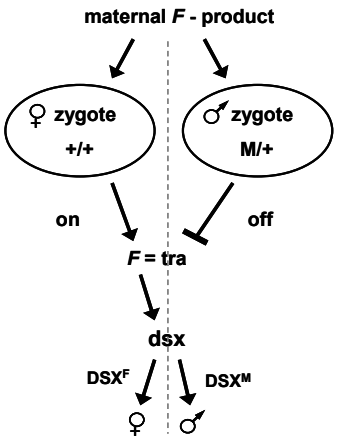


**Figure B1:** The male determining factor *M*, together with the maternal *F*-product, regulates the key gene *F* in the zygote. Without the presence of *M, F* will be positively regulated by the maternal *F*-product and sets *dsx* into female mode. In males however, *M* blocks the positive feedback loop between the maternal *F*-product and *F*, thus inactivates *F*, which sets *dsx* to male mode.

**Box 1.1 continued …**

*Distribution of different sex determining factors*

Housefly sex determining systems follow a clinal geographical distribution (Figure 1.2). The standard XX/XY system, with the *M* factor on the Y chromosome, is mostly found in northern or high altitude areas. In more southern and low altitude areas, the *M* factor can be found on any and even multiple autosomes. In populations with males homozygous for *M* on one of the autosomes, some or even all 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, 1989; Çakir & Kence, 1996; Dübendorfer *et al.,* 2002; Hamm *et al.,* 2005). Males with autosomal *M* factors have been observed to have lost the Y chromosome and are karyotypic XX or X0 (Denholm *et al.,* 1985; Çakir & Kence, 1996).

First evidence of a cline in Europe came from Franco *et al.* (1982) who examined housefly populations from Denmark to Sicily. Studies from England, Japan, Turkey and the US followed, all showing the same pattern with the XY system in the north changing gradually to an autosomal *M* system further south (Denholm *et al.,* 1985; Tomita & Wada, 1989; Çakir & Kence, 1996; Hamm *et al.,* 2005).

Two main hypotheses have been put forward to explain the clinal distribution of the sex determining factors. It first has been argued that the spread of the *M* factor might have been caused by linkage of the *M* factor with insecticide resistance genes, but a recent study refuted this hypothesis (Kerr, 1970; Franco *et al.,* 1982; Tomita & Wada, 1989; Hamm *et al.,* 2005). The other hypothesis states that autosomal *M* might lead to a fitness advantage in warmer regions but a fitness disadvantage in colder regions (Franco *et al.,* 1982; Çakir & Kence, 1996). This hypothesis has not been investigated yet.

*Temperature sensitive housefly strains*

In the laboratory two housefly strains have been discovered that both show temperature sensitivity in sex determination, but differ in the underlying mechanism (Vanossi Este & Rovati, 1982; Schmidt *et al.,* 1997a). $F^{man}$ is a loss of function mutation of *F* where under standard conditions homozygous $F^{man}/F^{man}$ individuals develop as males and heterozygous $F^{man}/F^{+}$ individuals as females. However at high temperatures some proportion of $F^{man}/F^{+}$ offspring develop into intersexes or fertile males (Schmidt *et al.,* 1997a). In the second strain the mutation *Ag*, which is thought to be a weak form of *M* on autosome I ($M^I$) is too weak for a zygotic male determining effect but strong enough to interfere with maternal *F* activity during oogenesis (Vanossi Este & Rovati, 1982; Dübendorfer *et al.,* 2002). Under standard conditions +/+ females produce exclusively daughters, *Ag*/+ females produce sons, daughters as well as some intersexes. Under high temperatures however *Ag* becomes weaker which leads to the development of *Ag*/+ daughters.

**Box 1.2: Evolutionary mechanisms leading to transitions between sex determining systems**

The probability of transitions between sex determining systems depends on the underlying genetic architecture of SD (e.g. the presence/absence of heteromorphic sex chromosomes), and occur when some mechanism destabilizes an existing sex determining system (Marin & Baker, 1998; Werren *et al.,* 2002). Evolutionary mechanisms known to potentially act as such destabilizing factor are genetic conflict, sex ratio selection, and indirect selection, which are briefly outlined in the following (for more details see Werren *et al.,* 2002; Kozielska, 2008).

*Genetic conflict*

Genetic conflict occurs on different levels and can be categorized in intragenomic, intergenomic as well as intralocus conflict (Hurst *et al.,* 1996; Rice & Chippindale, 2001). Intragenomic conflict arises within one individual between different loci caused by selfish genetic elements like meiotic drive elements or cytoplasmic factors (Rice & Chippindale, 2001; Werren *et al.,* 1988). Intergenomic conflict occurs between different individuals or between different loci, as in maternal-offspring conflict over the sex ratios or between males and females over mating frequency. Intralocus, also named intersexual conflict, arises between the two sexes within one locus when selection on a specific trait like e.g. body size differs between the sexes.

*Sex ratio selection*

Typically the evolutionarily stable sex ratio is expected to be 1:1 because of frequency dependent selection against the most common sex (Fisher, 1930). However, there are situations in which biased sex ratios are expected. Fisher's equal allocation theory predicts that mothers should invest equally in sons and daughters (Fisher, 1930). If costs for both male and female offspring are equal, the resulting sex ratio is 1:1. However, if one of the sexes is more costly than the other, the "cheaper" sex should be overproduced, thus leading to a biased sex ratio (Fisher, 1930; Trivers & Willard, 1973). In geographically structured populations it is assumed that biased sex ratios can lead to reduced competition between related individuals. While local competition for mates among brothers will lead to female biased sex ratios, local competition for breeding sites among sisters will lead to male biased sex ratios (Hamilton, 1967; Clark, 1978).

**Box 1.2 continued …**

*Indirect selection*

If individuals with different sex determining factors do not differ in their fitness, genetic drift can either lead to an infinite number of neutrally stable equilibria with multiple neutrally coexisting sex determining factors, or it can lead to the invasion of a new sex determiner in the population and result in a new SD system (Bull & Charnov, 1977; Kozielska *et al.,* 2006). Through hitchhiking, i.e. new sex determining factors tightly linked to a gene under direct positive selection can spread in the population and lead to a new SD system (Bull & Charnov, 1977; Jayakar, 1987).

# Part I: Theoretical Approach

# CHAPTER 2

# On the co-evolutionary dynamics of environmental- and genetic sex determination

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*In preparation for Proceedings of the Royal Society of London B*

## Abstract

Environmental sex determination (ESD) is phylogenetically widespread and has evolved multiple times from genetic sex determination (GSD). Several hypotheses have been proposed to explain the adaptive significance of ESD. For example, the Charnov-Bull hypothesis assumes a sex-specific effect of the environment on fitness and predicts that for a given environment, the sex with the highest reproductive value will develop. We present a model that does not assume environment dependent sex-specific fitness effects, but relies on selection for non-facultatively biased sex ratios due to local kin competition. Five different outcomes were observed: (i) the initial GSD state was stable; (ii) TSD evolved; (iii) a new type of GSD evolved at a second locus; (iv) GSD and TSD stably co-existed; (v) multifactorial GSD evolved. In line with earlier work, TSD evolved more easily when environmental fluctuations were small and when lifespan was long. The final outcome of the evolutionary dynamics can be difficult to predict and is governed by genetic conflict and genetic drift. Our main conclusion is that TSD can evolve more easily than expected even without environment dependent sex-specific fitness effects, thus our results offer a new, additional explanation for the evolution of ESD.

## Introduction

Sex determining mechanisms can be categorized into two types: genetic and environmental sex determination. With genetic sex determination (GSD) an individual's sex is determined by genetic factors; with environmental sex determination (ESD) some environmental factor (e.g. pH, day length, nutrients and temperature) determines sex during development, with some environments inducing the production of males and others producing females (Bull, 1983; Valenzuela *et al.,* 2003). ESD occurs in a wide range of taxa from amphipods, nematodes, echiurid worms, and fish to reptiles (Bull, 1983; Korpelainen, 1990). The most common environmental factor to influence sex determination is temperature and recent studies show that temperature dependent sex determination (TSD) has evolved multiple times independently (Janzen & Krenz, 2004). Apparently, sex determining mechanisms are more evolutionarily labile than expected, considering that sex determination is a fundamental process (Werren & Beukeboom, 1998). However, the evolutionary forces leading to these changes occur are far from clear.

The most widely accepted model for the selective advantage of ESD is the Charnov-Bull hypothesis (Charnov & Bull, 1977). It assumes sex-specific effects of an environmental factor, e.g. temperature, on fitness and predicts that for a given temperature, the sex with the highest reproductive value will develop. It is this flexibility in matching offspring sex to the environment which gives ESD an advantage over the more or less constant offspring sex ratios produced by GSD. There are a number of examples where the Charnov-Bull hypothesis was explicitly tested and where it was shown that one sex benefits more of one environment than the other (Conover, 1984; McCabe & Dunn, 1997; Warner & Shine, 2008). There are other cases where the hypothesis has not been explicitly tested but the environment dependent fitness patterns are in line with the Charnov-Bull hypothesis (Gutzke & Crews, 1988; Janzen, 1995; Langkilde, 2005). However there are also species with TSD in which no differential fitness effects were found (Janzen & Paukstis, 1991b; St. Clair, 1998) or cases concerning one species where one study found evidence favoring the hypothesis (Rhen & Lang, 1995) and the next study could not detect any differential fitness effects (Steyermark & Spotila, 2001) (for more examples see Valenzuela, 2004). This inconsistency in results led Shine (1999) to speak of an

"enigma" of the selective advantage of ESD in reptiles which up to now has not been solved yet.

What other factors could promote the evolution of environmental sex determination? One possible factor favoring ESD over GSD systems is that GSD is highly inflexible. Most GSD systems such as male or female heterogamety tend to produce a 1:1 sex ratio, and biases can only be achieved by costly means (e.g. "unwanted" abortion) (Pen & Weissing, 2002). This is disadvantageous in situations where a biased sex ratio, deviating from 1:1, is favored, like for example differential costs for offspring (Trivers, 1974), local mate competition (Hamilton, 1967) or local resource competition (Clark, 1978). TSD on the other hand is much more flexible in that sense and might be favored over GSD when sex ratio bias is favored (Korpelainen, 1990; Uller *et al.,* 2007 for review). In this paper we investigate the question whether the inflexibility of GSD in face of sex ratio selection may lead to the evolution of TSD.

We investigate this question by means of individual based simulations. We consider a patch structured population where sex ratio selection is mediated by local mate competition (LMC) and local resource competition (LRC). Both LMC and LRC predict that the most dispersing sex should be overproduced since this reduces competition among related offspring. In our model we therefore investigate situations with sex-specific dispersal and the question is whether that can induce the evolution of ESD in a population that consists mostly of GSD individuals.

To get a balanced view we do not only consider the potential advantage of ESD (higher flexibility) but also one of its disadvantages. It has been argued that ESD only produces a certain fixed sex ratio bias if the distribution of environmental condition affecting sex determination is more or less constant over space and time (Bull & Bulmer, 1989). Any fluctuations in this distribution will lead to a (temporal/local) deviation from the "desired" sex ratio bias. The potential advantage of TSD is reduced if environmental conditions fluctuate drastically across locations or change strongly over time (Charnov & Bull, 1977; Van Dooren & Leimar, 2003). Negative effects of the latter depend on longevity of the organism in question since they may average out in the cause of long lifespan (Bull & Bulmer, 1989). For these reasons we consider several

scenarios with various degrees of environmental fluctuations and different longevities.

## The Model

As motivated above we consider a patch-structured population with sex-specific dispersal. The sex of an individual is determined via a cascade of events that culminates in either genetic or environmental sex determination. Without loss of generality we consider temperature to be the relevant environmental factor. We consider various magnitudes of temperature fluctuations at the levels of the individual, the nest and the patch. Moreover we consider both short-lived organisms (non-overlapping generations) that experience a single environment and long-lived organisms (overlapping generations) where temperature fluctuations can average out, to some extent, over an organism's lifetime.

### Sex determination

At the highest level of the cascade we consider a control locus (C-locus) that determines whether sex is fully under genetic control or based on local temperature (Figure 2.1). This locus acts as a kind of switch between GSD and

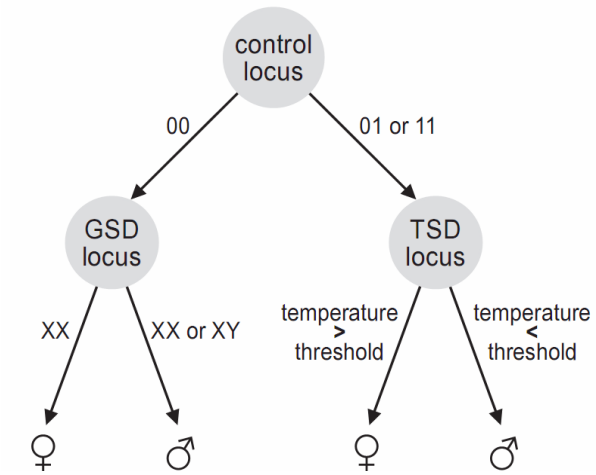


**Figure 2.1:** Sex determining cascade in the model. The control locus (CL) at the highest level of the sex determining cascade determines whether sex determination will be enforced by the genetic locus (GL) or the threshold locus (TL) and therefore the environment. Example given corresponds to male heterogamety, female heterogamety is modeled analogously.

TSD. It is modeled as a major effect gene with two alleles 0 and 1, where 1 (the TSD allele) is dominant over 0. In individuals with genotype 00 the GSD pathway is taken and the individual's sex is determined based on a GSD locus (G-locus). In individuals with 01 or 11 genotype the TSD pathway will be taken and sex is determined via a threshold locus (T-locus). In case of genetic sex determination sex is determined by male heterogamety (XY) or female heterogamety (ZW). In both cases the G-locus corresponds to a major effect gene with two alleles X and Y or Z and W. In case of male heterogamety, Y is a dominant male determining factor implying that XY or YY individuals turn into males and XX individuals into females. In case of female heterogamety, W is a dominant female determining factor. Note that in a mixed population with both TSD and GSD, individuals with YY respectively WW can arise in cases where the sex of these individuals is not determined by the genotype but by local temperature. In case of environmental (temperature) dependent sex determination an individual's sex is determined based on local temperature that is either higher or lower than a genetically determined threshold value, encoded by the T-locus. The alleles at the T-locus can take on any real numbered value, and the threshold corresponds to the mean of the two allelic values. Whenever the temperature experienced by an offspring (the egg temperature) is larger than the threshold the individual will develop into a female and otherwise into a male.

To allow the generation of new genetic variation we allow for mutations of alleles at the T-locus with probability $\mu_T = 0.01$ and a mutation step size drawn from a normal distribution with mean 0 and standard deviation $\sigma_T = 0.01$. We chose for a rather high mutation rate in order to observe changes within a reasonable number of generations, however in order to keep mutation effects in balance we kept the mutation step size relatively small.

## Temperature fluctuations

In case of TSD, the offspring sex ratio is strongly affected by temperature variation. We consider temperature fluctuations at three levels: the patch, the nest and the individual offspring (egg). To be specific the temperature experienced by an individual is given by the sum $T_{indiv} = T + T_P + T_F + T_E$. Here $T$ corresponds to a global average which, for simplicity, was normalized to 0, while for the stochastic variables $T_P$, $T_F$, $T_E$ values were drawn from normal

distributions with standard deviations $\sigma_P$, $\sigma_F$, $\sigma_E$ respectively (Table 2.1). Notice that $\sigma_E$ reflects temperature variation between the eggs within a nest. If this variation is 0, the whole clutch will experience the same temperature. Similarly, $\sigma_F$ corresponds to the temperature fluctuations between females within a patch, while $\sigma_P$ describes temperature variation between patches. Notice that in case of $\sigma_P > 0$ TSD may result in differences in offspring sex ratio across patches, and even extreme local sex ratios for sufficiently large values of $\sigma_P$. As mentioned above, we expect this might result in an advantage of GSD over TSD.

**Table 2.1:** Parameters of the model and parameter values used in simulations. SD refers to standard deviation.

| Variable | Notation | Numerical values |
|---|---|---|
| Number of patches | $N_P$ | 500 |
| Number of breeding sites per patch | $N_B$ | 4 |
| Clutch size | $N_C$ | 20 |
| Dispersal probability of females and males | $d_F$, $d_M$ | 0.001, 0.5, 0.99 |
| Adult survival probability | $S$ | 0, 0.9 |
| SD between patch temperature fluctuations | $\sigma_P$ | 0.001, 0.01, 0.1, 0.5 |
| SD between female temperature fluctuations | $\sigma_F$ | 0.01, 0.1 |
| SD between eggs temperature fluctuations | $\sigma_E$ | 0, 0.01 |
| mutation rate T-locus | $\mu_T$ | 0.01 |
| SD for mutation, T-locus | $\sigma_T$ | 0.01 |

## Life cycle and population structure

We consider a metapopulation consisting of $N_P$ patches. In each patch there are $N_B$ breeding positions, i.e. $N_B$ females can breed at each patch. Breeding females randomly choose a male from their patch, mate and produce a fixed number of $N_C$ offspring (Figure 2.2). The offspring have a certain dispersal probability $d_F$ for females and $d_M$ for males. After reaching a random patch (island model) they stay there for the rest of their life. Breeding females keep their breeding position as long as they live. Their survival probability is constant and given by $S$ (= 0 in case of non-overlapping generations). Whenever a breeding site becomes available, due to the death of a female, she is replaced by a randomly chosen female offspring from the same patch.

## Individual based simulations

We were interested in the question whether TSD can spread from an original state of GSD. To achieve this, 95% of the individuals were assigned 00 alleles at the C-locus (GSD) and 5% were assigned 01 at the C-locus (TSD) at the start of our simulations. At the G-locus half of the individuals were assigned the genotype XX (ZZ), the other half XY (ZW) respectively. At the T-locus originally all alleles had the value 0, and new variation had to evolve due to mutation.

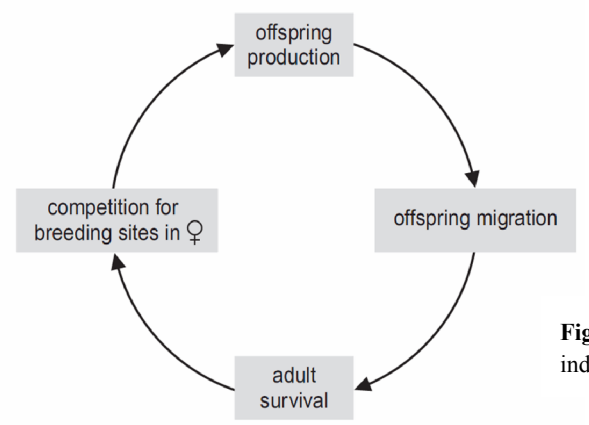


**Figure 2.2:** Life cycle of individuals in our model.

In the non-overlapping generation scenario (short-lived organisms) each simulation was run for 10 000 time steps (i.e. generations). In case of the overlapping generation scenario (long-lived organisms) each simulation was run for 50 000 generations to achieve a comparable generation number. Throughout we monitored the allele and genotype frequency at all three loci. Table 2.1 gives an overview of the parameter values used. Altogether this resulted in 512 different parameter combinations for which we ran ten replicate simulations each.

## Results

Our study was set up to investigate the hypothesis whether selection for sex ratio bias might induce the evolution of TSD. Indeed TSD spreads to fixation in many of our simulations, particularly in cases of large dispersal asymmetries between the sexes and low degree of temperature fluctuation and long lifespan.

However, perhaps surprisingly, various other evolutionary outcomes were also observed, sometimes even for the same parameter combination. Although each evolutionary trajectory is unique the outcomes of the more than 5000 simulation runs can be categorized as follows:

- GSD persists; TSD is driven to extinction.
- TSD fixates; GSD is driven to extinction.
- GSD and TSD more or less stably co-exist.
- A new GSD system evolves; the C-locus becomes the new GSD locus.
- A two-factor GSD system evolves; the C locus evolves into an additional GSD locus.

## Understanding the co-evolutionary dynamics

To help sharpen the reader's intuition we discuss the evolutionary dynamics leading to each of the five outcomes by studying in some detail the results for one single parameter combination (Figure 2.3). In this example the initial GSD system is XY, there is female-biased dispersal ($d_F$= 0.99, $d_M$=0.01), low to moderate temperature fluctuations ($\sigma_P = \sigma_F = \sigma_E = 0.01$) and a long-lived life history. Thus, a female-biased sex ratio is "optimal", tending to favor the sex ratio flexibility afforded by a TSD system, but temperature fluctuations tend to favor GSD. For this parameter combination these opposing forces seem to be nearly balanced, since all possible evolutionary outcomes are observed. Thus, the final outcome may be determined by second-order effects such as initial chance fluctuations in gene frequencies.

Before going into the details of Figure 2.3, here is how we think selection acts on the three gene loci. Selection on the G-locus tends to restore an even sex ratio: if the sex ratio is female-biased, the Y chromosome tends to increase in frequency, and conversely if the sex ratio is male-biased, the Y chromosome tends to decrease in frequency. Selection on the C-locus also tends to restore an even sex ratio, but in a more complicated fashion. If the alleles on the T-locus favor a female-biased sex ratio (negative threshold values), then the TSD allele on the C-locus increases in frequency if the overall sex ratio is male-biased, otherwise it will decrease in frequency. Conversely, if alleles on the T-locus favor a male-biased sex ratio (positive threshold values), then the TSD allele

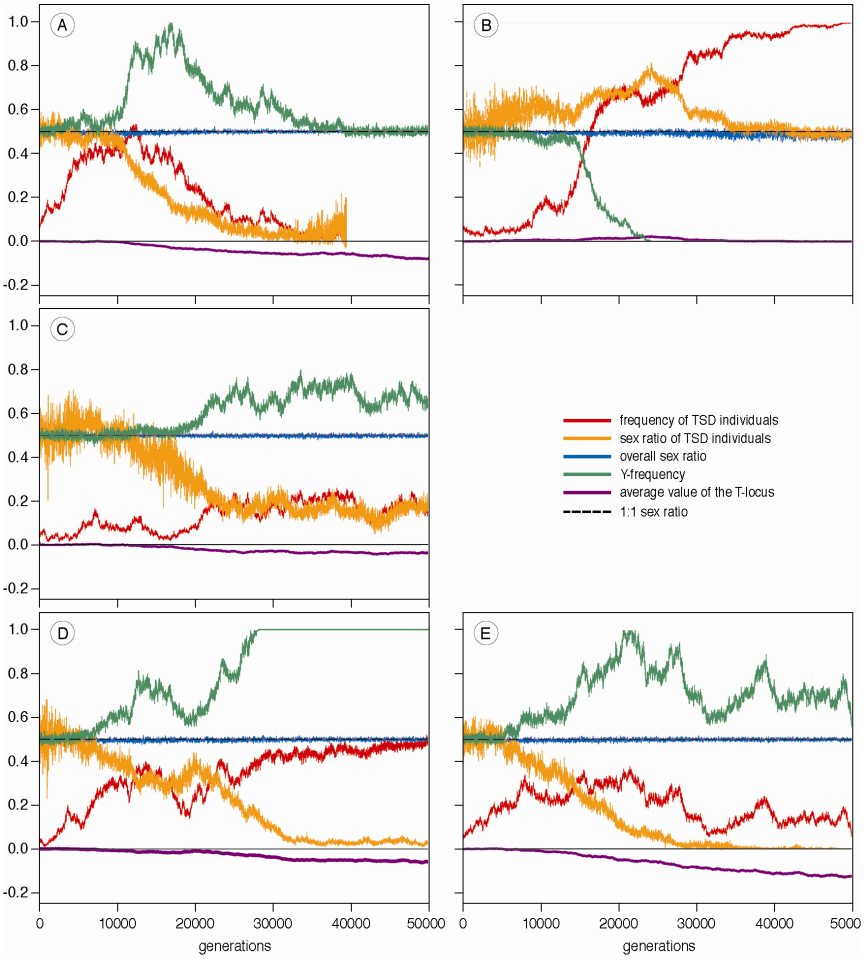


**Figure 2.3:** Illustration of the main outcomes of our simulations: (a) GSD; (b) TSD; (c) coexistence of TSD and GSD; (d) new GSD; (e) two level GSD. Note that the sex ratio is 1:1 in all cases except TSD (b) where the sex ratio is biased towards the dispersing sex, females in this case. The TSD sex ratio variance is inversely correlated with the number of TSD individuals. In figure (c) TSD persists at a frequency of about 20 percent. The female bias at the T-locus is counter acted by a higher frequency of Y at the G-locus. In figure (d) the C-locus becomes the new sex determination locus. The frequency of TSD increases while the Y chromosome goes to fixation, the T-locus evolves to extreme values leading to all females from "TSD" individuals and all males from "GSD" individuals. In figure e) "TSD" also persists in the population however does not show any temperature dependence any more. All simulations started with 95% GSD and 5% TSD individuals; $\sigma_P$, $\sigma_F$, $\sigma_E$ = 0.01; $d_F$=0.99; $d_M$=0.01; XY long lived.

increases in frequency if the overall sex ratio is female-biased, and decreases otherwise. Selection on the T-locus will favor a sex ratio biased towards the dispersing sex, but selection is weaker compared to selection on the G-locus and the C-locus since alleles on the latter two can have major effects while small mutational increments on the T-locus ensure alleles of small effect. Evolution at the T-locus therefore proceeds relatively slowly, and an equilibrium sex ratio biased towards the dispersing sex can only be realized if the G-locus is fixed for either X or Y and the C-locus is (nearly) fixed for the TSD allele. Thus, there is genetic conflict between the T-locus and the other two loci, but the T-locus can only "win" if there is no variation left on the other loci.

In Figure 2.3A, GSD ultimately persists after TSD (the red line) goes extinct. However, interestingly TSD initially spreads and is only lost after a prolonged period of coexistence. As TSD reaches almost a 50% frequency the sex ratio (blue line) becomes male-biased, triggering an increase in the frequency of Y (green line), almost until fixation. At the same time, threshold alleles at the T-locus evolve to negative values (purple line), causing a strongly biased sex ratio in individuals with TSD (orange line). This is followed by a decline in TSD, all the way to extinction, and Y evolves back to a 50% frequency. In Figure 2.3B, TSD first persists at low frequency until it increases in frequency and spreads to fixation. Alleles at the T-locus evolve slightly positive values, causing a slight female bias in TSD individuals. As TSD goes to fixation, the Y chromosome disappears from the population and a female-based sex ratio can be maintained in equilibrium. In Figure 2.3C, TSD coexists for a long time with GSD. Since alleles at the T-locus evolve to negative values, the sex ratio of TSD individuals is quite female-biased. This is compensated by a relatively high frequency of the Y chromosome, causing male-biased sex ratios in GSD individuals and an overall sex ratio very close to even. In Figure 2.3D, there is also coexistence between TSD and GSD for a while, but at some point the Y chromosome goes to fixation while the TSD allele converges to a frequency of about 50%. Meanwhile, negative threshold values evolve such that TSD individuals always develop into females. The end result is that individuals without the TSD allele always develop into males since they have two Y chromosomes, while individuals with a TSD allele become females. Thus, the C-locus has become a de facto GSD locus. Finally, in Figure 2.3E, the TSD allele remains at relatively low frequency while the Y chromosome stays above 50%. Since the threshold

evolves to a low value, individuals with the TSD allele always develop into females. Thus, in essence two GSD systems coexist: the G-locus is a GSD locus with male heterogamety, while the C-locus has female heterogamety.

## Degree of sex ratio bias under TSD

Our model was based on the idea that differential dispersal leading to selection for biased sex ratios would facilitate the evolution of TSD. Indeed, we only observed biased sex ratios in simulations where TSD evolved. However, the observed sex ratio bias was relatively small. According to analytical predictions by Pen (2006), in populations with patches of four breeding females and offspring control of the sex ratio, the expected sex ratio is 0.46 under female dispersal and 0.54 under male dispersal. In our simulations for the short lived scenario the sex ratios were 0.53 (+ 0.002 SE) for male dispersal and 0.47 (+ 0.002 SE) under female dispersal (averaged over the last 1000 generations in simulations where TSD evolved), slightly less biased than predicted. As shown previously in other models with selection for biased sex ratios and the transitions from one sex determining system to another, even small deviations from a 1:1 sex ratio can have drastic effects on the evolution of sex determination (Werren & Hatcher, 2000; Kozielska *et al.,* 2006; Uller *et al.,* 2007; Werren *et al.,* 2002).

## Effect of environmental variation and life history traits

We derived the frequency distribution of the different evolved sex determining systems in Figure 2.4 by classifying the results of our simulations as follows: *GSD* = TSD frequency < 5%, where we define TSD frequency as the frequency of individuals with either 01 or 11 alleles at the C-locus (as opposed to 00 for GSD individuals); *TSD* = TSD frequency > 95%; *"new GSD"* = the C-locus takes over the sex determining function in that "TSD" individuals only produce one sex due to extreme values at the T-locus, the Y or W chromosome is either extinct or fixated which means that the opposite sex is produced by GSD individuals, "TSD" frequency is about 50% ensuring a 1:1 sex ratio; *Co-existence of TSD and GSD* = TSD frequency is between 5-95% and both sexes are produced by TSD and GSD individuals; *Two level GSD* = TSD frequency between 5-95% here "TSD" individuals only produce one sex due to extreme

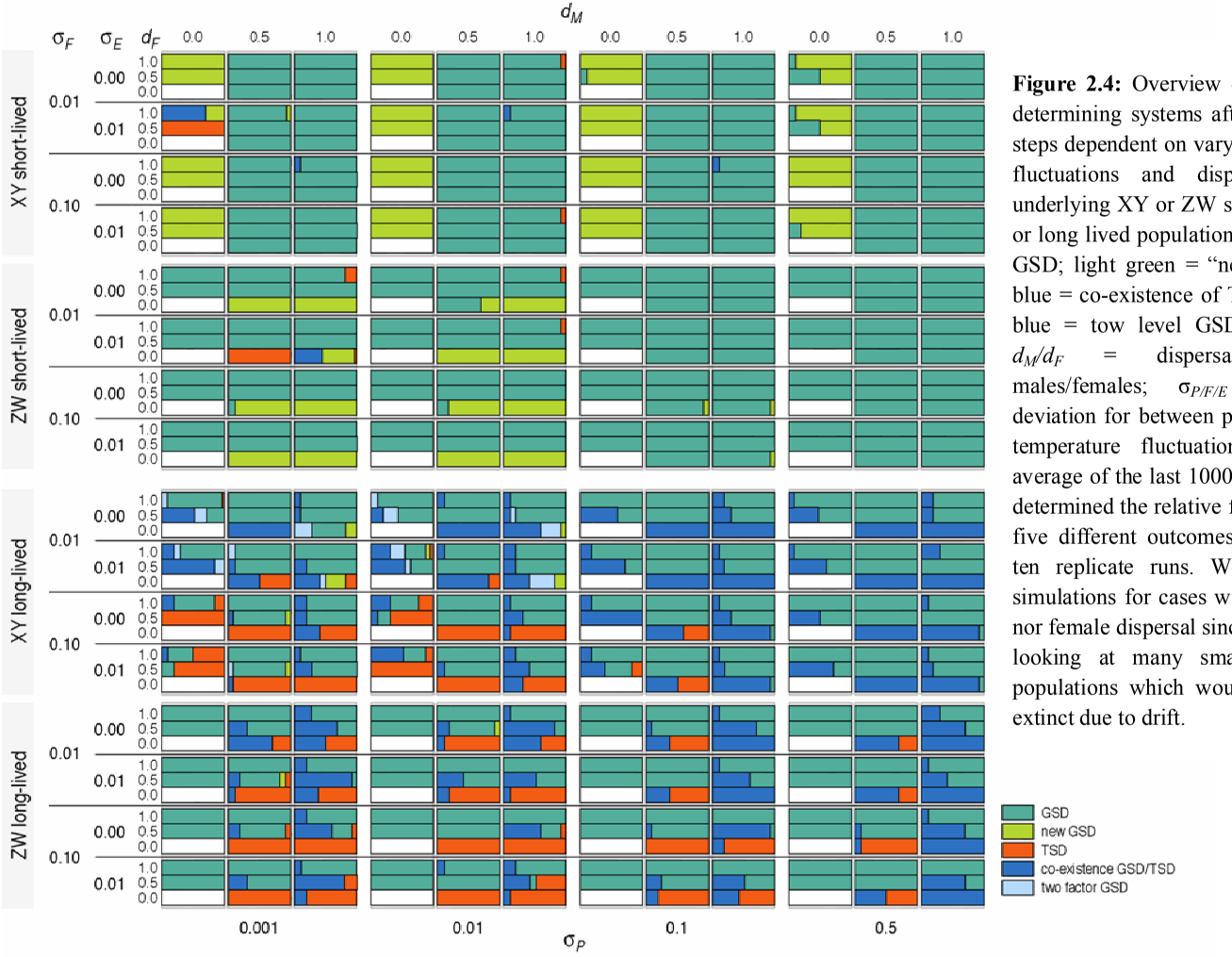


**Figure 2.4:** Overview of different sex determining systems after 50 000 time steps dependent on varying temperature fluctuations and dispersal regimes, underlying XY or ZW system and short or long lived populations. Dark green = GSD; light green = "new GSD"; dark blue = co-existence of TSD/GSD; light blue = tow level GSD; red = TSD; $d_M/d_F$ = dispersal probability males/females; $\sigma_{P/F/E}$ = standard deviation for between patch/female/egg temperature fluctuation. Taking the average of the last 1000 generations we determined the relative frequency of the five different outcomes averaged over ten replicate runs. We did not run simulations for cases with neither male nor female dispersal since that would be looking at many small independent populations which would probably go extinct due to drift.

allele values at the T-locus, the sex ratio bias is compensated by either high or low Y (W) frequencies.

When looking at Figure 2.4 one immediately observes the difference between the upper and the lower panels. While in the upper two panels, depicting the short-lived scenarios, TSD (red) evolved only rarely, it evolved much more often for the long lived scenarios (two lower panels). Zooming in to the level of one panel one can see a trend from left to right but also from top to bottom, showing that TSD frequency decreases with increasing between patch temperature fluctuations but increases with between female as well as between egg fluctuations. Thus, the probability that TSD evolves is larger when between-patch fluctuations are large relative to within patch fluctuations in temperature. The figure also shows that differential dispersal between the sexes promotes the evolution of TSD. With very few exceptions, TSD only evolves in situations where either only females or only males disperse. All these results are in line with existing hypotheses which we have discussed above. However, another striking difference one can observe is the effect of female versus male dispersal on the evolution of different sex determining systems between the XY and ZW system, most prominently in the short lived scenario. While mostly female dispersal promotes the evolution of a new sex determining system under XY, it is male dispersal under ZW. In most cases the end result meaning the heterogametic sex stays the same, sex determination is now just shifted one level up to the control locus.

Werren *et al.* (2002) showed that maternal-offspring conflict can lead to the evolution of dominant zygotic sex determination, where male heterogamety (XY) evolved when males more negatively effect fitness within the family, and female heterogamety (ZW) when females more negatively effected family fitness. Even though the models differ in the underlying assumptions and main mechanisms our results here might be explained by the same reasoning. First TSD increases and leads to bias in the sex ratio, which is counteracted by the G-locus in changing the frequency of the heterogametic sex chromosome, which in the case of XY leads to extinction of the Y-chromosome. Since the non-dispersing sex, here males, negatively affects family fitness through sib competition, selection leads to the evolution of male heterogamety. This is

realized in that at the level of the control locus 00 individuals ("GSD") now all become females and 01 individuals ("TSD") all males.

## Discussion

Our simulations show that selection for non-facultative sex ratio bias can lead to the evolution of TSD. As expected from previous hypotheses, a stable environment and long lifespan promote the evolution of TSD. Our "flexibility of sex ratio hypothesis" is not the first model to include selection for skewed sex ratios as possible driving mechanism for the evolution of ESD. The sib-avoidance hypothesis assumes that single sexes clutches from natural nests reduce deleterious effects of inbreeding and the group-structured adaptation in sex ratio hypothesis states that group fitness increases due to skewed sex ratios (Ewert & Nelson, 1991), however these hypothesis have never been modeled and lack support from empirical data (Ewert & Nelson, 1991; Shine, 1999). The most popular hypothesis which also finds some empirical support is the fitness benefits hypothesis of Charnov & Bull (1977). Here, however, the driving force of ESD evolution is the differential fitness benefits for the sexes depending on the environment and sex ratios evolve facultatively. Other models have looked at sex ratios either as mechanism to maintain ESD (Freedberg & Taylor, 2007) or models trying to explain extreme sex ratios found in some ESD species (Charnov & Bull, 1989; Freedberg & Wade, 2001; Reinhold, 1998).

Our model, not only shows the evolution of TSD is possible but also shows that through rapid transitions from one sex determining system to another, different sex determining systems in closely related species are possible. For the first time we present a model that also accounts for co-existence of TSD and GSD. We know of two cases where, within one species, populations with either TSD or GSD exist, the Atlantic silverside, *Menidia menidia* (Conover & Heins, 1987) and the snow skink, *Niveoscincus ocellatus* (Wapstra personal communication). Additionally, several species have been discovered whose sex is mainly determined genetically but can be overridden by temperature at extremes, e.g. the marked turtle, *Emys orbicularis* (Girondot *et al.,* 1994), montane lizard, *Bassiana duperreyi* (Shine *et al.,* 2002), crustacean, *Gammarus duebeni* (Dunn *et al.,* 2005), several amphibians (reviewed in Eggert, 2004). We also show that co-existence of two GSD systems (two level GSD) is possible which can also be found e.g. in the frog species *Rana rugosa*, where the

northern part of the population possesses XX/XY sex determining system whereas in the southern part the population possesses ZW/ZZ, which apparently evolved two times independently (Ogata *et al.,* 2008).

More and more studies show that genetic conflict can play a role in the evolution of sex determining systems (Bull & Charnov, 1977; van Doorn & Kirkpatrick, 2007; Werren & Beukeboom, 1998; Werren & Hatcher, 2000; Werren *et al.,* 2002). We also discovered interesting dynamics that shed light on the underlying inter-genomic conflict responsible for the outcome of our simulations. For example, we find that the GSD locus can diminish the sex ratio bias induced by TSD, thus hampering its' invasion. If selection for a certain sex determining system is weak we find that on the one hand effects like drift start playing a role, and on the other hand the conflict between the sex determining loci influences the patterns of the dynamics altering the end result. In this situation it is even possible to find several different end-results for one initial condition.

One possible drawback to our model is that we model a discrete locus at the top of the cascade that switches between GSD and TSD. A more reasonable approach might have been to model a continuous locus instead or a gene dosage system (as proposed by Deeming & Ferguson, 1988; Quinn *et al.,* 2007). However knowledge on the exact mechanism of sex determination in TSD species i.e. which gene actually is responsible for temperature dependence in reptiles (Shoemaker *et al.,* 2007; Valenzuela, 2008a) and therefore the mechanism for the change between one system to the other is unknown. Additionally it seems that gene expression patterns of genes responsible for gonadal development differ between different TSD species which might mean that TSD is realized by different means in different species (Valenzuela, 2008a). We chose for a simplistic approach although it might be interesting to modify the way that GSD and TSD interact in a future version of the model.

To confirm our prediction that biased sex ratios are indeed the driving force for ESD evolution we additionally tested our prediction with a sex allocation model. In that case we modeled a panmictic population where costs for male and female offspring varied, which is expected to lead to a sex ratio bias towards the cheap sex (Trivers, 1974). Also in that model TSD could evolve when costs were biased, thus indicating that the possibility for biased sex ratios

is indeed the driving factor. Since we had reptiles in mind when constructing our model we decided that differential dispersal (Tucker *et al.,* 1998; Ujvari *et al.,* 2008) would be more realistic than differential costs for offspring.

To find evidence for our hypothesis one could compare data on sex specific dispersal probabilities in TSD and GSD species. However, as factors like temperature fluctuations play a major role it will be hard to draw useful conclusions. Another approach would be to use species in which both SDMs exist. Here the transition from one system to the other might still be ongoing and a switch in both directions could still be possible. Thus by using one of these species and expose sets of individuals to different dispersal regimes one might find different SDMs between the groups. Another possible system to test our "sex ratio flexibility" hypothesis is *C. elegans*. Janzen & Phillips (2006) report of two *C. elegans* strains that exhibit a TSD like sex determining system and also state that sex ratio experiments with these strains might be of interest. With this system it should be possible to impose a LCM and/or LRC scenario with differing temperature fluctuations and monitor the response of TSD and GSD to different scenarios. The advantage of *C. elegans* is that it will be easy to observe many generations.

## Acknowledgements

We thank the Robert Bosch Stiftung for a scholarship to B. F.

# CHAPTER 3

# Divergent evolution of sex determination in the snow skink *N. ocellatus*: a theoretical analysis

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*In preparation for The American Naturalist*

## Abstract

In most organisms the sex of an individual is determined by its genes. In other organisms, many reptiles for example, sex is determined by environmental factors like temperature. Transitions between the two systems seem to be common, however the evolutionary significance is still unclear. On theoretical grounds several hypotheses have been proposed to explain the evolutionary significance of temperature dependent sex determination, however empirical support is scarce. Here in this study we make use of individual-based simulations to investigate several of these hypotheses by implementing empirical life history as well as environmental data of the snow skink, *N. ocellatus*. The snow skink is especially suitable for this kind of study since genetic as well as temperature dependent sex determination exist within this species in different populations.

## Introduction

In most species an individual's sex is uniquely determined by its genetic makeup (genotypic sex determination; GSD), but in some species an individual's sex is determined by properties of its environment (environmental sex determination; ESD) (Bull, 1983; Valenzuela *et al.,* 2003). The most common form of ESD is temperature-dependent sex determination (TSD), where an individual's sex is determined by the temperature experienced during a specific period of the embryonic development (Valenzuela & Lance, 2004). TSD is particularly common in reptiles (Janzen & Paukstis, 1991a), but also occurs in some fish (Conover, 1984) and invertebrates (Korpelainen, 1990). Phylogenetic analyses suggest that in reptiles multiple evolutionary transitions from GSD to TSD and vice versa have occurred (Janzen & Krenz, 2004), but the adaptive significance – if any – of these transitions remains mostly obscure.

Theoretically, whether ESD or GSD is favored by selection, depends on the balance of two opposing forces (Bull, 1983; Shine, 1999; Uller *et al.,* 2007). In favor of ESD is the flexibility it affords in adjusting the sex ratio. There are several hypotheses that specify exactly how a biased sex ratio translates into a benefit (Shine, 1999), but by far the most influential is the Charnov-Bull model (Charnov & Bull, 1977). This model is based on Trivers and Willard's (1973) idea that offspring fitness may co-vary in a sex-specific way with environmental conditions, such as maternal condition or temperature. Thus, ESD is a specific mechanism that facilitates adaptive matching between offspring sex and the environment. In contrast, GSD typically leads to approximately even sex ratio regardless of the environment. This consistency in even sex ratios is precisely the benefit of GSD under conditions that select against ESD, i.e., large between-year variation in environmental conditions that may cause maladaptive large sex ratio fluctuations and even (local) extinctions (Bulmer & Bull, 1982; Bull & Bulmer, 1989; Leimar *et al.,* 2004; Schwanz & Proulx, 2008). Which of the two forces dominates likely depends on the magnitude of the sex-specific fitness differences, the magnitude of environmental fluctuations, life history details and the details of the mechanism of sex determination.

It has proven quite hard to test these ideas, for a number of reasons. First, in species with TSD it's difficult to generate individuals of the "wrong" sex under

temperatures that usually generate the opposite sex. However, using hormonal manipulation to decouple sex and incubation temperature and the appropriate control treatments, Warner and **S**hine (2008) showed that males (and to a lesser extent females) from their "normal" temperature had higher reproductive success than individuals from incubation temperatures that normally overproduced the other sex. However, this study was carried out in captive colonies and whether the same results will hold in natural populations is unknown. Furthermore, the fitness difference was not related to the proposed selection behind evolution of TSD – matching offspring sex to hatching date (Warner & Shine, 2005; Warner *et al.,* 2009) – and could simply be the result of secondary acquired sex-specific thermal optima for developmental processes. An alternative approach would be to address differences in selection pressures in species or populations that differ in sex determining systems. Several species of lizard and fish show intraspecific variation in sex determining mechanisms. Perhaps the best known is the Atlantic silverside, a fish with a latitudinal cline from GSD to ESD.

The viviparous snow skink, *Niveoscincus ocellatus*, is a species which appears to have both GSD and TSD (Wapstra *et al.,* 1999; Wapstra & O'Reilly, 2001; Wapstra & Swain, 2001). Females from a coastal population have offspring sex ratios that are temperature-dependent, while females from a mountain population produce an insensitive even sex ratio (Wapstra *et al.,* 2004; Wapstra

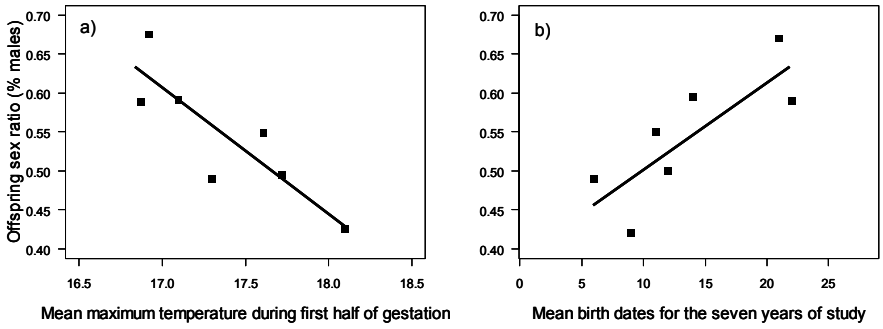


**Figure 3.1:** Offspring sex ratio in the lowland population dependent on a) the mean maximum temperature during the first half of gestation; b) mean birth dates (January) for the seven years of study. (Adapted from Wapstra *et al.,* 2008.)

*et al.,* 2009; Wapstra *et al.,* unpublished data) (Figure 3.1). Details of the underlying sex determining mechanisms are unknown. In the coastal population, thermal sensitivity results in seasonal sex ratio trends that might be adaptive because females appear to profit more from being born early than males as a result of sex-specific fitness consequences of body size (Wapstra *et al.,* 2004). In the mountain population this advantage appears to be weaker, and there are stronger between year fluctuations in temperature that might select against TSD. Clearly models are needed to investigate whether the advantages outweigh the disadvantages or vice versa.

Here we use an individual-based model approach to address whether models tailored to the life history and climatological differences between the two populations generate differences in sex determination that resemble those found in natural populations. Further, we use different sex determining systems in order to assess to what extent population divergence depends on ancestral sex determination. Our results suggest that observed differences are consistent with an adaptive scenario, in particular as we find temperature dependent sex ratio responses in the lowlands but not in the highlands. Interestingly these results are robust independent of the underlying sex determining system.

## The Model

### Temperature variation

Temperature data were collected at the lowland and highland field sites on a daily basis from January 1988 – January 2008. These data were used to calculate the long term mean yearly temperature ($T_M$), between year temperature variation ($\sigma_B$) as well as the within year temperature variation ($\sigma_W$) (Table 3.1). Temperature sensitivity of sex determination occurs throughout the first half of gestation (Wapstra *et al.,* unpubl data), which corresponds well with the gonadal differentiation in embryos occurs in the first half of gestation (Neaves *et al.,* 2006). We therefore considered a three months range, October-December for the lowlands and November-January for the highlands, to calculate $\sigma_B$. To estimate $\sigma_W$, we calculated the variation between average temperatures of 10 day intervals within the three months range for both sites separately. To determine the reliability of this estimate, we also determined the

**Table 3.1:** Overview of standard parameters used in the model.

| Parameters | Lowlands | Highlands |
|---|---|---|
| Longterm mean yearly temperature ($T_M$) | 17.41 | 15.43 |
| Between year temperature variation ($\sigma_B$) | 0.59 | 1.45 |
| Within year temperature variation ($\sigma_W$) | 3.22 | 4.27 |
| Maximum lifespan | 8 | 11 |
| Minimum age of maturation | 2 | 3 |
| Number of offspring per female | 2-4 | 2-6 |
| Number of offspring for females at minimum age maturation | 1-2 | 2-3 |
| Survival probability of early born offspring ($S_E$) | 0.54 | 0.48 |
| Survival probability of late born offspring ($S_L$) | 0.22 | 0.35 |
| Survival probability adults ($S_A$) | 0.55 | 0.45 |
| Probability of early born female offspring to breed with minimum age of maturation ($P_E$) | 0.5 | 0.9 |
| Probability of late born female offspring to breed the following season ($P_L$) | 0.1 | 0.7 |
| Probability of being assigned to early/late breeders ($P_B$) | 0.8 | 0.8 |
| Threshold (gene-product of one Z chromosome = 1) | 1.1 | 1.1 |
| Curve mean ($\mu_C$) | 17.41 | 15.43 |
| Standard deviation of the curve ($\sigma_C$) | 2.5 | 2.5 |
| Mutation probability of threshold, curve mean, and curve SD | 0.01 | 0.01 |
| Mutation step size for threshold, curve mean, and curve SD | 0.01 | 0.01 |

temperature variation for intervals between 5-20 days which resulted in a similar variation and were thus not included in the analysis.

In the model "yearly temperature" ($T_Y$) is calculated each time-step by drawing a value from a normal distribution with mean $T_M$ and with standard deviation $\sigma_B$. $T_Y$ is further used to calculate the female specific temperature ($T_F$) which resembles within year temperature fluctuations by drawing a value from a normal distribution with mean $T_Y$ and with standard deviation $\sigma_W$.

## Underlying sex determining systems

As the sex determining system in *N. ocellatus* is unknown we implement several different system in order to test the robustness of our results. First, we used a gene dosage system based on the system described for the dragon lizard, *Pogona vitticeps* (Quinn *et al.,* 2007). In this system intermediate temperatures lead to a 1:1 sex ratio, warm temperatures lead to female bias while cold temperatures lead to unviable offspring. The sum of gene-product produced by two Z chromosomes reaches the threshold level leading to male development (Figure 3.2). In females, which are ZW and thus only possess one Z chromosome but also under extreme temperature conditions the amount of gene-product is too small to reach the threshold and thus female development is induced.

In our model each individual has four loci which are necessary for sex determination. (1) The ZW-locus determines whether an individual contains ZW or ZZ chromosomes. (2) The curveMean-locus determines the mean of the curve ($\mu_C$) which is the point along the temperature axis with maximum amount of gene product produced. $\mu_C$ equals the longterm mean yearly temperature ($T_M$) at the start of the simulations (Figure 3.1). (3) The curveSD-locus determines the curve standard deviation ($\sigma_C$) with $\mu_C$ as mean. (4) The threshold-locus determines the threshold ($\theta_C$), thus the amount of gene-product that has to be produced as minimum to result in male development. To allow for new genetic variation, each curve trait locus has a probability of 0.01 to mutate and the mutation step size is drawn from a normal distribution with mean 0 and standard deviation of 0.1.

An offspring will become male if the amount of gene product produced reaches $\theta_C$, and otherwise become female. The amount of gene product depends on the number of Z chromosomes, the curve traits of an offspring, determined by the curve trait loci, as well as on the developmental temperature, thus $T_F$ (Figure 3.2).

In addition to the above mentioned system we also implement an XX/XY system in a similar manner. In the first scenario X is producing the gene product, where only two X chromosomes can produce enough product to reach the threshold and lead to female development, but dependent on temperature. In the second scenario Y was producing the gene product, thus the presence or

absence of Y determined whether an individual would develop as male or female, with the amount of gene product dependent on temperature.

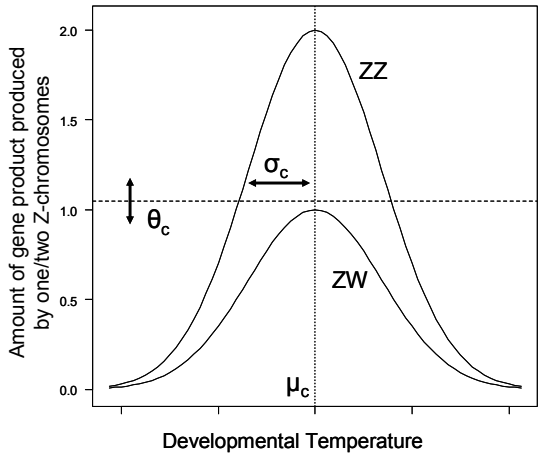


**Figure 3.2**: Amount of gene product produced as a function of developmental temperature. $\mu_C$ = curve mean; $\sigma_C$ = curve standard deviation; $\theta_C$ = threshold that has to be reached for male development.

## Maturation and clutch size determination

To facilitate model building, we divided the breeding and birth season into two categories, early and late. To determine whether a female and later her offspring are classified as early/late breeding or born respectively, $T_F$ is compared to $T_M$. If $T_F$ is higher than $T_M$, the female and her offspring are assigned to early breeders/born with a certain probability $P_B$, and otherwise to late breeders/born and vice versa. Offspring that are born early in the season have more time to develop and grow till winter compared to individuals that were born late in the season. Therefore early born females have a higher chance to breed when reaching minimum age of maturation than the late born females which will give early born females a fitness advantage compared to late born females. Female clutch sizes varied according to estimates from natural populations (Table 3.2). Females with minimum age of maturation generally have smaller clutches compared to older females. For males the probability to mature early, independent on whether they were born early or late in the season, is 90% in both populations, thus males fitness is not affected differentially by being born early or late in the season.

**Table 3.2:** Probabilities of clutch sizes for young and adult females in the two populations.

| Age of female | Lowland population | | Highland population | |
|---|---|---|---|---|
| | clutch size | probability | clutch size | probability |
| Minimum age maturity | 1 | 0.7 | 2 | 0.8 |
| | 2 | 0.3 | 3 | 0.2 |
| Adult | 2 | 0.5 | 2 | 0.1 |
| | 3 | 0.3 | 3 | 0.2 |
| | 4 | 0.2 | 4 | 0.4 |
| | | | 5 | 0.2 |
| | | | 6 | 0.1 |

## Life cycle

Each simulation started with 1000 male (ZZ) and 1000 female (ZW) individuals, with equal curve trait values and the age set to minimum age of maturation. Each simulation was run for 50 000 time steps (= generations), for lowland and highland population separately, i.e. we did not consider gene-flow between the populations. Since all our parameters are of course estimates we performed a sensitivity analysis to get insights in the dependency of our results on the chosen estimates. For the parameters of interest we therefore ran simulations with the according standard value +/- 25% and +/- 50% (Table 3.1).

Females mate with a randomly assigned male and produce offspring. For each of the four loci the offspring is randomly assigned one allele of the mother and one of the father, the alleles can mutate. The sex of the offspring is determined dependent on the number of Z chromosomes, its curve traits, the threshold and the $T_F$. Early born offspring, late born offspring and adults have a certain probability $S_E$, $S_L$, $S_A$ to survive to the next generation respectively until they reach maximum lifespan and will die. Offspring that have reached minimum age of maturation, have a certain probability ($P_{E/L}$) to be moved to the reproductives pool, older offspring are directly moved to the reproductives pool. From there, individuals are randomly assigned to the empty breeding spots, up to the maximum number of 1000 males and females respectively. At the end of each time step all individuals in the population age by one year and the cycle is re-started.

Throughout the simulations allele values and frequencies for all loci are monitored and the correlation between yearly temperature and sex ratio of the offspring is calculated.

### Statistical analyses

For each of the 20 replica-runs with standard parameter settings we calculated the correlation coefficient between sex ratio and temperature for the early and late born offspring of the last 300 generations. A students t-test was performed to test whether the strength of the correlation between the early and late born offspring for both the lowland and the highland population differed significantly. To test whether sex ratios were dependent on the population, date of birth, or a combination of both we performed an ANOVA. Both statistical analyses were performed in R (R Development Core Team).

## Results

All three implemented sex determination scenarios resulted principally in the same outcome. The differences between the lowland and highland populations were little less pronounced under the two XY scenarios compared to the ZW scenario. Since the differences in outcome between the systems were very small we focus mainly on the results of the ZW system.

As can be seen clearly in Figure 3.3, the simulations with the standard parameters (Table 3.1) result in different sex ratio responses comparing the lowland and the highland population. In the lowland population the sex ratio of early born offspring is female biased whereas the sex ratio of late born offspring is male biased (Figure 3.3). In the highland population an even sex ratio is observed in the early as well as late born offspring. When plotting sex ratios against temperature a clear negative correlation with more females being produced at warm temperatures is observed for the lowland population, whereas no correlation is found for the highlands. The correlation between sex ratio and temperature between the 20 replica runs is significantly higher in the lowland compared to the highland population (t-test: $df = 36$; $t = 3.29$; $p = 0.002$). An Anova revealed that sex ratio variation is significantly dependent on population, date of birth, as well as the combination of population and birth date (Table 3.3).

**Table 3.3:** Results of an Anova testing the effect of birth date and population on sex ratio. Both factors as well as the interaction of population and birth data have a significant effect on the sex ratio.

| | DF | Sum Sq | Mean Sq | F | P |
|---|---|---|---|---|---|
| Population | 1 | 0.020 | 0.020 | 7.76 | 0.007 |
| Birth date | 1 | 0.196 | 0.196 | 75.14 | <0.001 |
| Population x Birth date | 1 | 0.088 | 0.088 | 33.74 | <0.001 |
| Residual | 76 | 0.199 | 0.003 | | |

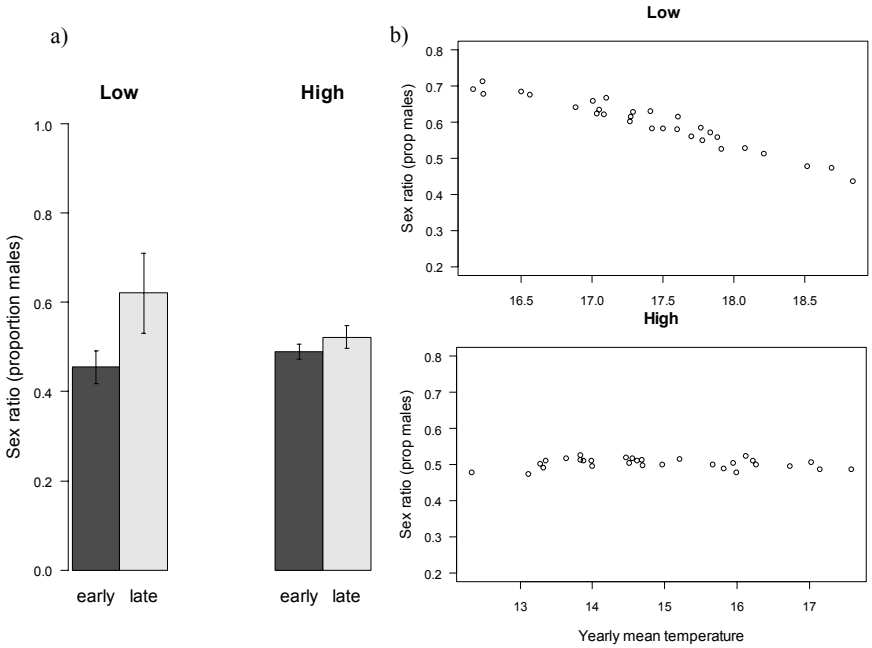


**Figure 3.3:** Comparison of sex ratios between the highland and lowland populations. a) Mean sex ratios of the lowland and highland population as well as between early and late born offspring of 20 replicate runs. Error bars indicate the standard deviations between the runs. Sex ratios are significantly dependent on the population, birth date as well as the interaction of both (Table 3); b) Sex ratio – temperature correlation over 30 generations in the lowland and highland population depicted for one example run. The correlation between sex ratio and temperature of the last 30 generations, calculated over 20 replica runs, is significantly higher in the lowland population compared to the highland population (t-test: df=36; t=3.29; p=0.002).

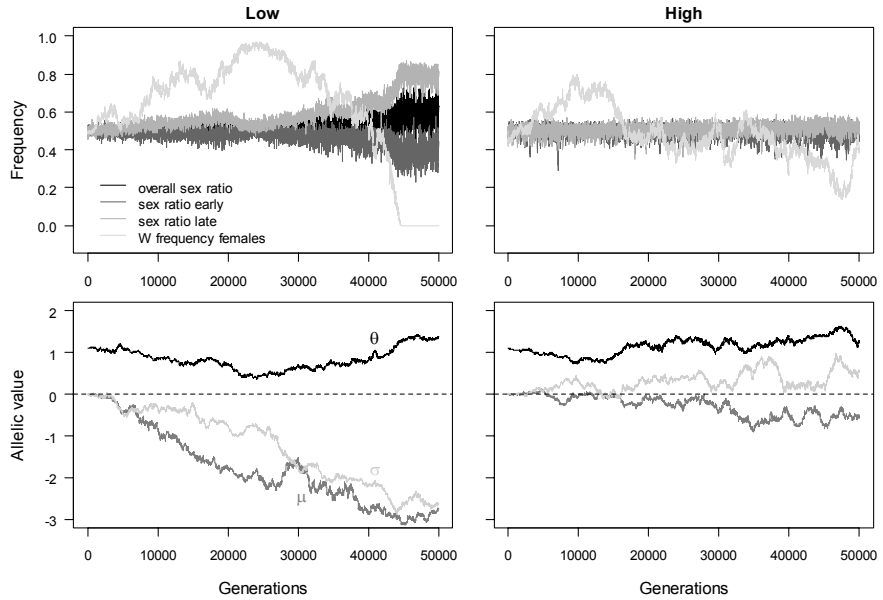


**Figure 3.4:** Typical simulation runs for the canonical lowland and highland parameter sets. Upper panels depict dynamics of sex ratios and frequencies of the W chromosome. Lower panels show dynamics of average threshold values ($\theta$) as well as the deviation from the mean at the curve mean ($\mu_C$) and curve SD ($\sigma_C$) locus. For the lowland population the $\sigma_C$ and $\mu_C$ become smaller, which leads to shift of the curve to the left, resulting in differential sex ratios for early and late born offspring, determined by temperature. In the highland population allele values fluctuate, however a balanced sex ratio independent of the birth timing, is given throughout the simulation.

When looking at the dynamics of the simulations (example of one run with standard parameters Figure 3.4) one can also see clear differences between the lowland and the highland population. In the lowland population the curve mean ($\mu_C$) and the standard deviation of the curve ($\sigma_C$) decrease constantly (Figure 3.4). The more the curve shifts to the left, the less females are produced at low temperatures until at some point mostly males are produced at low and mostly females at high temperatures. As the effect of temperature on sex determination becomes stronger the role of W becomes weaker and it goes extinct. The reason why sex ratios are not biased up to 100% male and 100% female at extreme temperatures is that additional variation to the yearly mean temperature is added by introducing within year temperature variation. This translates into between

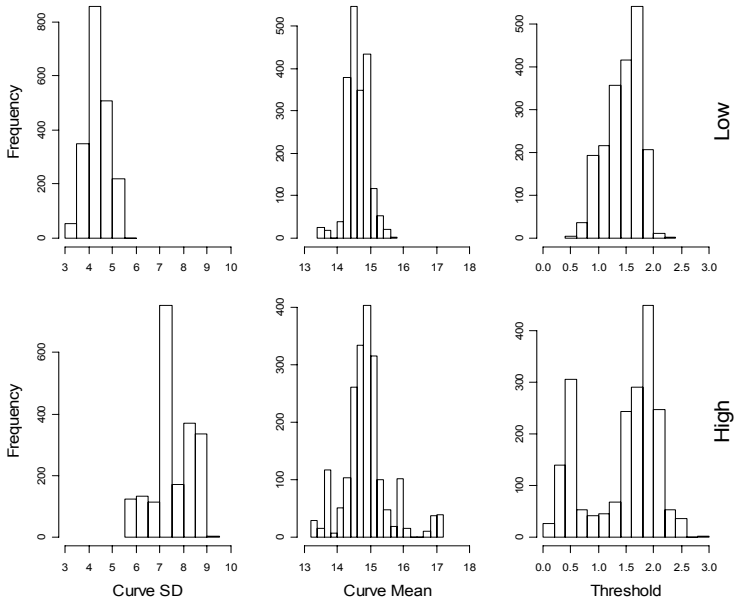


**Figure 3.5:** Allele distribution in the last generation at the curve SD, curve mean and threshold locus. Examples result from one simulation with standard parameters for the low- and highland populations.

female temperature variation, which means that in years with cold temperatures that would normally result in male offspring only, some females still produce female offspring and vice versa.

In the highland population several allele values fluctuate over time, however the overall sex ratio as well as sex ratio in the early and late born offspring stays balanced (Figure 3.4). The allele distribution in the lowland and highland population of the last adult generation are depicted in Figure 3.5. Here again, as mentioned above, it can be seen that in the lowland population $\sigma_C$ and $\mu_C$ have become smaller and shifted from a previous mean of 7.0 and 17.41 to approximately 4.2 and 14.6 respectively. The threshold on the other hand increased from 1.1 to approximately 1.5. For the highland population the mean at the three loci changed only slightly over the 50 000 generations. However, the allele distributions at the $\theta_C$ locus seems to follow a bimodal distribution which might indicate that the alleles are branching, and the population evolves towards allelic minima and maxima. The negative effect on the evolution of

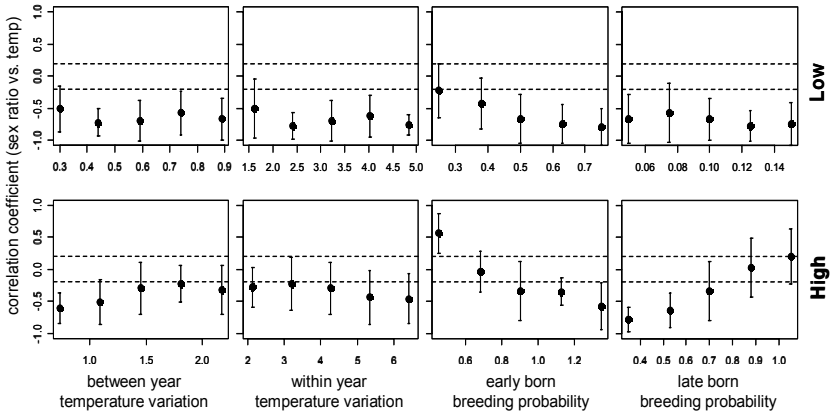


**Figure 3.6:** Results of the sensitivity analysis to test the influence of various parameters on the probability of TSD to evolve and the strength of the correlation between sex ratio and temperature. The mean correlation coefficient of the last 300 generations of 20 replicate runs was used to calculate the overall mean and standard deviation. The intermediate data point represents the results for the standard parameter value with the others being +/- 25%, +/- 50% of the standard value. The dotted line indicates the significant level of the correlation coefficient for N = 100.

TSD of between year temperature variation can be seen in the results of the sensitivity analysis for the highland population (Figure 3.6). We expect the same to hold true for the lowlands however the investigated temperature range is smaller in the lowlands compared to the highlands. A certain amount of within year temperature variation promotes TSD in the highlands, leading to a higher sex ratio variation between females within the population, thus preventing extremely biased sex ratios. The fitness difference in the probability of female offspring breeding in advance influences the probability of TSD evolution and also the strength of the sex ratio – temperature correlation (Figure 3.6). To test whether indeed the difference in breeding probability in early and late born offspring is the driving force behind TSD evolution simulations were run with similar parameter settings as before, except early and late born offspring had the same breeding probability and the same number of offspring. None of the parameter combinations resulted in a significant correlation between sex ratio and temperature, thus strongly suggesting that the fitness advantage of early born females leads to the evolution of TSD (data not shown). To test whether GSD would evolve from a TSD system, additional simulations

were run starting with a TSD system (curve mean moved to the lower limit of the mean within year temperature variation). For all parameter combination these runs resulted in very high correlation coefficients in both populations with lower standard deviation compared to the results with the standard mean (data not shown).

## Discussion

As outlined above the evolution of GSD or TSD seems to depend on the interaction and strength of various factors, like sex-specific fitness differences, environmental fluctuations, life history details and possibly also the underlying sex determining mechanism. Warner & Shine (2008) recently investigated the effect of sex-specific fitness differences in the Jacky dragon, *Amphibolurus muricatus*. By hormonally manipulating the sex of the offspring it was shown that individuals from their "normal" temperature had a higher reproductive success than individuals incubated at the opposite temperature. However, in that study it can not be ruled out that e.g. secondarily acquired sex-specific thermal optima play a role instead of the formerly proposed matching offspring sex to hatching data (Warner & Shine, 2005). With the snow skink, *N. ocellatus*, this problem can be circumvented as both TSD and GSD exist within one species. By making use of individual-based simulations we investigated the effect of sex-specific fitness differences, environmental fluctuations and life history details.

Our results show that by implementing empirical information on life history data and data on environmental conditions, we were able to simulate the evolution of the two different sex ratio response patterns observed in the two snow skink populations. Our results also show that the outcome with TSD in the lowland and GSD in the highland population, is robust independent of the underlying sex determining system. In addition we show that the driving force leading to the evolution of TSD in the lowland population is temperature dependent sex specific fitness differences. In the lowland population female offspring that are born early in the population have a higher chance of breeding the following season and have a higher survival probability compared to late born offspring (Table 3.1), which under standard settings leads to the evolution of TSD, a correlation between sex ratio and temperature respectively. Simulations in which early and late born offspring had the same chance of

breeding the following season as well as the same survival probability, did not lead to a correlation between sex ratio and temperature whatsoever (data not shown). The potential of differential fitness to promote the evolution of TSD is additionally shown by the sensitivity analysis (Figure 3.6), where in both populations higher differences between early and late born females in breeding probability, lead to higher sex ratio-temperature correlation, thus higher probability of TSD evolution. In the highland population early born female offspring also have a higher chance of breading sooner than late born female offspring and also a higher chance of survival, however the differences are less pronounced and also the temperature sex ratio correlations are lower compared to the lowland population. The sensitivity analysis also shows that these subtle differences might already be enough to select for TSD, however strong between year temperature fluctuations in the highlands lead to selection for GSD (Figure 3.6). This brings us to the next point. Besides showing the potential of differential fitness to promote the evolution of TSD, the results of the sensitivity analyses also seem to match previous hypotheses that strong environmental fluctuations leading to strong sex ratio fluctuations select for GSD (Bulmer & Bull, 1982; Leimar *et al.,* 2004). Within year temperature variation on the other hand, which can also be considered as temperature variation between females, has only a marginal effect on the strength of the sex ratio-temperature correlation. However, when looking at the average yearly sex ratio within year temperature variation plays an important role, and might explain the absence of 100% female and male sex ratios, at least for the simulation results. This is because within year temperature fluctuations add additional variation to the temperature range. Therefore in years with extreme mean temperatures, which by itself would lead to the development of single sexed offspring, some females experience less extreme temperatures and produce offspring of the opposite sex, preventing single sexed average sex ratios. Whether between female temperature variations prevent the occurrence of extreme average sex ratios in nature is unknown, but could be investigated by measuring body temperatures of single females during the gestation period, determining the temperature variation and also the sex ratio of the offspring.

A very interesting finding of our results is the bimodal allele distribution at the threshold locus in the highland population, which suggests that the alleles at this locus are branching (Figure 3.5). The implications of extreme alleles at the

threshold locus are that in this population a mixture of two GSD systems is evolving. For heterozygous individuals, thus with a low and high threshold allele, sex determination would function as depicted in Figure 3.2. In ZW individuals only one Z produces gene product, which is not enough to reach the threshold and therefore the individual becomes female. In ZZ individuals intermediate temperatures result in enough gene produce of the two Z chromosomes that the individual will become male. At extreme temperatures however, not enough gene product is produced and even ZZ individuals become females. Homozygous individuals for the low threshold alleles will become exclusively males as the gene product of one Z chromosome is enough to reach the threshold. On the other hand individuals homozygous for the high threshold alleles will become female as even two ZZ chromosomes will not be able to produce enough gene product to reach the threshold. Overall the mixture of these two systems still results in an even sex ratio independent of temperature. In another, more general model of ours, we recently investigated the probability of transitions from GSD to TSD and also encountered several parameter combinations in which mixed systems, such as this or mixtures of TSD and GSD evolved (Feldmeyer *et al.,* in prep). To detect such a system in nature will be very challenging as the second mechanism (the branched threshold locus in our example) is hard to detect and easily overlooked compared to differentiated sex chromosomes. In species without differentiated sex chromosomes one might be able to find sex determination linked to two loci.

In summary, our results show that it seems possible to model the occurrence of two sex determining systems in the snow skink by implementing empirically obtained life history as well as temperature data. Furthermore our results match the differential fitness hypothesis proposed by Charnov & Bull (1977) as well as hypotheses that temperature fluctuations negatively affect the evolution of TSD (Bulmer & Bull, 1982; Leimar *et al.,* 2004). Our approach might prove useful in other species with uncertain temperature effect on sex determination of which life history parameters as well as environmental conditions are studied, to investigate the probabilities of certain sex determining systems to evolve and possibly be presence in the focal species. We additionally could show that it is of minor relevance which underlying sex determining system is chosen to start the simulations, as our results are robust independent of the starting GSD system.

## Acknowledgements

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# Part II: Empirical Approach

# 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*

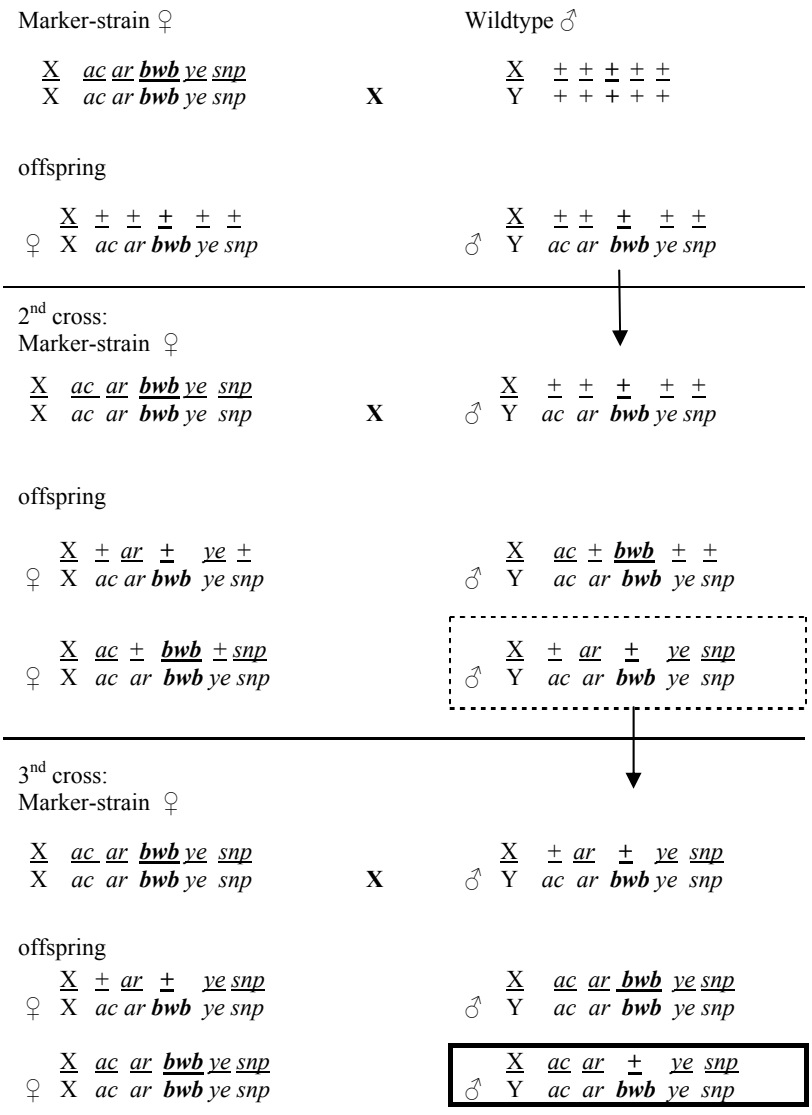
offspring

♀ 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).

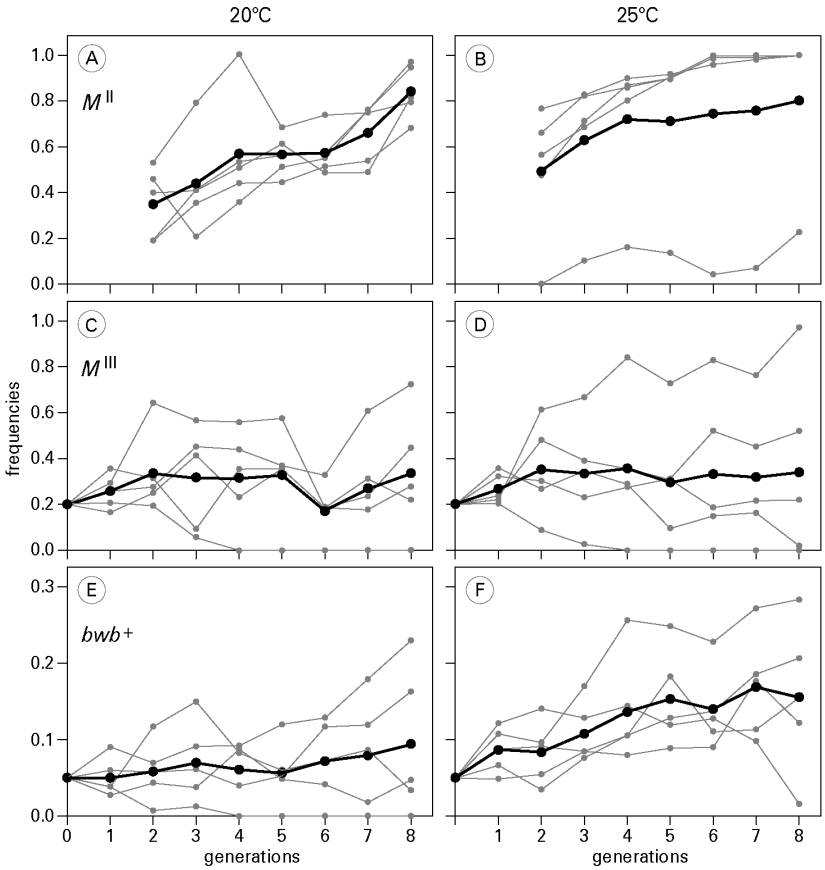



**Figure 4.1**: Frequencies of males with the $M^{II}$ factor (A and B), males with the $M^{III}$ factor (C and D) and the frequency of *bwb+* allele in control (E and F) at two different temperatures (20ºC and 25ºC) during the invasion experiment. Grey lines represent five different replicates and the 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 | $\chi^2$ | 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

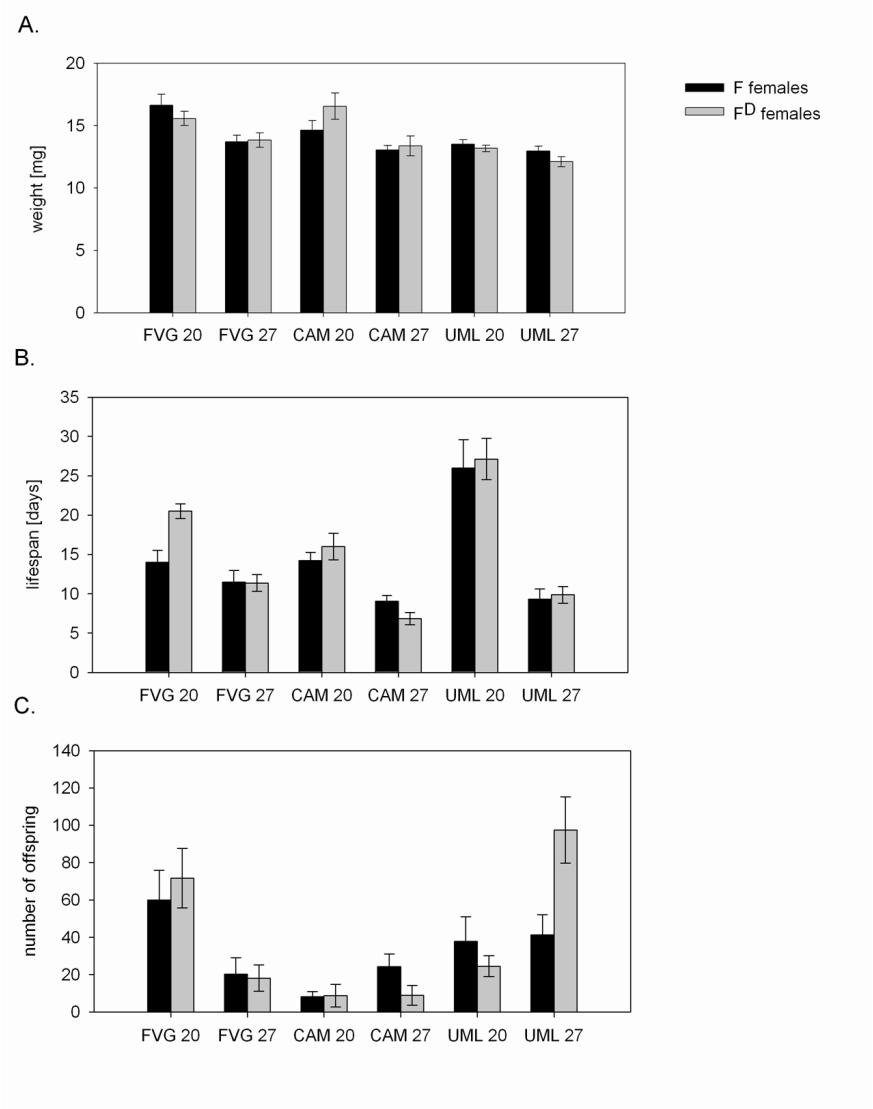



**Figure 4.2:** Average weight (A), lifespan (B) and lifetime offspring production (C) of females with F and FD from different populations at 20 and 27°C (as listed after the strain name). Error bars represent standard errors.

## 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.

## Acknowledgements

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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 $\chi^2$ - 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. $\chi^2$- 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)** | | | $\chi^2$ | **p** | **Temperature (°C)** | | | $\chi^2$ | **p** | **Cross** | **Sex** | **Temperature (°C)** | | | $\chi^2$ | **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.

# CHAPTER 6

# Are autosomal sex determining factors of the housefly (*Musca domestica*) spreading north?

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## Abstract

Multiple sex determining factors have been found in natural populations of the housefly, *Musca domestica*. Their distribution seems to follow a geographical cline. 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. Autosomal *M* factors were first observed some 50 years ago. It has been hypothesised that following their initial appearance, they are spreading northwards, replacing the standard XY system, but this has never been systematically investigated. To scrutinize this hypothesis, we here compare the current distribution of autosomal *M* factors in continental Europe, on a transect running from Germany to southern Italy, with the distribution reported 25 years ago. Additionally, we analyzed the frequencies of the $F^D$ factor, which has not been done before for European populations. In contrast to earlier predictions, we do not find a clear change in the distribution of sex determining factors: as 25 years ago, only the standard XY system is present in the north, while autosomal *M* factors and the $F^D$ factor are prevalent in Italy. We discuss possible causes for this apparently stable polymorphism.

## Introduction

Sex determination in the housefly, *Musca domestica*, is more variable than in most other species, which usually exhibit just a single sex determining mechanism (Bull, 1983; Dübendorfer *et al.*, 2002). Polymorphism for sex determining factors has been found in many natural populations of the housefly (Franco *et al.*, 1982; Denholm *et al.*, 1985; Tomita & Wada, 1989b; Feldmeyer *et al.*, 2008; Table 6.1). In "standard" strains, sex is determined by a male determining factor, *M*, which is located on the Y chromosome; therefore males are XY and females are XX. During development, the *M* factor blocks the female determining factor *F* located on autosome IV, the activity of which is necessary for female development. In many populations, *M* is located on one of the autosomes or even on the X chromosome (Denholm *et al.*, 1983). In such populations, usually a dominant constitutive mutation of *F* ($F^D$) is also present, which triggers female development even in the presence of several *M* factors in the same individual (see McDonald *et al.*, 1978; Franco *et al.*, 1982; Dübendorfer *et al.*, 2002; Table 6.1).

**Table 6.1:** Relation between genotype and gender in the housefly. The female determining factors ($F/F^D$) are located on autosome IV. The male determining factors (*M*) can be located on any chromosome. A "+" indicates the wild type state (no *M*) and a "∗" indicates that an *M* or + allele on this locus will not influence the sex.

| **Autosomes** | | **Sex chromosomes** | |
|---|---|---|---|
| IV | I-V | XX | XY |
| *F*/*F* | +/+ | ♀ | ♂ |
| *F*/*F* | *M*/∗ | ♂ | ♂ |
| $F/F^D$ | ∗/∗ | ♀ | ♀ |

The XY system is probably ancestral in the housefly, since it also most common in closely related species (Boyes *et al.*, 1964) and the first reports on autosomal sex determining (SD) factors appeared only around 1960 (reviewed by Franco *et al.*, 1982). Since then, the geographical distribution of different SD factors has been studied on most of the continents and appears to follow geographical clines. In general, the Y chromosome is more common at higher latitudes and altitudes and its frequency gradually decreases with decreasing latitude and altitude leading to populations with only autosomal sex determining factors

(autosomal *M* and $F^D$) closer to the equator and at low altitudes (Franco *et al.*, 1982; Tomita & Wada, 1989b; Çakir & Kence, 1996; Hamm *et al.*, 2005; Feldmeyer *et al*., 2008). It is not clear what forces are responsible for the distribution of different SD factors, but temperature seems to be an important factor (Feldmeyer *et al.*, 2008).

There is some evidence that autosomal sex-determining factors have spread in some populations replacing the standard XY system (Franco *et al.*, 1982; Tomita & Wada, 1989a, b). It has been hypothesized (Franco *et al.*, 1982; Denholm *et al.*, 1985; Tomita & Wada, 1989a, b; Çakir & Kence, 1996) that the observed distributions are a transient state. In particular, Franco and colleagues (1982) suggested that autosomal *M* factors are spreading north in Europe, but their hypothesis was based only on the change in frequency of the Y chromosome in a few populations before 1980. No systematic or recent studies have been done on the dynamics of different SD factors in natural populations of the housefly. The last study in continental Europe dates from 25 years ago (Franco *et al.*, 1982) in which cytological data were used to show a clear latitudinal cline with the standard XY system exclusively present in the north of Europe (Iceland, Denmark, the Netherlands, Germany and Switzerland) and entirely autosomal populations (lacking the Y chromosome) in southern Italy, at altitudes below 100m. In northern Italy mixed populations have been found with the frequency of the Y chromosome increasing with higher altitudes and latitudes.

The aim of this study was to investigate whether the distribution of SD factors in the housefly has changed in Europe over the last 25 years. Therefore, we sampled a number of European populations on a north-south transect from Germany to southern Italy, and compared the frequency of males that carry the Y chromosome and autosomal *M* factors with the data published by Franco and colleagues (1982). Additionally, we analyzed the frequencies of the $F^D$ factor and we publish the frequencies of *M* factors located on different chromosomes, which has not been done before for European housefly populations.

## Material and Methods

### Collection and rearing of the flies

We sampled populations along a north-south transect from north Germany to south Italy in July 2006 (see Figure 6.1 and Table 6.2 for details on the sampling locations). Most of the sampling sites were chosen to be close to the ones studied by Franco *et al*. (1982), as far as we could judge from the limited information. For Germany and Switzerland, they only gave the name of a state (Baden-Württemberg) or a canton (Mittelland) and our sampling sites lie within these areas. For Italy, Franco and colleagues published a map indicating sampling sites together with information on altitudes, but precise geographical coordinates were lacking. We judged their locations visually and used altitudes

**Table 6.2**: Geographical coordinates, altitudes (in meters above sea level) and average yearly temperatures of the sampling sites.

| Population code | Latitude N | Longitude E | Altitude m | Temperature °C |
|---|---|---|---|---|
| GE1 | 51° 19,4’ | 7° 10,9’ | 220 | 9.1 |
| GE2 | 48° 29,5’ | 9° 2,0’ | 347 | 9.0 |
| SW | 47° 17,8’ | 7° 51,8’ | 410 | 9.4 |
| IT1 | 45° 46,6’ | 8° 2,5’ | 794 | 8.8 |
| IT2 | 45° 42,3’ | 8° 14,1’ | 470 | 10.1 |
| IT3 | 45° 35,4’ | 7° 8,0’ | 1700 | 4.2 |
| IT4 | 45° 17,8’ | 8° 33,1’ | 121 | 12.3 |
| IT5 | 43° 29,2’ | 11° 33,1’ | 313 | 13.2 |
| IT6 | 43° 11,0’ | 10° 31,7’ | 18 | 15.4 |
| IT7 | 42° 32,6’ | 13° 49,3’ | 367 | 13.3 |
| IT8 | 40° 45,7’ | 16° 14,3’ | 562 | 13.3 |
| IT9 | 40° 32,5’ | 15° 6,4’ | 63 | 16.1 |
| IT10 | 39° 21,4’ | 16° 26,5’ | 1194 | 10.4 |
| IT11 | 38° 48,0’ | 16° 20,3’ | 690 | 13.9 |
| IT12 | 38° 40,6’ | 15° 54,6’ | 49 | 17.7 |

Sampling sites are ordered according to their latitude.

Letters in the code indicate the country of origin: GE – Germany, SW – Switzerland, IT – Italy.

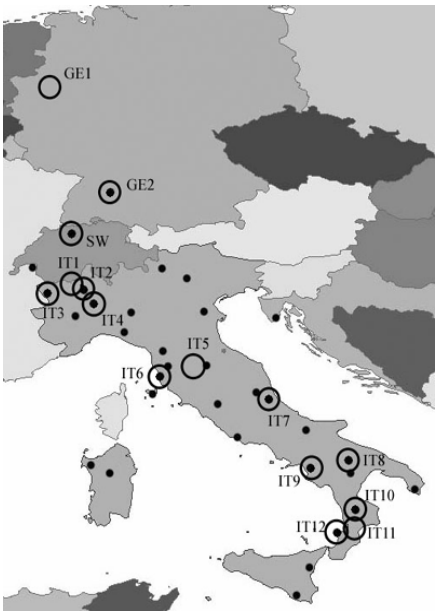


**Figure 6.1:** Sampling locations in the study of Franco and colleagues (1982; dots) and in the present study (circles). Locations from the present study are labelled with population codes as in Table 6.2.

within 110m, but usually within a 50m range. The exception is population IT5 where the altitude given by Franco *et al.* (1982) does not match the area indicated by them, so to match the altitude we sampled 50km west of their indicated location. Ultimately, our sampling sites were distributed approximately homogeneously along a north-south transect, with some areas having sampling sites at different altitudes.

For each location, we obtained data on average monthly minimum and maximum temperatures from WORLDCLIM (www.worldclim.org, see Hijmans *et al.*, 2005) which provides global estimates at a resolution of one square kilometre. We estimated average yearly temperatures as the mid-point between minimum and maximum temperatures (Table 6.2). Since all these measures of temperature are highly correlated ($p<0.0001$, Pearson's product-moment correlation test), we used only the average yearly temperature in our statistical analysis (see below).

Flies were sampled at farms and horse stables. At each location we caught approximately 50 adult males and females (except for IT3, where only 10 females were found). The flies were caught with sweeping nets, placed in plastic containers and provided with water and milk powder as food. They were also provided with egg laying medium (according to Hilfiker-Kleiner *et al.*, 1994) on which females laid eggs within a few days. Larvae were transferred to bigger containers after a few days and fed *ad libitum* on the same medium. Flies from all the locations (or their offspring) were successfully transported to the laboratory and populations were established and maintained in cages at population size of approximately 500 individuals.

## Analysis of the sex determining factors

(a) *M* factors: The presence of different *M* factors in males was determined by two generations of single-pair crosses with standard XX (without an $F^D$ factor) virgin females, from a marker strain that carries visible recessive mutations in homozygous state on each of the five autosomes (Tomita & Wada, 1989b). The sex ratio of F1 offspring shows whether the father was homozygous for at least one *M* factor (only sons are produced) or heterozygous for all *M* factors (daughters are also present among the offspring). Sex-linked inheritance of visible markers in the second generation of backcrosses to marker-strain females shows on which chromosomes *M* factors are located. This is a standard procedure in our laboratory and it gives a good estimation of the frequency of *M* factors located on different autosomes (for details see Denholm *et al.*, 1983). However, if a focal wild type male was homozygous for *M* (producing all-male offspring) and all of his sons appeared to have two (or more) *M* factors (e.g. *M* on autosome II and V), we could not unambiguously determine if the father was homozygous for *M* on only one or on both chromosomes, especially if the number of sons was small. For example, $M^{II}/M^{II}$; $M^{V}/M^{V}$, $M^{II}/+$; $M^{V}/M^{V}$ and $M^{II}/M^{II}$; $M^{V}/+$ males all produce $M^{II}/+;M^{V}/+$ sons when mated with standard females. This happened a few times (13 males in total, with a maximum of 4 males per population). For each chromosome involved in a population, we calculated both the minimal frequency of *M* (assuming that all ambiguous males were heterozygous for *M*), and the maximal frequency of the *M* factor (assuming that all ambiguous males were homozygous for *M*) on the given

chromosome. We then used the mid-point value between the two extremes as a population estimate.

We used 20 males from each population for the first series of crosses and 3 sons from each of them for the F1 backcrosses (although we did not obtain offspring from all males). Males used for analysis were either the ones caught in the field (IT3), or from the first generation in the lab (offspring of the wild caught flies; IT6, IT7, IT8, IT10, IT11, IT12), the third generation in the lab (GE1, GE2, SW, IT1, IT2, IT4, IT5) or the fourth generation (IT9). Because of the lack of visible markers on the X and the Y chromosome, in cases in which we assigned *M* to a sex chromosome, we cannot be sure whether it was located on the Y or the X (as has been found in Britain: Denholm *et al.*, 1983, 1985). If *M* was located on a sex chromosome we will call this chromosome Y, but we will discuss this issue in more detail later.

(b) $F^D$ factor: *F* and $F^D$ factors have been sequenced at the University of Zürich (M. Hediger and D. Bopp, personal communication). $F^D$ has two deletions compared to *F* in all populations analyzed (of European, Asian and African origin). We used primers designed for one of these deletions to distinguish between *F* (one band present) and $F^D$ (two bands) females. We used approximately 20 females from each population, either females caught in the field (populations: GE2, SW, IT5 and all 10 females from IT3) or from the first generation in the lab (all the other populations). Additionally, we took 2-3 females from each population and crossed them individually with a male homozygous for *M* located on autosome III. Females without $F^D$ produce only sons, but the ones with $F^D$ also produce daughters, because $F^D$ is dominant over *M*. After determining the sex of the offspring, we also analyzed the mothers molecularly and found without exception that the results of the molecular analysis were consistent with those obtained from the crosses. This shows that the deletion in the $F^D$ factor is also present in the populations we collected and justifies the use of the molecular technique for analyzing frequencies of $F^D$ in our populations.

## Statistical analysis

We performed a logistic regression analysis using the glm function with quasi-binomial errors in R (R Development Core Team, 2006) to investigate the

influence of latitude, altitude and temperature on the frequency of autosomal *M* males (with at least one autosomal *M* factor) and on the frequency of females with the $F^D$ factor. We started with a full model (including all two-way interactions between explanatory variables) and used backward selection to find the minimal adequate model. The significance of the difference between models was assessed with the likelihood-ratio approach, using F-tests to correct for under- and overdispersion (Krackow & Tkadlec, 2001).

A statistical comparison between the frequencies of different SD factors in the past and present is only possible to a limited extent, since Franco *et al*. (1982) only performed cytological observations. They used the frequency of XX males as a measure for the frequency of autosomal males. They checked the linkage of autosomal *M* factors with crosses similar to ours, but they do not provide the exact frequencies of different factors. They also do not provide data on frequencies of the $F^D$ factor. Moreover, due to the lack of data on the number of males tested by Franco and colleagues (1982), in each autosomal and standard population separately (except for GE2), we could only include eight populations (GE2, IT2, IT3, IT4, IT5, IT7, IT8 and IT10) in a statistical analysis to compare frequencies of autosomal males (without Y chromosome) between ours and their study. For this analysis, we performed a mixed-model logistic regression analysis in R using the lmer function with binomial errors from the lme4 package. The full model included population as a random effect and "study" (Franco *et al*., 1982 or this study) as a fixed effect. Significance of the effect of "study" was judged using the likelihood-ratio approach, using an F-test to correct for overdispersion (Krackow & Tkadlec, 2001). For each of the eight populations we also performed a binomial test, to see if there is a significant change in the frequency of XX males between the past and the present.

# Results

## Distribution of sex determining factors in 2006

We found *M* factors on the sex chromosomes and on each of the autosomes (Table 6.3, Figure 6.2a). *M* located on autosome III was the most frequent among autosomal *M* factors and the frequencies of *M* on autosome IV and V were very low. We did not detect any autosomal *M* in the German and Swiss populations and in one northern Italian population from the highest altitude (IT3). In populations with autosomal SD factors, often single males with multiple *M* factors, located on up to four different chromosomes, were observed (data not shown). Statistical analysis showed that altitude, latitude, temperature and interaction of temperature and latitude (and to a lesser extent interaction

**Table 6.3:** Estimated frequencies of females with $F^D$ factor and frequencies of *M* factors in males in samples from different housefly populations.

| Pop. code | # females | frequency of females with $F^D$ | # males | frequency of *M* on | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | sex chromosome | autosome | | | | |
| | | | | | I | II | III | IV | V |
| GE1 | 20 | 0.00 | 18 | 0.50 | 0 | 0 | 0 | 0 | 0 |
| GE2 | 19 | 0.00 | 20 | 0.50 | 0 | 0 | 0 | 0 | 0 |
| SW | 21 | 0.05 | 20 | 0.50 | 0 | 0 | 0 | 0 | 0 |
| IT1 | 20 | 0.44 | 20 | 0.52 | 0 | 0 | 0.12 | 0 | 0 |
| IT2 | 21 | 0.43 | 16 | 0.44 | 0 | 0.25 | 0.09 | 0 | 0 |
| IT3 | 10 | 0.10 | 11 | 0.50 | 0 | 0 | 0 | 0 | 0 |
| IT4 | 20 | 1.00 | 19 | 0.42 | 0.12 | 0.09 | 0.45 | 0 | 0 |
| IT5 | 22 | 1.00 | 20 | 0.62 | 0.02 | 0.17 | 0.50 | 0 | 0.09 |
| IT6 | 20 | 0.95 | 19 | 0.68 | 0.03 | 0.13 | 0.32 | 0 | 0 |
| IT7 | 23 | 0.78 | 18 | 0.17 | 0 | 0.03 | 0.53 | 0 | 0 |
| IT8 | 22 | 1.00 | 19 | 0.16 | 0.09 | 0.03 | 0.86 | 0.03 | 0.03 |
| IT9 | 22 | 0.86 | 18 | 0.06 | 0.08 | 0.17 | 0.46 | 0 | 0 |
| IT10 | 19 | 0.95 | 19 | 0 | 0.03 | 0 | 0.55 | 0.03 | 0 |
| IT11 | 23 | 0.96 | 17 | 0.03 | 0 | 0 | 0.76 | 0 | 0 |
| IT12 | 19 | 0.47 | 18 | 0.08 | 0 | 0 | 0.56 | 0 | 0 |

Frequencies of *M* are given separately for each chromosome (a value of 1.0 would indicate complete homozygosity for *M* on this chromosome). The sum of *M* frequencies over all chromosomes may exceed 1.0 when males carry multiple *M* factors.

Population codes as in Table 6.2 and Figure 6.1.

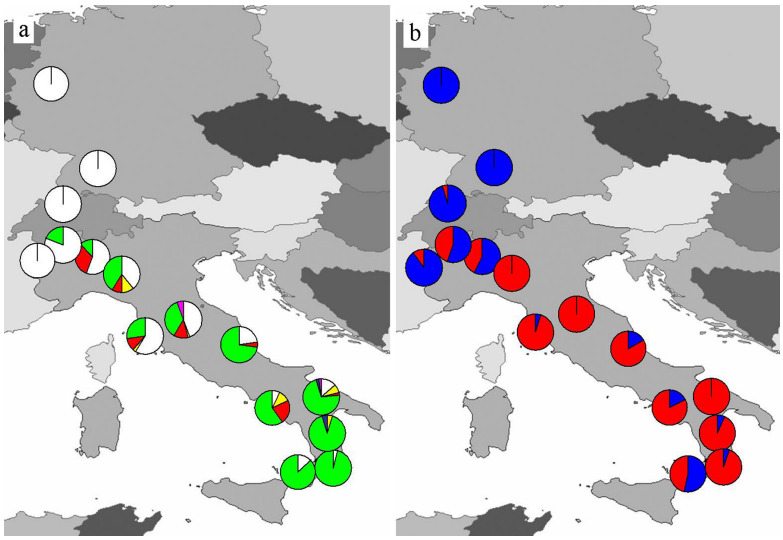


**Figure 6.2:** Distribution of sex determining factors in the housefly in 2006. (a) Relative frequencies of *M* factors located on different chromosomes: white – sex chromosome, yellow – autosome I, red – autosome II, green – autosome III, blue – autosome IV, pink – autosome V. (b) Frequencies of females with (red) and without (blue) the $F^D$ factor.

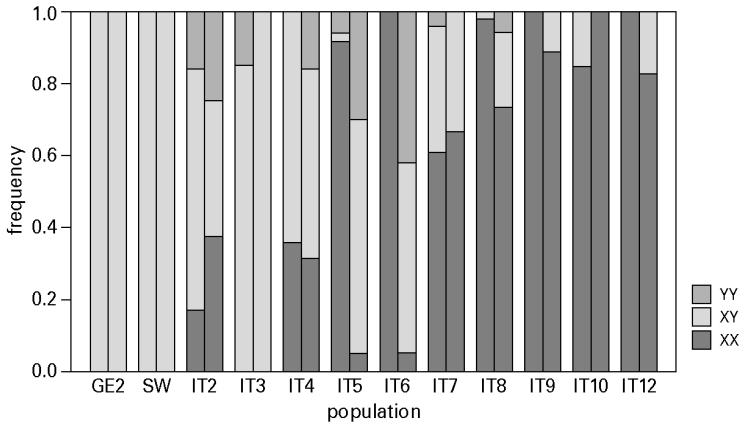


**Figure 6.3:** Comparison of karyotype frequencies in males in the past and the present (2006). For each population the left bar corresponds to the data from Franco *et al*. (1982) and the right bar to the data from this study. We inferred karyotypes from our crosses assuming that Y is the sex chromosome bearing the *M* factor (see Material and Methods). Three populations analyzed by us are not included in the figure since they were not studied by Franco and colleagues. Populations are ordered according to the decreasing latitude of the sampling sites (see Table 6.2).

between temperature and altitude) influence the frequencies of autosomal *M* males (Table 6.4).

We did not find $F^D$ in populations from Germany and only at low frequencies in Switzerland and at the highest location from northern Italy (IT3; Table 6.3, Figure 6.2b). In most of the Italian populations frequencies of $F^D$ females were above 0.75 and in three populations $F^D$ appeared to be at fixation. Statistical analysis showed that the frequency of females with $F^D$ is influenced by latitude, temperature and the interaction of the two (Table 6.4).

**Table 6.4:** Logistic regression analysis of (a) frequencies of autosomal *M* males and (b) frequencies of females with $F^D$. Parameter estimates (logit scale) and their standard errors (SE) are shown for the final models, after the removal of non-significant variables.

| Source of variation | Parameter | SE | Δdev | *F* | *P* |
|---|---|---|---|---|---|
| (a) Males | | | | | |
| Intercept | 277.4 | 33.2 | | | |
| Altitude (A) | –0.014 | 0.002 | 19.12 | 70.6 | <0.0001 |
| Latitude (L) | –5.521 | 0.652 | 28.39 | 104.9 | <0.0001 |
| Temperature (T) | –12.07 | 1.493 | 24.24 | 89.6 | <0.0001 |
| A*T | 0.0004 | 0.0002 | 1.53 | 5.6 | 0.042 |
| L*T | 0.222 | 0.029 | 22.28 | 82.3 | <0.0001 |
| (b) Females | | | | | |
| Intercept | 124.470 | 27.495 | | | |
| Latitude | –2.884 | 0.606 | 108.25 | 40.95 | <0.0001 |
| Temperature | –8.684 | 1.822 | 82.04 | 31.04 | <0.0005 |
| L*T | 0.204 | 0.042 | 85.32 | 32.28 | <0.0005 |

Temperature refers to the average yearly temperature. Δdev indicates the change in deviance resulting from removing the given variable from the final model. The F-tests for significance of removed variables have 1 and residual degrees of freedom of the final model (DF) for numerator and denominator, respectively.

Final models: (a) deviance=3.05, residual DF = 9; (b) deviance =27.28, residual DF = 11.

**Table 6.5:** Logistic mixed-model analysis of the frequencies of XX males in the study of Franco *et al*. (1982) and this study. The full model includes population as a random effect and study (data from Franco *et al*., 1982 or from our study) as a fixed effect under analysis.

| Model | DF | Deviance | *F* | *P* |
|---|---|---|---|---|
| Population (random) + study | 13 | 107.7 | | |
| Population (random) | 14 | 117.5 | 1.19 | 0.7 |

No significant difference between studies was found

## Comparison with the past

A comparison between our results and the results of Franco and colleagues (1982) shows that there is no clear evidence for the spread of autosomal *M* factors northwards during the last 25 years (Figure 6.3). In the two northernmost populations and in IT3, which lacked XX males in the past, we also did not find any autosomal *M* factor. Furthermore, all populations described by Franco and colleagues (1982) as mixed or autosomal were found to have autosomal *M* factors in 2006. However, in the populations which were described by Franco and colleagues as autosomal in 1982 (IT6, IT9 and IT12) we also found *M* on a sex chromosome. Statistical analysis based on the eight populations for which comparable data were available shows no significant systematic change in the frequencies of autosomal males in the last decades (Table 6.5). Statistical analysis for each population separately, shows a significant decrease in the frequency of XX males for two populations: IT5 and IT8 ($p<0.002$, which is also significant after Bonferroni correction for multiple tests).

The distribution of $F^D$ also seems to be relatively stable in time. $F^D$ frequencies were not analyzed by Franco *et al*. (1982), but the presence of $F^D$ can be deduced from the occurrence of at least one homozygous *M* male in all autosomal populations and the occurrence of XY females and YY males in mixed populations (Franco *et al.*, 1982), implying that 25 years ago $F^D$ (or a similar genetic element) was present across the entire range of Italy, as it is now. However, we did find $F^D$ in Switzerland, where it was not detected before 1982 suggesting that the $F^D$ factor has spread slightly northwards.

## Discussion

Our results show that autosomal *M* factors have not spread northwards in Europe over the last 25 years, in contrast to what was predicted by Franco *et al*. (1982). One may argue that we have overlooked low frequencies of autosomal *M* factors in Switzerland and Germany due to insufficient sample size. Although this may be true, very low frequencies of autosomal factors still support the hypothesis that the standard XY system is not being replaced by autosomal factors in northern populations. In line with our results, we suggest that after their initial spread in southern localities (see Franco *et al.*, 1982), autosomal *M* factors reached a stable distribution.

Our results indicate that some factors prevent the spread of autosomal *M* in populations north of Italy. In the transect we studied, the Alps may be considered as a barrier, although the biology of the housefly and its ease of spread with human transportation seem to preclude this physical barrier as being important for the potential long-term spread of autosomal *M* factors. In fact, the presence of the $F^D$ factor north of the Alps and the *M* factor on autosome II in flies collected in eastern France in 2004 (results not shown) suggests that geographical barriers do not prevent the northward spread of autosomal *M* factors. More likely, some climatic factors are responsible for the stability of the distribution of *M*. The most obvious climatic factor related with latitude is temperature, which has been shown to be a strong predictor of the frequencies of different sex determining factors in the housefly worldwide (Feldmeyer *et al*., 2008). However, it is not obvious how temperature might influence the evolution and distribution of SD mechanisms (discussed in detail in Feldmeyer *et al*., 2008).

Our statistical analysis reveals an effect of temperature, but also a significant interaction between temperature and latitude on the frequency of autosomal SD factors (Table 6.4). The interaction stems from the fact that at higher latitudes temperature has a positive effect on the frequencies of autosomal SD factors, whereas the opposite pattern is present at lower latitudes (not shown). This may suggest that autosomal SD factors reach the highest frequencies at intermediate temperatures. However, autosomal SD factors have been found at high frequencies in places where average temperatures are higher than at our sampling sites (Feldmeyer *et al.*, 2008). A more likely explanation is that

temperature interacts with other climatic factors (like humidity) that could be correlated with latitude (and altitude) in our study area. This could also explain why an *M* factor on autosome III and $F^D$ have been found at locations in England where the yearly range of temperatures is similar to Germany and Switzerland (Denholm *et al.*, 1985; data on temperatures from WORLDCLIM, not shown). Additionally, *M* factors located on different autosomes may be differently affected by temperature.

It has also been proposed that autosomal *M* factors have spread due to their linkage with insecticide resistance genes (Kerr, 1970; Franco *et al.*, 1982), since the isolation of autosomal *M* factors coincided with the appearance of insecticide resistance in natural populations of the housefly (Tomita & Wada, 1989b). Also, in a number of resistant populations autosomal *M* males have been found (Tsukamoto, 1983) and one laboratory experiment showed replacement of standard XY males by autosomal *M* males after several generations of selection for DDT resistance (Kerr, 1970). However, even though linkage with insecticide resistance genes could facilitate spread of autosomal *M* factors, it is not clear how it could contribute to the clinal distribution of SD factors in the housefly. One could argue that in warmer climates more generations of flies are produced and more applications of insecticides are used, allowing faster spread of *M* factors linked with insecticide resistant genes. However, since pesticides have been used in whole Europe for decades and resistance genes are widespread also in northern populations (Keiding, 1977; 1999), one would expect that, although slower, *M* factors would be increasing in frequency also in the north. As we showed in this study, this is not the case. Another argument is that there is no correlation between the frequency of autosomal *M* males and insecticide resistance in housefly populations from eastern United States (Hamm *et al.*, 2005). Therefore, linkage with insecticide resistance genes might explain spread of autosomal *M* factors is some cases, but it seems unlikely to provide a general explanation for the clinal distribution of SD factors in the housefly.

Interestingly, autosomal *M* factors are not fixed in most populations and multiple factors on several or even all chromosomes can be maintained in a single population. This polymorphism was one of the reasons underlying the opinion of earlier researchers that the sex determining mechanism in the

housefly is in a transient state (e.g. Franco *et al.*, 1982; Denholm *et al.*, 1985; Tomita & Wada, 1989b). However, theoretical models reveal that such a polymorphism can be stable not only for specific fitness values of different genotypes (Bull & Charnov, 1977; Jayakar, 1987), but also when different genotypes have the same viability and fertility (Kozielska *et al.*, 2006). Therefore, the conditions for a stable polymorphism may be much less restrictive than previously thought, and it may well be that the multifactorial SD system of the housefly is stable.

Unfortunately, we do not have data on the frequencies of different autosomal *M* factors in the past to see whether these frequencies have changed. Franco and colleagues (1982) did not find any *M* factors located on autosomes I, IV or V, but they do not provide the number of males investigated. If these factors were present in the past at low frequencies as they are now (Table 6.3), Franco *et al*. (1982) might not have detected them in small sample sizes. They reported that *M* was more common on autosome III than on autosome II. The same pattern is seen in this study and in several other studies (Tomita & Wada, 1989b; Denholm *et al.*, 1990; Hamm *et al.*, 2005; except for Tanzanian populations, Feldmeyer *et al.*, 2008.). This suggests that *M* on autosome III confers the largest fitness gain to its bearer, but this may only be a conditional effect (e.g. frequency- or temperature-dependent) since the *M* on autosome III did not replace other *M* factors during the last decades in the Italian populations.

Another explanation for the high polymorphism in genomic location of *M* factors is that the *M* factor is part of a transposable element, as is known for the *M* factor in *Megaselia scalaris* (Traut & Willhoeft, 1990). In this species transposition rate differs depending on which chromosome *M* is located (Green, 1980). This might not only explain why *M* factors are more common on some autosomes than others, but also the clinal distribution of *M* factors, since transposition rate is known to be dependent on temperature and often increases with increasing temperature (Lampe *et al.*, 1998; Ohtsubo *et al.*, 2005; but see Hashida *et al.*, 2003). Molecular studies are necessary to establish whether the *M* factor is always the same gene located on a transposable element or whether *M* factors on different chromosomes are different genes blocking the female determining factor *F* (see Dübendorfer *et al.*, 2002).

Our crosses suggest that the frequency of the Y chromosome has increased over the last decades in some Italian populations. We found an *M* factor on the sex chromosomes in some populations that were described as purely autosomal by Franco and colleagues (1982; Figure 6.3). It is difficult to assess what the cause of these changes in particular populations is; some local factors may be involved. For population IT5, the difference between past and present frequencies of XX males might reflect the fact that we could not locate accurately the sampling site of Franco and colleagues (1982; see Material and Methods). Moreover, it should be noted that due to the absence of visible markers on the sex chromosomes of the housefly, our crosses did not allow us to determine whether the *M* factor was present on the Y or on the X chromosome (as found in England: Denholm *et al.*, 1983; 1985). Without additional information, the data obtained from the crosses could easily lead to the incorrect classification of XX*M* males as XY males. Therefore, we performed additional cytological investigations, using orcein staining, a standard technique used in cytological studies of the housefly (Hiroyoshi, 1964; Franco *et al.*, 1982; Denholm *et al.*, 1983; 1985). Our preliminary results (not shown) confirm that males from the northernmost populations (GE1, GE2, SW and IT3) are of karyotype XY. Unfortunately, we could not unambiguously distinguish between XX, XY and YY karyotypes in the other populations, because the length polymorphism of the housefly sex chromosomes (also know from other strains: Boyes *et al.*, 1964; Boyes, 1967; Milani, 1971; Franco *et al.*, 1982; Denholm *et al.*, 1983; 1985; Hediger *et al.*, 1998b) did not allow a reliable distinction between X and Y chromosomes. Therefore, we cannot exclude the possibility that the X chromosome (rather than the Y chromosome) bears the *M* factor in the southern populations.

In conclusion, even if the distribution of the Y chromosome in European populations is difficult to assess, our main conclusion that autosomal *M* factors have not spread northwards in the last 25 years still holds. This suggests that the polymorphism of the SD factors in natural housefly populations is not transient but stable. Additional studies, both at the ecological and the molecular level, are required to unravel the factors responsible for the stable coexistence of various SD factors. Undoubtedly, better understanding of the housefly SD system will also provide general insights into the evolution of sex determination, which is still poorly understood in other taxa as well.

## Acknowledgments

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# CHAPTER 7

# Climatic variation and the geographical distribution of sex determining mechanisms in the housefly

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## Abstract

**Questions:** (1) Are the geographic clines of sex determining factors in the housefly of the northern hemisphere mirrored by similar clines on the southern hemisphere?

(2) What climatic factors can best explain the geographical distribution of sex determining factors in the housefly?

**Data:** Frequencies of sex determining factors of houseflies collected in Africa and corresponding literature data on houseflies studied on other continents. Global climate data from public databases.

**Results:** Housefly populations on the southern hemisphere repeat the pattern earlier found on the northern hemisphere: higher frequencies of autosomal *M* and $F^D$ factors closer to the equator. Seasonality in temperature variation is the best predictor for the distribution of the male sex determining factor whereas female sex determining factors are best explained by variation in humidity and yearly mean temperature.

## Introduction

Sex determining mechanisms vary considerably across taxa and seem to evolve quite rapidly, for reasons that are still poorly understood (Bull, 1983; Bull, 1985; Marin & Baker, 1998; Kraak & Pen, 2002; Werren *et al.,* 2002). However, the vast majority of variation occurs above the species level. Since the housefly (*Musca domestica*) harbors several different sex determining mechanisms it is a particularly interesting model species for studying sex determination. All individual houseflies possess a female determining factor (the *F* factor) which turns on the female developmental pathway, unless a so-called *M* factor is also present and blocks the action of *F*, thus triggering developmental into a male. In "standard" males, the *M* factor is located on the Y chromosome (Dübendorfer *et al.,* 1992), but *M* factors can also be located on any of the five autosomes or even on the X chromosome (Table 7.1) (Denholm *et al.,* 1983; Dübendorfer *et al.,* 2002).

**Table 7.1:** Relation between genotype and sexual phenotype in the housefly. The female determining factors ($F/F^D$) are located on chromosome 4; the male determining factors (*M*) can be located on any chromosome. + = wildtype state (no *M*); ● = the same phenotype will develop irrespective of the presence or absence of *M*.

| **Autosomes** | | **Sex chromosomes** | |
|---|---|---|---|
| 4 | 1-5 | XX | XY |
| *F/F* | +/+ | ♀ | ♂ |
| *F/F* | ●/*M* | ♂ | ♂ |
| $F/F^D$ | ●/● | ♀ | ♀ |

In populations where autosomal *M* factors are prevalent, the Y chromosome is often absent and males are either XX or sometimes XO (Denholm *et al.,* 1985; Denholm *et al.,* 1990; Çakir & Kence, 1996). In some populations males may be homozygous for an autosomal *M* factor or possess multiple *M* factors on different autosomes. In such populations, females often possess a special dominant version of the *F* factor, designated $F^D$, which is not blocked by *M* factors (Tomita & Wada, 1989; Hilfiker-Kleiner *et al.,* 1993).

Interestingly, the different sex determining systems in the housefly show clear latitudinal clines and altitudinal clines. This was first reported by Franco *et al.* (1982), who examined houseflies from 53 localities in Europe, from Denmark in the north to Sicily in the south, and discovered that frequencies of autosomal *M* factors increased towards the south and decreased with higher altitude. Additional studies from England (Denholm *et al.,* 1985), Japan (Tomita & Wada, 1989b), Turkey (Çakir & Kence, 1996) and the United States (Hamm *et al.,* 2005) showed similar patterns with XY males in the north or at high altitudes and males with autosomal *M* factors dominating at lower latitudes and altitudes. It is not clear whether these clines represent stable distributions or whether they are a transient phenomenon. Some authors argued for the latter because before 1948 no study on the housefly revealed any other system than the standard XY system (Franco *et al.,* 1982; Denholm *et al.,* 1985; Tomita & Wada, 1989b; Çakir & Kence, 1996).

We have evidence (Kozielska *et al.,* 2008) that frequencies of autosomal *M* factors have not changed much for several decades in Europe. This is not entirely unexpected, since recent theoretical models have shown that multiple *M* factors may stably coexist (Kozielska *et al.,* 2006). However, it is still unclear why the clines exist in the first place. Some authors have suggested that autosomal *M* factors "hitchhike" with insecticide resistant genes (Kerr, 1970; Franco *et al.,* 1982; Tomita & Wada, 1989b), but more recent studies did not find supporting evidence for this (Shono & Scott, 1990; Hamm *et al.,* 2005). Obviously, any factor which shows pronounced clinal variation could in principle be involved in causing the clinal distribution of sex determining mechanisms. The most obvious factor which varies predictably with both latitude and altitude is temperature, and temperature variation has been invoked as a possible explanation by several authors (Franco *et al.,* 1982; Çakir & Kence, 1996). However, other climatic variables might explain geographic variation as well, as shown in studies on clinal variation in body size. For example wing length in birds as a measure of body size correlates with humidity and temperature (James, 1970). Seasonality on the other hand seems to best explain body size in muskrats (Boyce, 1978), and seasonality in resource availability might explain the body size pattern in several insect species (Chown & Klok, 2003; Blanckenhorn & Demont, 2004). Recently it has been shown that

body size variation in a seed-feeding beetle (*Stator limbatus*) is best explained by host plant size, humidity and seasonality (Stillwell *et al.,* 2007).

No systematic quantitative analysis has yet been performed to investigate to what extent variation in temperature or other climatic factors can explain the distribution of sex determining mechanisms in the housefly. In this paper, we present such an analysis, based on previously published data and on newly collected data. All previous studies of geographical distributions of sex determining mechanisms in the housefly have been carried out on populations in the northern hemisphere. If temperature or other climatic factors are important in determining the distributions, we would expect to find the opposite pattern in the southern hemisphere, i.e. relatively more autosomal *M* factors in the north than in the south. To test this prediction we additionally collected houseflies from several subequatorial populations in Africa and examined them for the presence of autosomal *M* factors and $F^D$ factors.

## Materials and methods

### Sampling and analyses of African housefly populations

We collected houseflies at farms, horse stables and markets at five locations in Tanzania and six locations in South Africa. At every location, approximately 100 adult flies were caught with a sweeping net and stored in boxes supplied with water, milk powder and egg-laying medium (according to the protocol of Hilfiker-Kleiner *et al*., 1994). For transport to our laboratory in the Netherlands, 150-200 larvae from each sampling location were stored in 50ml tubes that contained medium. In the laboratory, larvae, flies and eggs were grown under conditions as described by Hilfiker-Kleiner *et al*. (1994) with the following modifications of their protocol: ambient temperature was set at 20ºC, relative humidity at 60% and flies were kept under constant light.

For each sampling location 15 males were crossed with virgin females from a mutant strain recessive for visible traits on each autosome (*ali curly* (*ac*) on linkage group 1; *aristapedia* (*ar*) on 2; *brown body* (*bwb*) on 3; *yellow eyes* (*ye*) on 4; *snip wings* (*snp*) on 5). Since mutant females have the standard *F* factor, they only get sons when crossed with males homozygous for an *M* factor, and mixed-sex offspring when crossed with males heterozygous for *M* factors. Thus, by inspecting the F1 sex ratio of each male, we could estimate the frequency of

homozygous males. For 10 out of the 15 males for each location, we selected 3 male F1 offspring and crossed each of them with a mutant virgin female to determine on what chromosomes male-determining *M* factors were located (see Franco *et al.,* 1982 for a more detailed description of this technique).

To determine whether females were carrier of a dominant female-determining factor $F^D$, for each sampling location up to 15 females were crossed with males of a laboratory strain that were homozygous for an autosomal *M* factor. Female offspring of such crosses necessarily carried an $F^D$ factor, since $F^D$ overrides the male determining effects of up to three simultaneously present *M* factors (McDonald *et al.,* 1978, Franco *et al.,* 1982).

**Table 7.2** Studies of the geographical distribution of housefly sex determining mechanisms used in our pooled analyses.

| Study | # locations | # males | # females |
|---|---|---|---|
| 1. Denholm *et al*., 1985; UK | 6 | 430 | - |
| 2. Tomita and Wada, 1989; Japan | 18 | 1105 | 739 |
| 3. Çakir and Kence, 1996; Turkey | 34 | 1050 | - |
| 4. Hamm *et al.*, 2005; USA | 4 | 308 | - |
| 5. This study; Africa | 11 | 99 | 126 |

## Compilation of published studies

We compiled relative frequencies of males with autosomal *M* factors and females with $F^D$ from four additional published studies (see Table 7.2 and Figure 7.1). These studies used either cytological techniques to determine the presence/absence of the Y chromosome, or used crosses similar to those described above. In the cytological studies (Denholm *et al.,* 1985; Çakir & Kence, 1996), autosomal *M* factors were inferred from the absence of Y chromosomes. This procedure can obviously underestimate frequencies of autosomal *M* factors, since males with Y chromosomes can also have autosomal *M* factors. For the studies relying on crosses (Tomita & Wada, 1989b; Hamm *et al.,* 2005), we also regarded males to be "autosomal" only in the absence of a Y

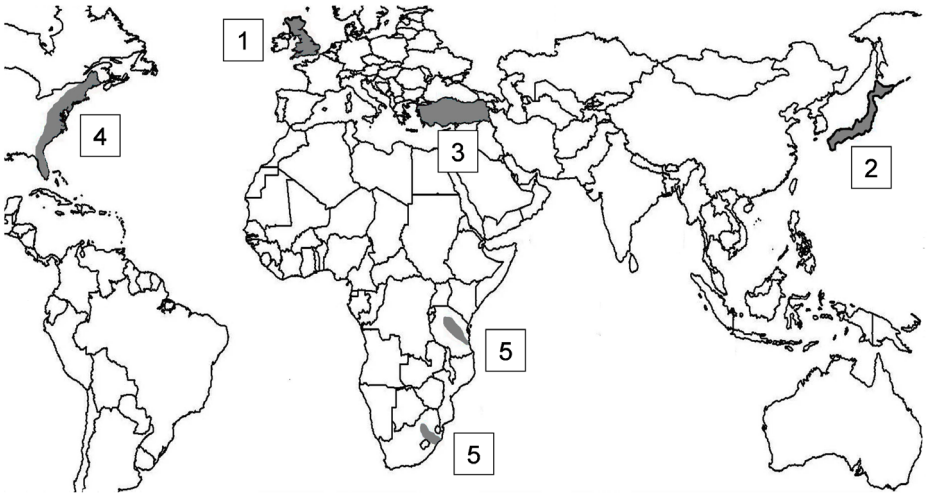


**Figure 7.1** Geographical locations and references (see Table 7.2) of housefly studies that were used in the analysis.

chromosome, in order to make these studies comparable to the cytological studies.

## Sources of geographical and climatic data

In our study of African houseflies, we used GPS to estimate the latitude and altitude of the study locations. For the published studies, latitudes and altitudes were either explicitly provided in the original study (Turkey: Çakir & Kence, 1996; USA latitude only: Hamm *et al*., 2005), or we estimated latitude and/or altitude based on the description of the sampling location provided in the original study (Japan: Tomita & Wada, 1989b; UK: Denholm *et al.*, 1985) with Google Earth (www.earth.google.com). To correct for idiosyncratic differences between countries, for each sampling location we used latitude relative to the mean latitude of the sampling locations per country/study as a predictor variable, rather than absolute latitude.

For each study location we obtained estimates of annual mean temperature, annual relative humidity, annual daily temperature range, annual number of days with ground frost, and annual percentage of sunshine hours during daylight from the World Water and Climate Atlas of the International Water

Management Institute (www.iwmi.cgiar.org/WAtlas). More detailed information on monthly averages of minimum and maximum temperatures were obtained from WORLDCLIM (www.worldclim.org, see Hijmans *et al.*, 2005), which provides global estimates at a spatial resolution of one square kilometer.

We derived two proxies for seasonality as predictor variables in the statistical analysis (Table 7.3). The first estimate (Season1) was calculated as the (dimensionless) coefficient of variation of the average monthly temperatures. The second (Season2), we calculated as the average of the difference between the highest and lowest monthly maximum temperature and the difference between the highest and lowest monthly minimum temperature.

As proxy that might reflect temperature during the active season we included the highest monthly mean temperature (Tactive).

**Table 7.3** Abbreviations and explanations of predictor variables used in the analysis.

| Abbreviation | Description |
|---|---|
| Alt | Altitude |
| Lat | Latitude relative to mean latitude per study |
| Tmean | Annual mean average daily temperature |
| Tmin | Annual mean minimum daily temperature |
| Tmax | Annual mean maximum daily temperature |
| Tactive | Mean temperature of warmest month |
| DailyTR | Annual mean daily temperature range |
| Humidity | Annual mean relative humidity |
| Frost | Annual number of days with ground frost |
| Sunshine | Annual percentage sunshine hours |
| Season1 | Coefficient of variation of average monthly temperatures |
| Season2 | Average of [difference between highest and lowest monthly maximum temperature] and [difference between highest and lowest monthly minimum temperature]. |

## Statistical analysis

Relative frequencies of autosomal *M* males and $F^D$ females were modeled as proportions with logistic regression in R (R Development Core Team, 2007), using the glm procedure with the “family=binomial” option.

We used a model selection approach to find the "best" (most parsimonious) collections of explanatory variables, employing a modified version of Akaike's Information Criterion, corrected for overdispersion and small sample sizes (Burnham & Anderson, 2002):

$$\mathrm{QAIC}_c = 2k - 2L/\hat{c} + 2k(k+1)/(n-k-1)$$

In this formula, $k$ is the number of estimated model parameters, $L$ the log-likelihood of the model, $n$ the number of data points, and $\hat{c} = \chi^2_{\mathrm{GOF}} / \mathrm{df}_{\mathrm{full}}$ is a variance inflation factor to adjust for overdispersion of the model, $\chi^2_{\mathrm{GOF}}$ being the chi-squared goodness-of-fit statistic and $\mathrm{df}_{\mathrm{full}}$ the residual degrees of freedom of the full model. The final term on the right-hand side of the $\mathrm{QAIC}_c$ equation corrects for small sample sizes.

Given the number of predictor variables, an all subset selection approach starting with all predictors was computationally infeasible. We therefore started our model selection algorithm with all one-variable models and all possible additive two-variable models and selected the models with the lowest $\mathrm{QAIC}_c$ values. In cases where a two-variable model had a lower $\mathrm{QAIC}_c$ value than any of the one-variable models we tested for both variables of the two-variable model separately whether they could be deleted without significantly increasing the residual deviance, using F-tests ($F$ = (Δdeviance/Δdf)/(deviance$_{\mathrm{full}}$/df$_{\mathrm{full}}$) with df$_{\mathrm{fulll}}$ numerator and Δdf denominator degrees of freedom). If neither of the variables could be removed, we then selected the best model from all possible models with three predictors. Again we tested whether one or more of the predictors could be sequentially removed from the model without significantly reducing the fit of the model. If three predictors remained, we went on to test all models with four variables, and so on. However, it turned out that in all analyses the best three-variable models were always reducible, thus terminating our model selection algorithm.

**Table 7.4** Frequencies of males with autosomal *M* factors and females with $F^D$ in Tanzanian and South African sampling locations. Chrom *M* = chromosomes on which *M* factors were found; % Auto *M* = percentage of males carrying the *M* factor exclusively on autosomes (all other males had *M* on the Y chromosome but also on an autosome); % $F^D$ = percentage of females carrying the $F^D$ factor; n = number of individuals tested.

| Location | Chrom *M* | % Auto *M* (n) | % $F^D$ (n) |
|---|---|---|---|
| Tanzania, Same | 2 | 100 (10) | 100 (13) |
| Moshi | 2 | 100 (10) | 100 (11) |
| Makuiuny | 2,Y | 80 (10) | 100 (13) |
| Arusha | 2 | 100 (10) | 100 (14) |
| Karatu | 2,Y | 80 (10) | 85 (13) |
| South Africa, Zinkwazi Beach | 2,3 | 100 (9) | 29 (7) |
| Umhlali | 1,2,3,5 | 100 (10) | 79 (14) |
| Hammarsdale | 2,3 | 100 (9) | 92 (13) |
| Ashburton | 1,2,3 | 100 (5) | 13 (8) |
| Mooi River | 2,3 | 100 (6) | 29 (7) |
| Warden | 3,Y | 70 (10) | 15 (13) |

## Results

### New African data

In Tanzania, in three out of five sampled populations, all males had autosomal *M* factors and no Y chromosome, while in the remaining two populations 80% of the males had autosomal *M* factors. The autosomal *M* factors were always located on chromosome 2. In South Africa, the overall frequency of males with autosomal *M* factors was about the same as in Tanzania (see Table 7.4), but the *M* factors were found on all chromosomes except chromosome 4. However, males from Tanzania were significantly more often homozygous for *M* factors than males from South Africa (TZ: 62%; SA 26%; logistic regression: $P = 0.02$). Females with $F^D$ were found in all populations (Table 7.4). However, in South Africa the frequency of $F^D$ was significantly lower than in Tanzania, where all females carried the $F^D$ factor in all but one population.

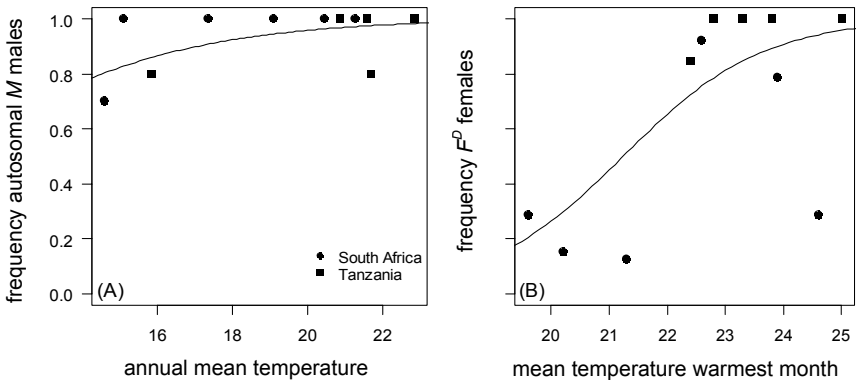


**Figure 7.2** Best predictors of frequencies of sex determining factors in African houseflies. Relationship between the single variables of the most parsimonious logistic regression models and frequencies of **(A)** males with autosomal *M* factors and **(B)** females with $F^D$ factors.

**Table 7.5** Logistic regression model selection for autosomal *M* frequencies (males) and $F^D$ frequencies (females) in African houseflies. Depicted are the five one-variable models with the lowest $QAIC_c$ values plus the two-variable model with the lowest $QAIC_c$. F-tests refer to deletion of a single variable. DF = residual degrees of freedom; bold = final model. See Table 7.3 for explanation of variables.

| Model | DF | $QAIC_c$ | *F* | *P* | Model | DF | $QAIC_c$ | *F* | *P* |
|---|---|---|---|---|---|---|---|---|---|
| *Males* | | | | | *Females* | | | | |
| **Tmean** | | 16.22 | | | **Tactive** | | 13.16 | | |
| NullModel | | 16.50 | | | Tmin | | 15.34 | | |
| Tmax | | 17.17 | | | NullModel | | 15.59 | | |
| Season1 | | 17.91 | | | Tmean | | 15.66 | | |
| Frost | | 17.94 | | | DailyTR | | 15.69 | | |
| Tmean+Tactive | 9 | 18.17 | 0.3 | 0.86 | Tactive+Tmax | 9 | 15.70 | 0.06 | 0.82 |

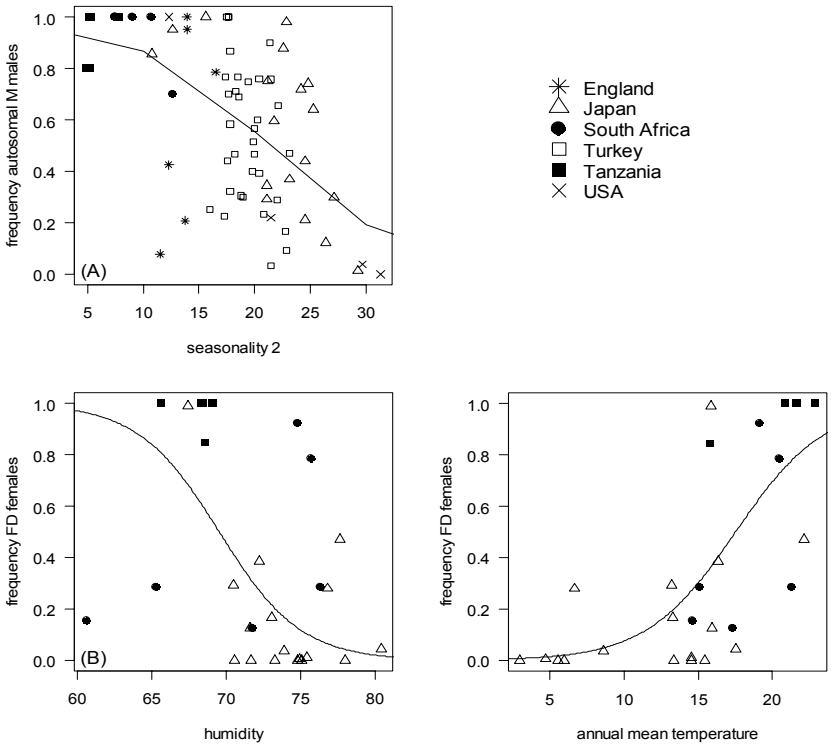


**Figure 7.3** Best predictors of frequencies of sex determining factors in houseflies, pooled data. Relationship between the single variables of the most parsimonious logistic regression models and frequencies of **(A)** males with autosomal *M* factors and **(B)** females with $F^D$ factors.

Model selection showed that the distribution of African autosomal *M* males is most parsimoniously explained by yearly mean temperature alone (Table 7.5, Figure 7.2). The distribution of $F^D$ females on the other hand was best explained by temperature during the warmest month of the year (Table 7.5, Figure 7.2).

## Analysis including data from previous studies

The analysis of the combined data is presented in Table 7.6 (see also Figure 7.3). The most parsimonious model for the frequency of autosomal *M* males contained the single seasonality variable Season2 (described in Table 7.3). Model selection showed that the distribution of $F^D$ in females is most parsimoniously explained by a two-variable model with predictors humidity and yearly mean temperature.

**Table 7.6** Logistic regression model selection for autosomal *M* frequencies (males) and $F^D$ frequencies (females) for pooled data. Depicted are the one-variable models with the lowest $QAIC_c$, three of the best two-variable and three three- variable models and the results of F-tests by deleting the right-most variable. Note that the two-variable models have lower $QAIC_c$ than the one-variable models, but for males the second variable can be removed without significant affecting the model. The same holds true for the two-variable and three-variable models in females. DF = residual degrees of freedom; bold = final model. See Table 3 for explanation of variables.

| **Model** | **DF** | **$QAIC_c$** | ***F*** | ***P*** | **Model** | **DF** | **$QAIC_c$** | ***F*** | ***P*** |
|---|---|---|---|---|---|---|---|---|---|
| *Males* | | | | | *Females* | | | | |
| **Season2** | | 92.24 | | | Humidity | | 23.63 | | |
| Season2+DailyTR | 71 | 88.17 | 1.09 | 0.30 | **Humidity +Tmean** | 26 | 18.65 | 6.82 | 0.02 |
| Season2+Sunshine | 71 | 89.16 | 0.50 | 0.48 | Humidity+Sunshine | 26 | 19.35 | 0.54 | 0.47 |
| Season2+Humidity | 71 | 92.16 | 0.05 | 0.83 | Humidity+Frost | 26 | 20.17 | 7.41 | 0.01 |
| | | | | | Humidity+Tmean+Sunshine | 25 | 17.74 | 0.29 | 0.59 |
| | | | | | Humidity+Tmean+DailyTR | 25 | 18.06 | 0.57 | 0.46 |
| | | | | | Humidity+Tmean+ Season2 | 25 | 18.28 | 0.00 | 0.97 |

## Discussion

This study set out to address two main questions regarding the geographical distribution of sex determining factors in the housefly. The first question was whether the increasing frequency of autosomal *M* factors towards the equator on the northern hemisphere would be matched by a similar pattern on the southern hemisphere. The second question was which of a number of climatic and spatial variables could best explain the observed distributions of sex determining factors.

Our results show that unlike in studies of European, North American and Asian housefly populations, where males without autosomal *M* factors are common, all males carried at least one autosomal *M* factor in the Tanzanian and South-African populations, and the frequency of Y chromosomes was very low (Table 7.4). Nevertheless, the frequency of males homozygous for autosomal *M* factors was considerably higher in Tanzania than South-Africa, indicating that in African populations autosomal *M* factors are more frequent towards the equator, just like they are in populations on the northern hemisphere. Similarly, the frequency of $F^D$ factors in females was much higher in Tanzania than in South-Africa (Table 7.4). Thus, the answer to the first question, whether the increasing frequency of autosomal *M* factors towards the equator on the northern hemisphere would be matched by a similar pattern on the southern hemisphere is yes.

Our analysis further showed that neither latitude nor altitude per se were good predictors, but that climatic factors had more explanatory power. The analysis suggests that autosomal *M* and $F^D$ distributions are affected by different climatic factors (Tables 7.4 and 7.5). The distribution of autosomal *M* males is best explained by a measure of seasonality (the average of the differences between the minimum and maximum values of the monthly minimum and maximum temperatures), whereas the distribution of $F^D$ females is best explained by humidity and annual mean temperature.

Because our study is entirely correlational, we have not established a causal link between climatic factors and the distribution of male and female sex determining factors. We therefore will discuss and speculate about plausible mechanisms that might be responsible for the clinal distribution of autosomal *M* and $F^D$, and that might be investigated further.

Seasonality, i.e. within year changes in climatic factors, is an important determinant when it comes to adaptation of organisms to their environment (Danks, 2006). Seasonal responses, e.g. diapause, cold hardiness and reproductive pattern, have to go in line with a variety of climatic conditions (Danks, 2006). In the housefly there is evidence of a seasonal trend in the frequency of intersexes which are more frequent in winter than in summer (Milani, 1967). In this paper we showed that frequencies of autosomal *M* factors are best explained by variation in a measure of seasonality. It is conceivable that these two observations are somehow connected. Autosomal *M* factors might confer higher fitness at high temperatures but at the same time cause greater developmental instability with respect to fluctuations in temperature. Although there is no direct evidence that autosomal *M* factors are temperature sensitive, two housefly laboratory strains have been discovered with temperature dependent expression of sex determining factors. One strain carries a maternal effect mutation, *Arrhenogenic* (*Ag*) (Vanossi Este & Rovati, 1982; Dübendorfer *et al.,* 2002). If the mother is heterozygous for *Ag* she produces mostly sons and intersexes at lower temperatures and mostly daughters at higher temperatures whereas females without *Ag* only produce daughters (Schmidt *et al.,* 1997a). In a second strain the mutation *masculinizer* (*man*) occurs, which has the properties of a null allele of *F* (Schmidt *et al.,* 1997b). All individuals homozygous for *man* develop into males whereas all individuals heterozygous for *man* develop into females at low and into males and intersexes at high temperatures. Thus, in these strains temperature directly acts on the sex determining system but, so far, these variants have only been found in the laboratory. Whether autosomal *M* factors are also temperature sensitive, albeit perhaps to a lesser degree, remains to be investigated.

It is also conceivable that *M* changes chromosomes via translocation e.g. via transposable elements. This mechanism seems quite plausible since it has been demonstrated in the scuttle fly *Megaselia scalaris*, where the *M* factor resides within a transposable element (Traut & Willhoeft, 1990). Theoretical models show that the fixation probability of transposable elements in a population not only depends on the transposition rate but also correlates negatively with generation time (Le Rouzic & Capy, 2005). In the case of the housefly this would imply that autosomal *M* is more frequent in warmer regions as more generation cycles are possible per population. Over time one would expect the

autosomal *M* factor to spread into colder regions. However Kozielska *et al*. (2008) found that the distribution of autosomal *M* in Europe has not changed over the last 50 years. This suggests that generation time per se cannot be the sole explanation and there has to be an additional mechanism.

An alternative mechanism could be temperature induced segregation distortion by *M* factors. It is well known theoretically that segregation distorters can increase in frequency even at the expense of individual fitness (Haig & Bergstrom, 1995; Weissing & van Boven, 2001). Jayakar (1987) has shown that sex determining factors linked to segregation distorters may lead to a shift in sex determining mechanisms. In *Drosophila melanogaster*, segregation distorters have been found that are temperature sensitive (Mange, 1968; Hartl, 1975; Hiraizumi, 1993): in some strains a temperature of 25ºC was associated with strongly aberrant segregation ratios, while the degree of distortion was lower at both higher and lower temperatures. There is weak evidence that segregation distortion sometimes occurs in the housefly (Clark, 1999), but this has not been linked to temperature.

Adult houseflies have an optimal humidity-temperature range in which they are most active (West, 1951). Both humidity and temperature extremes do have lethal effects on adult houseflies as well as on any other developmental stage (Hewitt, 1908; West, 1951). Humidity has a strong effect on eggs and larvae as they dry out if there is not enough moisture, whereas too much humidity leads to drowning. Larvae in a later stage however need a less moist environment to be able to pupate (Hewitt, 1908). It is possible that $F^D$ besides being unsusceptible towards *M* could also lead to increased viability of any of the fly developmental stages in warmer and less humid places.

Our main goal of this study was to find out whether the distribution of male and female sex determining factors in the housefly can be explained by variation in climatic variables. As previously suggested by Stillwell *et al*. (2007) for body size, mean temperature should not be the only variable taken into consideration when studying latitudinal clines. We find that seasonality and humidity in addition to mean temperature explain the clines in male and female sex determining factors. How this association comes about at the mechanistic level remains to be elucidated.

## Acknowledgements

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# CHAPTER 8

# A microsatellite linkage map of the housefly, *Musca domestica*; implications for recombination rate and sex chromosome evolution

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*In preparation for Insect Molecular Biology*

## Abstract

We present the first molecular marker linkage map for *Musca domestica* containing 35 microsatellite plus six visible markers. We report the development of 33 new microsatellite markers of which 19 are included in the linkage map. 236 F2 individuals from three mapping crosses were genotyped. Linkage maps of each cross were combined into a single composite map consisting of five linkage groups representing the five autosomes of the housefly. None of the markers mapped to either the X or the Y chromosome. The map covers a total of 229.6 cM with an average marker spacing of 4.4 cM spanning approximately 80.2% of the genome. We found up to 29% recombination in male houseflies confirming previous studies. Recombination frequencies on autosomes depended on the presence/absence of the male determining factor *M*, as predicted for early stage sex chromosomes. The linkage map will aid further genome studies, in particular those aimed at revealing the nature and location of the housefly autosomal sex determining factors and testing hypotheses of sex chromosome evolution.

## Introduction

The housefly (*Musca domestica*) is a cosmopolitan species and an important disease vector for cattle and humans (Fotedar *et al.,* 1992). It is also of interest for the evolution of sex determination, as this species harbors several different sex determining systems (Dübendorfer *et al.,* 2002). Despite its medical and economic importance, and even though it has been studied for decades, remarkably little genomic mapping information is available of the housefly. Thus far, linkage studies in the housefly are constrained to back crosses with mutants carrying visible mutations (Hiroyoshi, 1961; Tsukamoto *et al.,* 1961; Wagoner, 1967; Hiroyoshi, 1977), and have mostly been used to localize sex determining factors, but also for mapping of other genes (Wagoner, 1969; Franco *et al.,* 1982; Denholm *et al.,* 1985; Tomita & Wada, 1989b; Denholm *et al.,* 1990; Çakir & Kence, 1996; Kozaki *et al.,* 2002; Hamm *et al.,* 2005; Kandemir *et al.,* 2006; Feldmeyer *et al.,* 2008; Kozielska *et al.,* 2008). Whereas several studies on mitochondrial genes, as well as population genetic studies based on mitochondrial sequences are available (Roehrdanz, 1993; Marquez & Krafsur, 2002; 2003; Cummings & Krafsur, 2005), we know of only one population genetic study where microsatellite markers have been used (Krafsur *et al.,* 2005). With the advancement of molecular marker techniques, a first microsatellite linkage map of the housefly can now be constructed.

Up to date, the overall consensus among housefly researchers has been that there is little or no recombination in male houseflies (Rubini *et al.,* 1980), similar to *Drosophila* where male recombination is completely absent (Morgan, 1914). Hiroyoshi (1961) found no recombination at all, whereas Sullivan (1961) and Milani (1967) observed some recombination in mutant strains with visible mutations, suggesting that recombination in males might be population dependent (Milani, 1967). In a later study, Lester *et al.* (1979) reported up to 31% male recombination in an Australian housefly strain. Rubini *et al.* (1980), however, attributed the rare occurrence of recombinants of heterozygous males and the appearance of mosaics to mitotic recombination. Few years later, Hiroyoshi *et al.* (1982) also found male recombination in low frequency in several Japanese populations. One aspect that all these studies on male recombination have in common, as also noted by Hiroyoshi *et al.* (1982), is that they investigated populations with autosomal sex determining factors. In the

housefly a diverse array of sex determining factors exist. In so called "standard" populations females are XX and males are XY (Dübendorfer *et al.,* 2002). All individuals are homozygous for the female determining factor (*F*) on chromosome IV. Males additionally possess the male determining factor (*M*) on the Y chromosome which suppresses *F* and leads to male development (Hediger *et al.,* 1998a). In some populations individuals are homozygous for *M* on an autosome and sometimes males carry multiple *M* factors on different autosomes (Franco *et al.,* 1982; Tomita & Wada, 1989b; Çakir & Kence, 1996; Hamm *et al.,* 2005; Feldmeyer *et al.,* 2008; Kozielska *et al.,* 2008). In most populations with autosomal *M*, in particular in populations with homozygous autosomal *M* males, females carry a dominant female determining factor $F^D$, which is insensitive to suppression by *M*, leading to female development even in the presence of *M* (Dübendorfer *et al.,* 2002). IN this study, we investigate recombination rates in three populations, one with XY males and two with autosomal *M* carrying males.

The evolution of sex chromosomes is thought to follow a certain pattern (Charlesworth *et al.,* 2005). After a so-called neo-sex chromosome has acquired sex determining function, recombination will gradually reduce along the chromosome starting from the sex chromosome locus. Due to lack of recombination the sex determining chromosome will gradually degrade. At some point another gene on a different chromosome might take over sex determining function either by transposition of the sex determining gene or evolution of a novel sex determining gene. The "old" sex chromosome may eventually vanish, if it does not contain essential genes anymore. This process is believed to have general application to many organisms with chromosomal sex determination and may also act in the housefly where *M* factors can be found on different autosomes in different populations, turning these autosomes into neo sex chromosomes. With our data we can compare recombination frequencies between autosomes with and without sex determining factors.

Hence, the aims of this paper are threefold. We present the first genetic linkage map of the housefly using molecular markers. By combining microsatellites with traditional visible markers on each of the five autosomes we directly assign the molecular markers and linkage groups to a particular chromosome. In addition, we provide further evidence for male recombination

in houseflies and we consider chromosomal recombination frequency in the context of sex chromosome evolution theory. We expect that our linkage map will be instrumental for future genome studies, such as revealing the nature of autosomal sex determining factors, for annotation of the housefly genome, and for further testing hypotheses of sex chromosome evolution.

## Experimental procedures

### Crosses

We studied the segregation of 35 microsatellites in combination with six visible markers in three different crosses. The microsatellites are a combination of the newly developed microsatellites that we report here, and microsatellites that have been published earlier (Endsley *et al.,* 2002; Chakrabarti *et al.,* 2004). For each cross we used a mutant marker strain (012345-1) recessive for visible traits on each of the five autosomes (*ali curly* (*ac*) on linkage group 1; *aristapedia* (*ar*) on 2; *brown body* (*bwb*) on 3; *yellow eyes* (*ye*) on 4; *snip wings* (*snp*) on 5) (see Tomita & Wada, 1989b). This strain has been used by several authors to determine the position of the male determining factor *M* in natural populations by back crossing wild type males with mutant females (Tomita & Wada, 1989b; Feldmeyer *et al.,* 2008; Kozielska *et al.,* 2008). Since the visible mutations have been cytologically assigned to chromosomes (Wagoner, 1967) we can directly associate the markers with linkage groups and chromosomes.

The wild type males in our crosses came from populations which contained both autosomal *M* factors and *M* on the Y chromosome. We individually crossed wild type males to mutant females. F1 male offspring were backcrossed to mutant females. Because of sex-linked inheritance of the phenotype, the F2 reveals the location of the *M* factor (for more details see Denholm *et al.,* 1983). For the linkage analysis we chose the strain FVG, collected in Faverges, France (2004), with autosomal *M* on chromosome II (this cross will be called M2-cross) and the strain WAD, collected in Warden, South Africa (2005), with autosomal *M* on chromosome III (this cross will be called M3-cross). Since females from the mutant strain are homozygous at almost all loci, these two crosses result in “male only” maps as recombination information will stem exclusively from males. The third cross was constructed by mating a female from the strain UML, collected in Umhlali, South Africa (2005), to an XY male

of the mutant strain (MY-cross). Males and females of the resulting F1, thus brothers and sisters, were mated to create the F2 generation. This cross resulted in recombination information for both female and male. We genotyped 58 offspring of the M2-cross, 98 offspring of the M3-cross and 80 offspring of the MY-cross, resulting in an overall number of 236 individuals for the combined linkage map.

## Microsatellite development and genotyping

Genomic DNA of male houseflies was collected from four different strains; two laboratory strains (WHO, World Health Organization and the 012345-1 mutant strain, both obtained from Zürich) and two wild caught strains (FVG, Faverges, France and MID, Midlaren, The Netherlands). Males of these strains carried the Y chromosome. DNA was extracted using a standard proteinase K/salt-chloroform protocol and pooled for all stains.

An enriched library was made by Ecogenics GmbH (Zürich, Switzerland) from size selected genomic DNA ligated into SAULA/SAULB-linker (Armour *et al.,* 1994) and enriched by magnetic bead selection with biotin-labelled (GA) 13 and (TAC) 8 oligonucleotide repeats (Gautschi *et al.,* 2000). Of 951 recombinant colonies screened, 271 gave a positive signal after hybridization. Plasmids from 192 positive clones were sequenced of which 168 yielded microsatellite sequences. Fourty-three out of the 168 sequences were duplicates leaving 125 sequences that were analyzed with the software Tandem Repeat Finder (Benson, 1999) to identify the repeat motif, length and position of the repeat sequence. The microsatellite motives were tandem repeats of either CT (52%) or AG (48%). Primers were designed using the software PRIMER3 (Rozen & Skaletsky, 2000). Fourty-three sequences (34%) were either too small or the repeat flanking region was too small for primer design, leaving 82 sequences for which primers could be designed.

A total of six individuals (three females and three males from three different strains) were initially screened for marker amplification and polymorphism on a 5% agarose gel. Thirty-eight primer pairs failed to amplify or gave dubious amplification patterns and were discarded for further analysis. From the remaining fourty-four markers the forward primers were labeled with a fluorescent dye (FAM, HEX or NED). PCR reactions were performed in 1X

PCR buffer magnesium free (Promega) with 2.5 mM $MgCl_2$, 0.2mM dNTPs (Roche), 0.2µM of each primer, 0.4 units of Taq polymerase (Promega) and approximately 5ng of template DNA. The PCR profile was 1 cycle of 15 min at 95°C followed by 25 cycles of 30 sec at 94°C, 90 sec at the primer specific annealing temperature (Table 8.1), 60 sec at 72°C, and a final cycle of 10 min 72°C. Reactions were carried out in an Eppendorf mastercycler gradient machine. PCR products were analyzed on an ABI 3730 automatic sequencer with ROX-500 as size standard. The size of the fragments was calculated using GeneMapper 4.0 software (Applied Biosystems).

Of the 44 loci tested, eleven turned out to be monomorphic or gave unreliable results and 33 were polymorphic and suitable for use (Table 8.1). The nomenclature for the microsatellites is equivalent to Endsley *et al.* (2002), with Md referring to *M. domestica*, followed by the repeat type and the microsatellite sequence number. Additionally we developed one more microsatellite marker from available microsatellite sequences in GeneBank (Table 8.1).

## Linkage analysis

We constructed a linkage map for each of the three crosses separately using JoinMap 3.0 (Van Ooijen & Voorrips, 2001). We used the population type code "CP" in JoinMap to allow for heterozygous and homozygous diploid parents and assigned genotype codes for each locus depending on the segregation type (for details see the JoinMap manual). All markers were tested for significant deviation from Mendelian segregation by $\chi^2$ analysis ($p < 0.01$). Markers that deviated significantly from Mendelian expectations were included in linkage groups if their presence did not alter the order established without them. Marker placement was determined using a minimum LOD score threshold of 4.0. The Kosambi mapping function that incorporates the possibility of crossover interference was used to convert recombination frequencies into map distances (Kosambi 1944). After establishing separate linkage maps per cross we joined the linkage maps by using the "combine groups for map integration" command of JoinMap for groups that had enough overlapping markers and linkage was

**Table 8.1:** Newly developed microsatellite markers for the housefly, *M. domestica*. $T_a$ = annealing temperature.

| Name | GenBank Accession | Repeat length | Primer sequence | | $T_a$ |
|---|---|---|---|---|---|
| | | | Forward | Reverse | |
| MdCT220 | FJ231915 | 13 | TGCTGTTGTGACCTCGACTC | AAATGAAAAATTCCGCCAAG | 56 |
| MdCT222 | FJ231914 | 44 | GGCAATGACCTCTTGACCTT | AAACTCATAGCCTGCGTTCG | 56 |
| MdAG224 | FJ231912 | 18 | ACTGCCCTTCTCCACTTCCT | TTTGACCGAAGGTATGACCA | 56 |
| MdAG227 | FJ231910 | 23 | TATTGCAGCTCCCCCATAAG | TGGTCAATGGTTTCAGGTCA | 56 |
| MdAG228 | FJ231909 | 15 | CTCCAACCAGCCACCATATC | TTTTGGGTTCACGAGAGAGG | 56 |
| MdCT238 | FJ231905 | 19 | TGCAATGGAAAGACAACAGG | GTGGCGTTGATTTTCCTGAC | 58 |
| MdCT268 | FJ231922 | 13 | CTTCATCAGACCCACAATTTCA | TTAGCAAACGCCAACATCTG | 56 |
| MdCT289 | FJ231930 | 16 | TCGGCATATGAACGATTTGA | CGGTGACCCGCTACTCTTTA | 58 |
| MdCT297 | FJ231934 | 22 | AGACAAAGTTTCCAAGTGAGAATATG | TAGAGCGTTGCTCGCTTACA | 56 |
| MdAG290 | FJ231931 | 13 | CGACTGATTGTCAGCATGGA | CCATCTGCAAAAAGAACAATACA | 56 |
| MdCT291 | FJ231932 | 22 | CATCCGTCGGTTCATTCATT | ATGCAATCTTCTCGGCTCAC | 56 |
| MdCT302 | FJ231937 | 22 | AGTTTTCTCCGGCAGTCGT | GTCCAGTGTCACCAAATCCA | 56 |
| MdCT322 | FJ231943 | 19 | AACAATTTATGCCGGCTCAG | TCTTCAGGTCCTCTGCAACC | 58 |
| MdAG324 | FJ231944 | 14 | TTCCCATGAAAAATGTCAGC | CCACTCATTCTGGTACCTCCA | 56 |
| MdAG328 | FJ231945 | 15 | GTGGGGTGTGCACAAGAAG | CCCGTGTAGAAAGTGTGCAA | 56 |
| MdAG329 | FJ231946 | 18 | CTGCAATGATGTGAGGTTGG | AACAATTTATGCCGGCTCAG | 60 |
| MdCT339 | FJ231949 | 15 | GGCGCACACTCTACATAGCA | GAGCGTTTGAGAGCTTAGCA | 56 |
| MdAG357 | FJ231952 | 31 | TCGTAAGACTGGCGAAAAGAA | AGACTCCTCGGTCATCAAAAA | 56 |
| MdCT364 | FJ231955 | 16 | CACCCGTGTAGAAAGTGTGC | GGGGTGTGCACAAGAAGAAG | 56 |
| MdAG372 | FJ231960 | 19 | GTCCGACTTCTGGTCGAAAG | CATTTTCCGCTTCTGCTTGT | 60 |
| MdCT373 | FJ231961 | 15 | CGGATGGTGAGAATTGTTTTC | CAAGGGAGCTGAGAGAAACG | 56 |
| MdAG422 | FJ231976 | 21 | TAGAGCGTTGCTCGCTTACA | CTAGACAAAGTTTCCAAGTGAGAAT | 56 |
| MdCT234 | FJ231907 | 20 | GCTACAAACGGAATGACGA | TCGCGATCCTGGAAAATTAG | 56 |
| MdCT243 | FJ231903 | 17 | CGGTGGCAGATAAACTTCCT | CAGAAAATGAGCAGTGGTCAAA | 58 |
| MdAG247 | FJ231900 | 12 | CCTCCCACAAATGAATGGTC | ATTTTGAAGAAAGCCGCTCA | 56 |
| MdCT269 | FJ231923 | 16 | CGATGTAGAAGCTGGCTGTG | GCCTGCCTTCAGCTCTTCTA | 58 |
| MdCT276 | FJ231926 | 17 | TTCAAGGCGACTACTGCAAA | ACGACGTTTCGGTCTTGTCT | 56 |
| MdAG318 | FJ231941 | 23 | ATGAGCGTTTTGGATGTTCC | TTTCCGTTTAGATCGCATCC | 56 |
| MdCT319 | FJ231942 | 15 | GCGATTTCCGTCTCTCAGTC | TGGGTATGTCTCGCTTCCTT | 56 |
| MdAG336 | FJ231948 | 20 | ACAAACTGCTGGACAACGAA | GAACTTACACCGCAACAGCA | 56 |
| MdAG341 | FJ231951 | 24 | TGCCACAGAAGCATAAGAGG | TAGGCGCGAAGGGACTAATA | 56 |
| MdCT399 | FJ231969 | 19 | TTCGTATTCCAAAATCGGTTC | TTTTATCGGTTGGTGTCGTG | 56 |
| MdCT413 | FJ231973 | 21 | TCTTTCGCTCTCTCTCTCTCTAAAA | ACAAACCCAACCCTGAGAGA | 56 |
| MdCAG78[1] | AF380993 | 24 | GCAAGGTGAAAAAGGTCCAG | CGGGAGYAGCATCCATTTTC | 56 |

[1]sequence previously published in GenBank

sufficient. This was not possible for linkage group (=chromosome) IV as only the M2-cross yielded more than two linked markers on this group.

We note that the conventional way of constructing a linkage map is to analyze both sexes separately when recombination frequencies differ. As the number of linked markers to construct a "female only" map was too small and the number of linked markers increased by including female recombination information we included both sexes in one map (for the MY- and the combined map). To obtain pairwise recombination frequencies for all markers per linkage group, and also separate for each sex (MY-cross) we used the LINKMFEX.exe module of the LINKMFEX v2.3 program (R. Danzmann, University of Guelph, http://www.uoguelph.ca/~rdanzman/software/LINKMFEX). To determine whether recombination frequencies of autosomes carrying sex determining factors differ from autosomes without them, we performed a Wilcoxon rank sum test in R (R Development Core Team, 2006) by introducing a variable coding for autosomes with *M* or *F* (1) versus autosomes without (0). Since the number of similar marker pairs between the crosses is small, as well as the overall number of markers on certain linkage groups, we compared the average recombination frequency of all possible marker pairs per chromosome and cross.

## Map length and coverage

Two approaches were used to estimate the map length of *M. domestica*: 1) $G_{e1}$: to compensate for the two chromosome ends beyond the outer most marker of the linkage group 2s (s = average spacing of the linkage map) were added to the length of each group (Fishman *et al.,* 2001); 2) $G_{e2}$: each linkage group was multiplied by the factor $(m + 1)/(m\text{-}1)$, where $m$ is the number of markers in each linkage group, irrespective of markers mapping to the same location. The estimated map length is the sum of the revised length of all linkage groups (Chakravarti *et al.,* 1991). The final estimated map length ($G_e$) is the average of the two estimated map lengths. The observed map length was calculated as the length of the framework map ($G_{of}$). Map coverage then was calculated as $G_{of}$ / $G_e$.

## Results

A total of 236 F2 progeny and backcross parents from three crosses (referred to as M2, M3, and MY) were genotyped for 58 microsatellite markers. Of the 33 newly developed microsatellite markers 20 turned out to be informative in at least one of the crosses analyzed. Seventeen of the previously published 25 microsatellite markers (Endsley *et al.,* 2002; Chakrabarti *et al.,* 2004), plus one marker developed from a GenBank sequence, were informative in at least one of the crosses (Table 8.2). A total of 35 microsatellite markers, six visible mutations plus "sex" as seventh visible trait mapped into five linkage groups, which correspond to the five autosomes of the housefly. None of the microsatellite markers mapped to the X or the Y- chromosome. Three markers, MdCT222, MdAG228 and MdCA06, did not map to any of the linkage groups.

**Table 8.2:** Overview of informative markers per cross. Prefix Md has been omitted. Underlined markers were analyzed in more than one cross. Markers in parentheses did not map to any of the linkage groups. Earlier published markers are described in Endsley *et al.* (2002) and Chakrabarti *et al.* (2004).

| Cross | No.offspring | Polymorphic markers | |
|---|---|---|---|
| | | Newly developed | Earlier published |
| M2 | 58 | CT238, CT291, CT289, CT297, CT322, CT339, CT364, CT373, AG224, AG227,AG290,AG324, AG422 (AG228, AG357) | CA104, CA117, CA119, CA121, CA148, CA154, CA155, CA224, CA226, HF25, HF31, HF33, HF44 (CAG34) |
| M3 | 98 | CT238, CT289, CT297, CT302, CT339, CT364, CT373, AG224, AG324, AG328, AG357, AG372, AG422 (AG227, CT291, AG329, CT222) | CA104, CA154, CA170, HF33, HF44 |
| MY | 80 | CT268, CT291, CT297, CT302, AG329, AG422, CAG78 (CT322, AG224, AG290) | CA104, CA117, CA170, CA202, CA224, CAG34, HF31, HF44 (CA06) |

**Table 8.3:** Observed and estimated map lengths and coverage for each of the three crosses separately and the combined linkage map. Values are based on all five autosomes for the M2-cross and the combined map, but for the M3- and MY-cross no linkage group IV was available.

| | M2 | M3 | MY | Combined |
|---|---|---|---|---|
| Observed map length (cM) | 78 | 64 | 165 | 184 |
| Estimated genome length (cM) | 110.5 | 92.8 | 252.12 | 230.91 |
| Coverage (%) | 70.59 | 68.97 | 65.45 | 79.68 |
| Number of markers | 26 | 18 | 15 | 35 |

For the M2-cross (where males carry the *M* factor on autosome II) five linkage groups were found, representing all five autosomes ranging in size from 6-34 cM and consisting of 3-11 markers per group. The total linkage distance covered by these markers was 78 cM with an average spacing of 3.0 cM between markers for the whole framework map (Table 8.3). For the M3-cross (*M* factor on autosome III) linkage groups for autosomes I-III and V were found, ranging in size from 3-30 cM and consisting of 5-6 markers per group. The total map size was 64 cM with an average spacing of 3.2 cM between markers. The M2 and M3-crosses yielded recombination frequencies for males only, since the females are homozygous for almost all markers (see Experimental procedures for details). Although potentially possible, we did not construct maps separately for each sex in the MY-cross, because the number of markers per linkage group in females was mostly too small. For the MY-cross we found linkage groups for autosomes I-III and V, ranging in size from 12-62 cM and consisting of 3-8 markers per group. The total linkage distance covered was 165 cM, which is on average 2.3 times the size of the autosomal *M* maps, and with an average marker spacing of 9.2 cM. After joining the three maps, the combined map consisted of five linkage groups ranging in size from 7-62 cM, containing 3-14 markers per group and a total map size of 184 cM with an average spacing of 4.5 cM between markers (Figure 8.2).

The estimated map length for the combined map was 230.9 cM, which is the average of the two methods (see Experimental procedures), respectively 228.9 and 232.9 cM (Table 8.3). The combined map covers about 79.7% of the genome, calculated as the observed length of 184 cM divided by the estimated length of 230.9 cM.

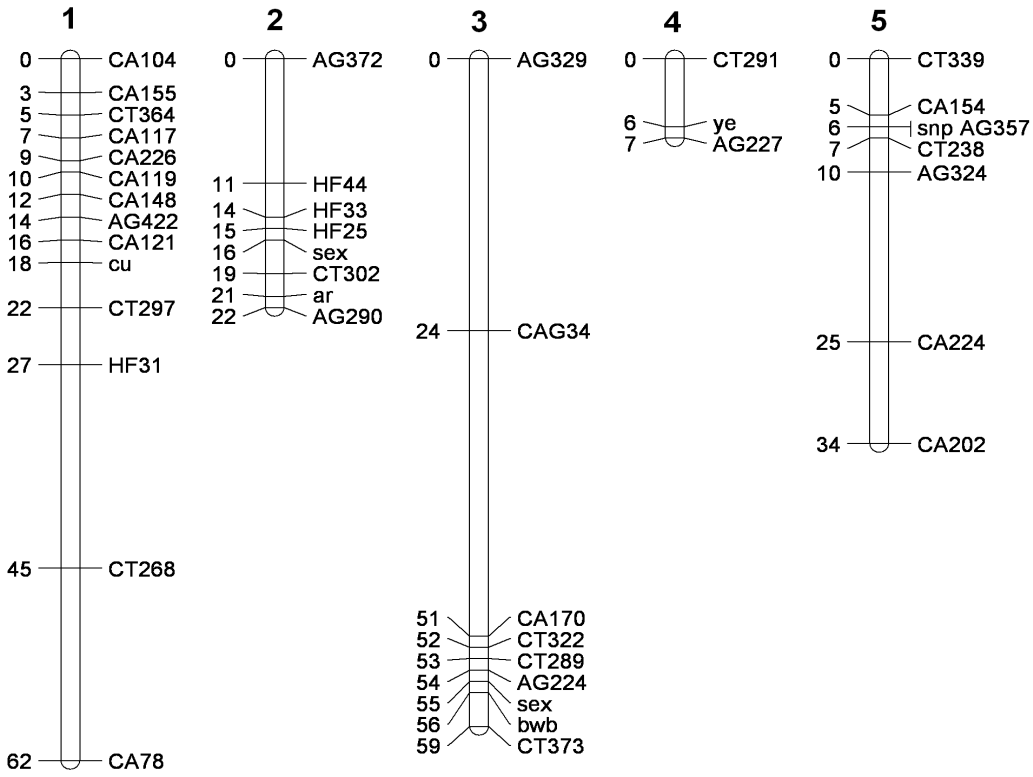


**Figure 8.1:** Linkage map of the housefly, from combining three different mapping populations. Markers are indicated on the right; map distances (in Kosambi cM) on the left of a chromosome. Linkage groups are arranged by chromosome number according to Wagoner (1967). The number of linkage groups corresponds to the number of autosomes, no markers were found on the sex chromosomes. Each chromosome contains one visible marker (*cu, ar, bwb, ye, snp*, see Experimental procedures), the marker "sex" occurs twice as it was once mapped with a strain that contained *M* on the second and once with a strain that contained *M* on the third autosome, and all other markers are microsatellites.

Based on 19 marker pairs distributed over four autosomes and mapped in both sexes, the average female recombination rate was estimated to be 1.8 times higher than in males (23% compared to 12%). Single pairwise recombination rates in males between markers with LOD>3 varied between 0-29%. Average pairwise recombination rates for all mapped markers ranged from 0.06 – 0.23 per autosome (Table 8.4). Autosomes carrying one of the sex determining factors (*M* or *F*) show significantly reduced recombination frequency compared to autosomes without sex determining factors (Wilcoxon rank sum test, W = 26299, $p<0.0001$), consistent with predictions of sex chromosome evolution.

**Table 8.4:** Average male recombination frequencies for all possible pairwise marker combinations per autosome for each cross. Values in parentheses indicate the number of marker pairs. Average recombination frequencies on chromosomes containing a sex determining factor is significantly reduced, indicated in bold (Wilcoxon rank sum test, W = 26299, $p<0.0001$).

| Cross | Autosome | | | | |
|---|---|---|---|---|---|
| | I | II | III | IV | V |
| MY | 0.16 (21) | 0.22 (3) | 0.23 (6) | - | 0.21 (3) |
| M2 | 0.17 (45) | **0.10** (15) | 0.19 (15) | **0.08** (3) | 0.21 (21) |
| M3 | 0.18 (10) | 0.18 (10) | **0.06** (15) | - | 0.19 (15) |

## Discussion

We present the first genetic linkage map of the housefly, *Musca domestica*, using microsatellite markers. With the help of visible markers that had previously been assigned to the five autosomes we were able to place 35 microsatellite markers on to five linkage groups representing the five autosomes. We did not find any markers linked to either the X or the Y chromosome, which is most likely due to technical reasons. Similar to the medfly (Stratikopoulos *et al.,* 2008), the X and Y chromosome of the housefly consist mainly of heterochromatin (Hediger *et al.,* 1998b). Heterochromatic regions are known to be refractory to cloning and sequencing strategies (International Human Genome Sequencing Consortium, 2004), which would explain their absence in our library.

The distribution of microsatellite loci along the linkage map appears to be non-random. In all five linkage groups we find clusters of markers towards one end of the linkage group. Non-random distribution of microsatellite markers along linkage groups has also been observed in for example rice, zebrafish and the medfly (Shimoda *et al.,* 1999; La Rota *et al.,* 2005; Stratikopoulos *et al.,* 2008). In rice the accumulation of microsatellites in certain regions of the genome is correlated with gene-rich regions (La Rota *et al.,* 2005), but in zebrafish it was attributed to the accumulation of CA/GT sequences in these chromosomal regions (Shimoda *et al.,* 1999).

The recombination density found in this study is quite low with 0.8 cM / Mb (total map size of 229.6 cM estimated in this study divided by 295 Mb according to Gao & Scott (2006), but is comparable to other Dipteran insects where recombination densities range between 0.1-3.1 cM / Mb (reviewed and discussed in Wilfert *et al.,* 2007).

Studies on housefly male recombination have found varying results, ranging from no recombination (Hiroyoshi, 1961; Rubini *et al.,* 1980) up to 31% (Lester *et al.,* 1979). With our crosses we confirm the occurrence of recombination in males as we find recombination frequencies up to 29%, thus supporting the claim of Lester *et al.* (1979) to revise the assumption of recombination absence in male houseflies. We did not only find recombination in crosses with autosomal *M* males but also in the MY-cross on all autosomes (Table 8.4). It is not possible to discern whether this is due to crossing two different strains, i.e. two unrelated genomes disrupt recombination suppression in males, or whether recombination actually occurs widespread in "standard" XY populations.

The occurrence of male determining factors on different autosomes in various populations make the housefly a suitable organism for studying sex chromosome evolution. All hitherto studies of male recombination have in common that they investigated populations with autosomal *M*. From studies investigating frequencies of sex determining factors we know that $F^D$ and autosomal *M* often co-occur in populations (Tomita & Wada, 1989b; Kozielska *et al.,* 2008, Feldmeyer *et al.,* 2008). This has two important implications for the evolution of recombination suppression as envisaged in the hypothesis of Charlesworth *et al.,* (2005). First, autosomal *M* chromosomes can recombine in females with $F^D$ which makes it harder for any male recombination suppressor

to settle on the *M* carrying autosome. Second, $F^D$ carrying females are the heterogametic sex and one would rather expect females to evolve reduced recombination rates. Charlesworth *et al.* (2005) predict that one should be able to detect differences in recombination frequency between differentially advanced sex chromosomes. We have indications that the recombination frequency is reduced on chromosomes with sex determining function as both *M* carrying autosomes II and III, and the *F* carrying autosome IV were found to have reduced recombination. Our analysis is still limited in resolution since we can only compare recombination frequencies of a limited number of shared markers between the crosses. In addition, we use all possible pairwise combinations instead of only adjacent marker pairs which violates independence of used recombination rates. Ideally one should compare recombination frequencies of autosomes with and without *M*, which may allow to (1) estimate the relative time that has passed since the autosome has acquired sex determining function, assuming that the lower the recombination frequency, the longer *M* is located on the autosome; (2) investigate whether similar changes take place on different chromosomes with sex determining function; (3) study sex chromosome evolution in a male versus female heterogametic system.

We hope that this linkage map will serve as starting point for further gene mapping studies such as identifying economically important insecticide resistance genes, locating and characterizing sex determining factors, and to further test hypotheses of sex chromosome evolution. Finally we express the wish that the map will increase the chance of a future housefly genome project (Gao & Scott, 2006).

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# Summary and Conclusions

Temperature is the result of the motion of molecules, and increases as the molecules move faster. As organisms are largely made of molecules, this simple principle has important implications on all levels of biological organization. From simple chemical reaction rates, via individual organisms, to entire ecosystems, all are affected by temperature one way or another (Johnston & Benett, 1996). This thesis is concerned with one specific effect of temperature, namely on the sexual development of animals.

In many cold blooded species, like many reptiles and fish, the sex of an individual is determined by temperature during embryogenesis with some temperatures leading to female and others to male development. This is called temperature dependent sex determination (TSD). For example in turtles, high incubation temperatures lead to female and low temperatures to male development. In crocodiles the opposite pattern is observed with high temperatures resulting in male and low temperatures in female development. In contrast, in most other organisms, such as all mammals and birds, the sex of an individual is determined by its genotype. Such a system is called genetic sex determination (GSD). Recent work on the molecular basis of sex determination has revealed that the dichotomy between GSD and TSD may not be as sharp as previously believed. The underlying gene networks are quite similar and small changes can convert a GSD system into TSD and vice versa. In addition, more and more species are being discovered where both genes and temperature affect sexual development, and where multiple sex determining (SD) systems coexist within one species. Phylogenetic analyses strongly indicate that multiple transitions between GSD and TSD, in both directions, have occurred. Finally, the geographic distribution of sex determining mechanisms also follows temperature gradients, TSD being confined mostly to warmer regions of the globe, and different types of GSD sometimes follow geographical clines as well. These evolutionary and geographical patterns are still far from being well-understood, and it is the goal of this thesis to bring us closer to such an understanding.

In general, the effects of temperature on sex determination can be classified in two categories. On the one hand temperature can be viewed as proximate trigger that directly affects organisms themselves, such as in TSD. On the other hand temperature can be seen as ultimate cause, setting the selective environment for

the evolution of and transition between different sex determining systems, thereby also affecting the geographical distribution of sex determining systems in nature. In this thesis the main emphasis lies on the second category with the focus on the ultimate effect of temperature leading to the transition between different sex determining systems and their distribution. However, ultimate issues cannot properly be addressed without considering the underlying proximate mechanisms as well.

This thesis is divided into two parts. In the first part a modeling approach is taken to address questions about evolutionary transitions between GSD and TSD and the conditions which favor coexistence of different SD systems. However, this part is not entirely separated from reality, since some of the models are parameterized with empirical data from the snow skink, *Niveoscincus ocellatus*. The second part of this thesis is concerned with empirical research on the housefly, *Musca domestica*, a global species with geographical clines in frequencies of different GSD systems. With experiments and fieldwork a suite of hypotheses regarding the origin of these geographical distributions were systematically tested.

## Part I: Theoretical modeling approach

There are several hypotheses regarding the evolution of TSD from GSD (Ewert & Nelson, 1991; Shine, 1999), but by far the most influential hypothesis is embodied in the model of Charnov and Bull (1977). The basic idea is that the fitness of males and females is differentially affected by variation in environmental conditions. Specifically, under some conditions females have higher expected fitness than males, while other conditions benefit males more. TSD is a mechanism that allows for flexible adjustment of sex ratios in situations where biased sex ratios are selectively favored. GSD does not readily allow for such flexible facultative sex ratio adjustments, thus giving TSD a selective advantage in these cases. However, the very flexibility of TSD can also be a liability when fluctuations in temperature between years are sufficiently large. In that case, years with extreme temperatures may lead to very biased sex ratios among the offspring, and thereby cause (near) population extinction. This is less of a problem in long-lived species, where the fluctuations over a lifetime tend to cancel each other out.

In **Chapter 2** a model is presented that does not assume environment dependent sex-specific fitness effects, but is based on selection for unfacultatively biased sex ratios due to local kin competition. Sex ratio theory predicts that the more dispersive sex should be overproduced since it is least likely to compete with relatives for resources or space. The basic idea is that under these conditions TSD is likely to evolve because it allows for unfacultatively biased sex ratios and therefore has an advantage over GSD. In the model the degree of environmental fluctuations and longevities was systematically varied to understand the interplay and importance of the different factors. Starting with a GSD population it was investigated whether and under which conditions TSD might evolve. Five different outcomes were observed: (i) the initial GSD state was stable; (ii) TSD evolved and took over; (iii) a new type of GSD evolved at a second locus; (iv) GSD and TSD stably coexisted; (v) multiple coexisting GSD systems evolved. In line with earlier work, TSD evolved more easily when environmental fluctuations were small and when lifespan was long. In the simulations rapid evolutionary transitions between GSD and TSD were common even without the assumption of temperature dependent sex-specific differential fitness, thus offering a new, additional explanation for the evolution of TSD. Another important insight from the model is that multiple sex determining systems can evolve from identical initial conditions, which may be governed by random factors such as genetic drift.

Although conceptual models as in Chapter 2 are important to gain general insights into the evolution of TSD, more specific models tailored to specific species are needed to test these insights. Most species are unsuitable to test hypotheses on the evolution of TSD because they possess a single sex determining system. The snow skink *Niveoscincus ocellatus* is an exception since both GSD and TSD populations exist within this species in different geographical regions. Moreover, and a large amount of knowledge about the life history of this species has accumulated over the years. Females in the TSD population that are born early during the breeding season have a higher probability of reproducing one year earlier compared to late born females. In the GSD population the probabilities of breeding early still differ between early and late born females, but to a lesser extent. In both populations male fitness is independent of birth date. While early and late born females in the GSD population show fitness differences, large between-year temperature

fluctuations in their range might lead to a selective advantage of GSD. In **Chapter 3** models parameterized with data from the skinks life history and climatic data were used to predict which SD system would evolve in the different skink populations. The results show that the model correctly predicts the sex determining system for each population based on local temperature variation and demography of the skinks.

Three main conclusions can be drawn from this theoretical part of the thesis. First, selection for unfacultatively biased sex ratios can lead to transitions between GSD and TSD, even without assuming temperature dependent sex-specific fitness effects. These results might offer a new explanation for the evolution of TSD and might explain why several studies failed to find sex specific fitness effects in certain species. Second, the simulations did not only result in transitions from GSD to TSD but also in multi-factorial GSD, and coexistence of GSD and TSD, hence providing a potential explanation for observed instances of coexistence. Third, tailoring an evolutionary model to a specific species can be useful to make predictions on the evolution of sex determining systems in this species.

## Part II: Empirical Approach

In the second part of this thesis the main goal was to study the effect of temperature variation on the distribution of sex determining (SD) factors in the housefly, which was investigated by a series of experiments and statistical analyses. The default sex of houseflies is female since all individuals carry the female determining factor *F*. Individuals that carry an additional male determining factor *M*, which suppresses the function of *F*, turn into males. In the so called standard XY system, which is most common at high latitudes and high altitudes, *M* is found on the Y chromosome. In populations at lower latitudes and altitudes the *M* factor can be found on any, or even multiple of the five autosomes. In populations with autosomal *M* factors females often posses a mutated version of *F*, the $F^D$ factor, which is insensitive to *M*.

But what causes these clines? The most obvious factor that varies along latitude is temperature. This lead several authors to suggest that the distribution of SD factors is somehow caused by variation in temperature (Franco *et al.*,

1982; Çakir & Kence, 1996); to what extent this is the case is the subject of this part of the thesis.

A simple hypothesis that could explain the distribution of SD factors is that temperature affects the fitness of flies, in a way that depends on their sex determining factors. Therefore the goal of **Chapter 4** was to experimentally investigate whether houseflies with autosomal *M* or $F^D$ have higher fitness at higher temperatures compared to standard XY flies. To determine whether autosomal *M* factors could invade the standard XY population, some males with the *M* factor on autosomes II and III were introduced into standard XY populations under different temperatures, and their frequency was tracked over several generations. The results were less straightforward than expected. Although *M* on autosome II replaced the Y, *M* on autosome III did not increase in frequency and no effect of temperature was detected. To compare the fitness of females with and without $F^D$, several fitness parameters of female flies with either sex determining factor were measured at different temperatures. There was great variation between populations, but again, as in the male experiment, no effect of temperature was detected.

In the previous chapter none of the investigated fitness parameters showed an influence of temperature. For **Chapter 5** the goal was to study an additional fitness component, the frequency of intersexes (low fitness individuals possessing both male and female characteristics) among offspring. It has been reported that the frequency of intersexes in the housefly increases in winter (Milani, 1967), which lead to the hypothesis that their frequency increases at cold temperatures. Therefore intra- and inter-population crosses with standard XY as well as autosomal *M* populations with different frequencies of $F^D$ were conducted and distributed over different temperatures. For each of the crosses offspring were examined for intersex characteristics. No effect of temperature on the frequency of intersexes at any of the temperature treatments was detected. However, population sex ratios were affected by temperature in some of the experiments. This suggests that sexual development might be influenced by temperature but further experiments are needed to investigate this more closely.

Another way of investigating the dynamics of and selection on housefly SD factors besides studies in the laboratory over short time spans is to study long term changes in distribution patterns in the field. Autosomal *M* factors were first reported some 50 years ago, and it was hypothesized that they are spreading northward, replacing the standard XY system. **Chapter 6** reports on the collection of houseflies from populations across Europe, that had been studied 25 years ago (Franco *et al*., 1982), in order to investigate how and if the distribution had changed over time. In contrast to earlier predictions no clear change in the distribution of autosomal *M* factors in comparison with the distribution 25 years ago was detected. This implies that autosomal *M* factors do not have a uniform advantage over the standard *M* factor, since otherwise it would have completely replaced the latter. The apparent stability of the cline suggests that XY has an advantage in the north, while autosomal *M* factors seem to have an advantage in the south.

For **Chapter 7** the goal was to further investigate the proposition that the geographical distribution of housefly SD factors is caused by variation in temperature. If this is indeed the case one would expect to find similar distribution patterns in the southern hemisphere compared to the once found in the north. Therefore samples were collected from several locations in South Africa and Tanzania. The results show that housefly populations on the southern hemisphere repeat the pattern earlier found on the northern hemisphere, higher frequencies of autosomal *M* and $F^D$ factors are observed closer to the equator or lower altitudes.

Not only temperature but also other climatic variables change systematically along latitudinal and altitudinal clines. Nowadays climatic databases are publically available, making it possible to obtain data on a number of climatic factors on a global scale. Using the newly collected data on SD factor frequencies of the southern hemisphere plus data of previously published studies and data on several climatic variables a meta analysis was conducted to investigate which climatic variable(s) can best explain the clinal distribution of the SD factors. The results show that seasonality in the degree of temperature variation rather than mean temperature best explains the distribution of the male SD factor, in the sense that autosomal *M* factors are more common in places with less seasonal temperature fluctuation. For female SD factors on the other

hand the combination of humidity and yearly mean temperature best explains their distribution, low humidity and high mean temperature being positively correlated with the frequency of $F^D$.

For **Chapter 8** the main goal was to extent the available molecular tools for studying the housefly by developing new microsatellite markers and constructing a linkage map. The markers mapped to five linkage groups, corresponding with the five autosomes of the housefly, but none of the markers mapped to either the X or the Y chromosome.

Theory on sex chromosome evolution predicts that recombination rates on chromosomes acquiring sex chromosome function should decrease along the chromosome starting from the location of the sex determining gene. The reason for this is, that genes accumulate on the Y chromosome for example, which are beneficial for males, however disadvantageous for females. Reduced recombination rates prevent these genes from ending up in females. For the housefly this implies that recombination rates on autosomes containing the *M* or *F* factor should also show a decrease in recombination rates compared to autosomes without. To test this prediction, recombination rates of autosomes containing a SD factor were compared with autosomes without, by making use of markers of the specific autosomes based on the results of the linkage analysis. A statistical analysis revealed significantly reduced recombination rates on autosomes with SD factors, in line with theoretical expectations.

Given our results of the housefly experiments can we now claim that we understand the geographical distribution of the sex determining factors and the role of temperature? On the one hand the finding that the distribution of SD factors in the southern hemisphere follows the patterns found in the north hints towards a role of temperature in shaping these clines. On the other hand experiments failed to show an effect of temperature on the fitness of houseflies with different SD factors. This is perhaps not too surprising in the light of the subsequent meta analysis, which suggests that not temperature per se but rather seasonality or the combination of humidity and mean temperature might be responsible for the clinal distribution of SD factors. Whether the correlations from the meta analysis reflect causal effects remains to be investigated in future experiments.

# Zusammenfassung und Schlussfolgerungen

Temperatur ist das Ergebnis von Molekülbewegungen sie und nimmt zu, wenn sich die Moleküle schneller bewegen. Da Organismen zu einem Großteil aus Molekülen bestehen, hat dieses einfache Prinzip wichtige Auswirkungen auf allen Ebenen biologischer Organisation. Von einfachen chemischen Reaktionsraten über einzelne Organismen, bis hin zu gesamten Ökosystemen, sind alle auf die eine oder andere Weise von Temperatur beeinflusst (Johnston & Benett 1996). Diese Doktorarbeit beschäftigt sich mit einem speziellen Einfluss von Temperatur, nämlich dem Einfluss von Temperatur auf die Geschlechtsbestimmung bei Tieren.

In vielen kaltblütigen Arten, wie z.B. vielen Reptilien und Fischen, wird das Geschlecht eines Individuums während der Embryogenese durch Temperatur bestimmt, indem manche Temperaturen zu Weibchen-, und andere zu Männchenentwicklung führen. Dies wird temperaturabhängige Geschlechtsbestimmung genannt ("temperature dependent sex determination", TSD). Bei Schildkröten zum Beispiel führen hohe Inkubationstemperaturen zu Weibchen, und niedere Temperaturen zu Männchen. Bei Krokodilen findet man das gegenteilige Muster, hier resultieren hohe Temperaturen in Männchen-, und niedere Temperaturen in Weibchenentwicklung. Im Gegensatz dazu wird in den meisten anderen Organismen, wie z.B. Säugern und Vögeln das Geschlecht eines Individuums durch seinen Genotyp bestimmt. Dieses System wird genetische Geschlechtsbestimmung genannt (genetic sex determination, GSD). Neue Studien an der molekularen Grundlage der Geschlechtsbestimmung haben gezeigt, dass die Dichotomie von GSD und TSD möglicherweise nicht so ausgeprägt ist, wie bisher angenommen. Die zugrunde liegenden Gennetzwerke sind sich relativ ähnlich und kleine Änderungen können ein GSD- in ein TSD-System konvertieren und umgekehrt. Zusätzlich werden immer mehr Arten entdeckt, in denen sowohl Gene als auch Temperatur die Geschlechterentwicklung beeinflussen, und in denen mehrere Geschlechtsbestimmungssysteme innerhalb einer Art koexistieren. Phylogenetische Analysen liefern starke Hinweise, dass mehrere Wechsel zwischen GSD und TSD, in beide Richtungen, erfolgten. Letztlich folgt die geographische Verteilung von Geschlechtsbestimmungsmechanismen auch Temperaturgradienten, TSD ist hauptsächlich auf wärmere Regionen des Globus begrenzt und verschiedene GSD Typen folgen auch manchmal einem geographischen Gradienten. Wir sind

noch immer weit davon entfernt, diese evolutionären und geographischen Muster vollständig zu verstehen, und es ist das Ziel dieser Arbeit, uns diesem Verständnis ein Stück näher zu bringen.

Im Allgemeinen können die Effekte von Temperatur auf die Geschlechtsbestimmung in zwei Kategorien unterteilt werden. Auf der einen Seite kann man Temperatur als proximaten Auslöser ansehen, der Organismen direkt beeinflusst, wie z.B. bei TSD. Auf der anderen Seite kann man Temperatur als ultimaten Einfluss ansehen, der die selektive Umgebung für die Evolution und den Wechsel verschiedener Geschlechtsbestimmungs-mechanismen schafft und dabei auch die geographische Verteilung von Geschlechtsbestimmungssystemen in der Natur beeinflusst. In dieser Arbeit liegt der Hauptschwerpunkt auf der zweiten Kategorie, auf dem ultimaten Einfluss von Temperatur, der zu Wechseln zwischen verschiedenen Geschlechtsbestimmungssystemen und deren Verteilung führt. Man kann sich jedoch nicht richtig mit ultimaten Fragen befassen, ohne nicht auch die zugrunde liegenden proximaten Mechanismen in Betracht zu ziehen.

Diese Arbeit ist in zwei Teile unterteilt. Im ersten Teil wird anhand eines Modell-Ansatzes auf Fragen zu evolutionären Wechseln zwischen GSD und TSD eingegangen; es werden auch die Bedingungen diskutiert, die die Koexistenz verschiedener Geschlechtsbestimmungssysteme begünstigen. Dieser Teil ist jedoch nicht vollständig realitätsfern, da einige der Modelle mit empirischen Daten des Gefleckten Skinks, *Niveoscincus ocellatus*, parametrisiert sind. Der zweite Teil dieser Arbeit befasst sich mit empirischen Untersuchungen an der Hausfliege, *Musca domestica*, einer global verbreiteten Art mit geographischen Gradienten in der Häufigkeit verschiedener GSD Systeme. Mit Experimenten und Feldarbeit wurden verschiedene Hypothesen zum Ursprung dieser geographischen Verteilung systematisch getestet.

## Teil I: Theoretischer Ansatz

Es gibt mehrere Hypothesen zur Evolution von TSD aus GSD (Ewert & Nelson, 1991; Shine, 1999), wobei die bei weitem einflussreichste Hypothese in einem Modell von Charnov und Bull (1977) mit inbegriffen ist. Die Grundidee ist, dass die Fitness von Männchen und Weibchen unterschiedlich von Schwankungen verschiedener Umweltbedingungen beeinflusst wird. Genauer

esagt haben Weibchen unter manchen Bedingungen eine höhere erwartete Fitness als Männchen, während andere Bedingungen Männchen begünstigen. TSD ist ein Mechanismus, der es erlaubt, in Situationen, in denen eine Verschiebung des Geschlechterverhältnisses selektiv bevorzugt ist, diesen flexibel anzupassen. GSD erlaubt eine solche flexible fakultative Verschiebung des Geschlechterverhältnisses nicht so einfach, und ermöglicht TSD damit einen selektiven Vorteil in den oben genannten Fällen. Diese Flexibilität von TSD kann jedoch auch von Nachteil sein, wenn Temperaturschwankungen zwischen den Jahren sehr groß sind. In diesen Fällen können Jahre mit Extremtemperaturen eine große Verschiebung des Geschlechtsverhältnisses unter den Nachkommen hervorrufen, und dadurch (beinahe) zum Aussterben der Population führen. In Arten mit langer Lebenserwartung ist dies weniger ein Problem, da sich die Fluktuationen über die Lebensdauer hinweg ausgleichen.

In **Kapitel 2** wird ein Modell präsentiert, in dem keine umweltabhängigen geschlechtsspezifischen Fitnesseffekte angenommen werden, sondern das auf Selektion der nicht-fakultativen Verschiebung des Geschlechterverhältnisses durch lokale Verwandtenkonkurrenz basiert. Die Theorie zum Geschlechterverhältnis sagt voraus, dass das sich am meisten verbreitende Geschlecht überproduziert werden sollte, da es am unwahrscheinlichsten mit Verwandten über Ressourcen und Raum konkurriert. Die Grundidee ist, dass unter diesen Bedingungen TSD wahrscheinlich evolviert, da es eine nicht-fakultative Verschiebung des Geschlechterverhältnisses erlaubt und damit einen Vorteil gegenüber GSD hat. In diesem Modell wurde die Stärke der Umweltschwankungen und der Lebenserwartung systematisch verändert, um das Zusammenspiel und die Wichtigkeit der verschiedenen Faktoren zu verstehen. Beginnend mit einer GSD Population wurde untersucht, ob und unter welchen Bedingungen TSD eventuell evolviert. Fünf verschiedene Ergebnisse wurden erhalten: (i) der ursprüngliche GSD Zustand war stabil; (ii) TSD evolvierte und breitete sich aus; (iii) ein neuer GSD Typ evolvierte auf einem zweiten Lokus; (iv) GSD und TSD koexistierten stabil; (v) mehrere koexistierende GSD Systeme evolvierten. Wie aus früheren Arbeiten bekannt, evolvierte TSD leichter, wenn Umweltfluktuationen gering waren und die Lebenserwartung groß. In den Simulationen waren schnelle evolutionäre

Wechsel zwischen GSD und TSD häufig, sogar ohne die Annahme von temperaturabhängiger geschlechtsspezifischer Fitness. Sie bieten dadurch eine neue, zusätzliche Erklärung für die Evolution von TSD. Eine weitere wichtige Einsicht des Modells ist, dass mehrere Geschlechtsbestimmungssysteme aus identischen Anfangsbedingungen evolvieren können, gesteuert von Zufallsfaktoren wie genetischer Drift.

Obwohl konzeptionelle Modelle, wie in Kapitel 2, wichtig sind, um allgemeine Einsichten in die Evolution von TSD zu gewinnen, sind spezifischere Modelle - zugeschnitten auf spezifische Arten - nötig, um diese Einsichten zu testen. Die meisten Arten sind ungeeignet, um Hypothesen über die Evolution von TSD zu testen, da sie nur ein Geschlechtsbestimmungssystem besitzen. Der Gefleckte Skink *Niveoscincus ocellatus* ist eine Ausnahme, da sowohl GSD- als auch TSD Populationen innerhalb dieser Art in unterschiedlichen geographischen Regionen vorkommen. Darüber hinaus hat sich eine große Menge an Wissen über den Lebenszyklus dieser Art über die Jahre angesammelt. Weibchen der TSD Population, die am Anfang der Brutsaison geboren werden, haben eine höhere Wahrscheinlichkeit ein Jahr früher zu reproduzieren, als Weibchen die später geboren werden. In der GSD Population unterscheidet sich die Wahrscheinlichkeit früher zu brüten zwischen früh- und spätgeborenen Weibchen auch, jedoch in geringerem Ausmaß. In beiden Populationen ist die Fitness der Männchen unabhängig vom Geburtenzeitraum. Obwohl früh- und spätgeborene Weibchen in der GSD Population Fitnessunterschiede haben, führen wahrscheinlich hohe Temperaturfluktuationen in ihrem Verbreitungsgebiet zu einem selektiven Vorteil von GSD. In **Kapitel 3** wurden Modelle parametrisiert mit Skinklebenszyklusdaten (life history) und Klimadaten genutzt, um voherzusagen, welches Geschlechtsbestimmungssystem in den verschiedenen Skinkpopulationen evolvieren würde. Die Ergebnisse zeigen, dass das Modell basierend auf lokaler Temperaturvariation und Demographie der Skinks, die Geschlechtsbestimmungssysteme richtig vorhersagt.

Drei Hauptschlussfolgerungen können aus diesem theoretischen Teil der Arbeit gezogen werden. Erstens: Selektion auf ein nicht-fakultativ verschobenes Geschlechterverhältnis kann zu Wechseln zwischen GSD und TSD führen,

sogar ohne die Annahme von temperaturabhängigen geschlechtsspezifischen Fitnesseffekten. Diese Ergebnisse könnten eine neue Erklärung für die Evolution von TSD darstellen und könnten erklären, warum einige Studien in bestimmten TSD Arten scheiterten, geschlechtsspezifische Fitnesseffekte zu finden. Zweitens: Die Simulationen resultierten nicht nur in einem Wechsel von GSD zu TSD, sondern auch in Koexistenz von verschiedenen GSD Systemen und Koexistenz von GSD und TSD, und bieten somit eine mögliche Erklärung für die beobachteten Fälle von Koexistenz. Drittens: Ein evolutionäres Modell auf eine bestimmte Art zu zuschneiden, kann nützlich sein, um Vorhersagen über die Evolution von Geschlechtsbestimmungssystemen bei dieser Art zu treffen.

## Teil II: Empirischer Ansatz

Im zweiten Teil dieser Arbeit war das Ziel, den Einfluss von Temperaturvariation auf die Verteilung von Geschlechtsbestimmungsfaktoren durch eine Reihe von Experimenten und einer statistischen Analyse zu untersuchen. Bei der Hausfliege sind Weibchen die "Grundeinstellung" in der sexuellen Entwicklung, da alle Individuen den Weibchenbestimmungsfaktor *F* tragen. Individuen, die einen zusätzlichen Männchenbestimmungsfaktor *M* tragen, der *F* blockiert, werden Männchen. Beim sogenannten Standard XY System, das in höheren Breiten und größeren Höhen am häufigsten ist, befindet sich *M* auf dem Y- Chromosom. In Populationen niederer Breiten und Höhen kann der *M* Faktor auf jedem, oder sogar mehreren, der fünf Autosomen gefunden werden. In Populationen mit autosomalem *M* Faktor besitzen die Weibchen oftmals eine mutierte Form des *F* Faktors, $F^D$, der unempfindlich gegenüber *M* ist.

Was führt zu dieser Verteilung? Der offensichtlichste Faktor, der mit Breitengraden und Höhe variiert, ist die Temperatur. Dies brachte einige Autoren zu der Annahme, dass die Verteilung der Geschlechtsbestimmungsfaktoren der Hausfliege irgendwie durch Temperatur verursacht wird (Franco *et al.*, 1982; Çakir & Kence, 1996). Inwieweit dies tatsächlich zutrifft, ist das Thema dieses Teils der Arbeit.

Eine einfache Hypothese, die die Verteilung von Geschlechtsbestimmungsfaktoren erklären könnte, ist, dass Temperatur die Fitness der Fliegen beeinflusst, abhängig von ihren Geschlechtsbestimmungsfaktoren. Deshalb war das Ziel von **Kapitel 4** experimentell zu untersuchen, ob Hausfliegen mit autosomalem *M* oder $F^D$ größere Fitness bei hohen Temperaturen haben, als Standard XY Fliegen. Um herauszufinden, ob sich autosomale *M* Faktoren in einer Standard XY Population ausbreiten können, wurden Männchen mit *M* Faktoren auf den Autosomen II und III unter verschiedenen Temperaturen in Standard XY Populationen eingebracht und ihre Häufigkeit über mehrere Generationen verfolgt. Die Ergebnisse waren nicht so geradlinig wie erwartet. Obwohl *M* auf Autosom II Y verdrängte, erhöhte sich die Häufigkeit von *M* auf Autosom III nicht, und es konnte kein Effekt von Temperatur festgestellt werden. Um die Fitness von Weibchen mit und ohne $F^D$ zu vergleichen, wurden mehrere Fitnessparameter bei verschiedenen Temperaturen untersucht. Es gab große Unterschiede zwischen den Populationen, aber wie bereits in den Experimenten mit Männchen, wurde kein Einfluss von Temperatur festgestellt.

Im vorherigen Kapitel zeigte keiner der untersuchten Fitnessparameter einen Einfluss von Temperatur. In **Kapitel 5** war das Ziel einen weiteren Fitnessaspekt zu untersuchen, die Häufigkeit von Zwittern (Individuen mit geringer Fitness, die sowohl männliche als auch weibliche Charakteristika besitzen) bei Nachkommen. Es ist berichtet worden, dass die Häufigkeit von Hausfliegenzwittern im Winter zunimmt (Milani, 1967), was zur Hypothese führte, dass deren Häufigkeit mit kalten Temperaturen zunimmt. Darum wurden Intra- und Interpopulationskreuzungen mit Standard XY und autosomalen *M* Populationen mit unterschiedlicher Häufigkeit von $F^D$ durchgeführt und diese über mehrere Temperaturen verteilt. Von jeder dieser Kreuzungen wurden die Nachkommen nach Zwittercharakteristika untersucht. Bei keiner der Temperaturbehandlungen konnte ein Effekt von Temperatur auf die Häufigkeit von Zwittern festgestellt werden. Das Geschlechterverhältnis in einigen der Populationen zeigte jedoch einen Einfluss von Temperatur. Dies deutet darauf hin, dass die Geschlechtsentwicklung eventuell von Temperatur beeinflusst sein könnte, aber weitere Experimente sind nötig, um dies genauer zu untersuchen.

Eine weitere Möglichkeit neben Kurzzeitstudien im Labor, um die Dynamiken von, und Selektion auf Hausfliegengeschlechtsbestimmungs-

faktoren zu studieren, ist es, Langzeitveränderungen in freier Natur zu untersuchen. Autosomale *M* Faktoren wurden zum ersten Mal vor 50 Jahren beschrieben, und es wurde damals angenommen, dass sie sich nordwärts ausbreiten und das Standard XY System verdrängen. **Kapitel 6** berichtet über das Sammeln von Hausfliegenpopulationen in Europa, die vor 25 Jahren bereits studiert worden waren (Franco *et al*. 1982), um zu untersuchen, wie und ob sich diese Verteilung über die Jahre hinweg verändert hat. Im Gegensatz zu früheren Vorhersagen konnte keine klare Veränderung der Verbreitung von autosomalem *M* im Vergleich mit der Verbreitung vor 25 Jahren festgestellt werden. Dies deutet darauf hin, dass autosomale *M* Faktoren keinen allumfassenden Fitnessvorteil gegenüber dem Standard *M* Faktor haben, da sie diesen ansonsten komplett verdrängt hätten. Die scheinbare Stabilität des Gradienten deutet eher darauf hin, dass XY im Norden einen Vorteil hat, während autosomale *M* Faktoren im Süden einen Vorteil zu haben scheinen.

Für **Kapitel 7** war das Ziel die Behauptung zu untersuchen, dass die geographische Verteilung der Hausfliegengeschlechtsbestimmungsfaktoren durch Temperaturvariation bedingt ist. Falls dies tatsächlich der Fall ist, würde man ein ähnliches Verteilungsmuster in der südlichen im Vergleich mit der nördlichen Hemisphäre erwarten. Dafür wurden Proben von mehreren Stellen in Südafrika und Tansania gesammelt. Die Ergebnisse zeigen, dass sich das gezeigte Verteilungsmuster der nördlichen Hemisphäre im Süden wiederholt, mit mehr autosomalen *M* und $F^D$ Faktoren zum Äquator hin oder bei niederen Höhen.

Nicht nur Temperatur, sondern auch andere Klimavariablen ändern sich systematisch entlang von Breiten- und Höhengradienten. Heutzutage sind Klimadatenbanken öffentlich zugänglich, die es ermöglichen, global Daten von zahlreichen Klimafaktoren zu erhalten. Anhand der neu erworbenen Daten über die Häufigkeit von Geschlechtsbestimmungsfaktoren in der südlichen Hemisphäre plus Daten aus bereits publizierten Studien und Daten einiger Klimavariablen wurde eine Metaanalyse durchgeführt, um zu testen welche Klimavariable(n) die Verteilung der Geschlechtsbestimmungsfaktoren am besten erklären kann (können). Die Ergebnisse zeigen, dass Saisonalität, gemessen als Stärke von Temperaturschwankungen, die Verteilung der Männchenbestimmungsfaktoren besser erklären kann als Durchschnitts-

temperatur; d.h. autosomale *M* Faktoren sind in Gegenden mit geringeren saisonalen Temperaturschwankungen häufiger. Für Weibchenbestimmungsfaktoren auf der anderen Seite erklärt eine Kombination aus Luftfeuchtigkeit und Jahresmitteltemperatur die Verbreitung am besten, also geringe Luftfeuchtigkeit und hohe Jahresmitteltemperaturen sind mit der Häufigkeit von $F^D$ Faktoren positiv korreliert.

In **Kapitel 8** war das Ziel die vorhandenen molekularen Werkzeuge für Hausfliegenstudien zu erweitern, indem neue Mikrosatelliten entwickelt wurden und eine „Linkage Map" erstellt wurde. Die Marker ordnen sich in fünf „linkage groups" an, die den fünf Autosomen der Hausfliege entsprechen, keiner der Marker war auf dem X oder Y Chromosom lokalisiert.

Die Theorie über Geschlechtschromosomenevolution sagt voraus, dass die Rekombinationsraten auf Chromosomen, die Geschlechtschromosomfunktion übernehmen, geringer werden sollten. Dies beginnt an der Position des Geschlechtsbestimmungsgens und breitet sich später über das gesamte Chromosom aus. Der Grund dafür ist, dass sich Gene z.B. auf dem Y Chromosom ansammeln, die für Männchen von Vorteil sind, für Weibchen jedoch von Nachteil. Geringere Rekombinationsraten verhindern, dass diese Gene in Weibchen gelangen. Für die Hausfliege bedeutet dies, dass auch die Rekombinationsraten von Autosomen mit *M*- oder *F*- Faktoren im Vergleich mit Autosomen ohne Geschlechtsbestimmungsfaktoren verminderte Rekombinationsraten zeigen sollten. Um diese Vorhersage zu testen, wurden Rekombinationsraten von Autosomen mit und ohne Geschlechtsbestimmungsfaktor verglichen. Die Position der Marker auf den spezifischen Autosomen wurde auf den Ergebnissen der „linkage" Analyse basiert. Eine statistische Analyse zeigte signifikant geringere Rekombinations-raten von Autosomen mit Geschlechtsbestimmungsfaktoren, im Einklang mit theoretischen Erwartungen.

Können wir jetzt anhand unserer Ergebnisse der Fliegenexperimente behaupten, dass wir die geographische Verteilung der Geschlechtsbestimmungsfaktoren und die Rolle von Temperatur verstehen? Auf der einen Seite weisen die Ergebnisse, dass die Verteilung der Geschlechtsbestimmungsfaktoren auf der südlichen Hemisphäre dem gleichen Muster folgen wie im Norden, auf eine Rolle von Temperatur in der Gestaltung der Gradienten hin. Auf der anderen

Seite gelang es in unseren Experimenten nicht, einen Einfluss von Temperatur auf die Fitness von Hausfliegen nachzuweisen. Hinsichtlich unserer Ergebnisse der nachfolgenden Metaanalyse, die darauf hindeutet, dass nicht alleinig Temperatur, sondern eher Saisonalität oder die Kombination aus Luftfeuchtigkeit und Jahresmitteltemperatur für die graduelle Verteilung der Geschlechtsbestimmungsfaktoren verantwortlich sind, ist dies wohl nicht zu überraschend. Ob die Korrelation der Metaanalyse einen kausalen Effekt widerspiegelt, bleibt Untersuchungsgegenstand zukünftiger Experimente.

# Samenvatting en Conclusies

Temperatuur wordt veroorzaakt door de bewegingen van moleculen; naarmate moleculen sneller of langzamer bewegen neemt de temperatuur toe of af. Omdat ieder organisme nu eenmaal uit moleculen bestaat heeft dit simpele principe invloed op ieder biologisch organisatieniveau. Van eenvoudige chemische reactiesnelheden, via individuele organismen tot hele ecosystemen: ieder niveau wordt in meer of mindere mate door temperatuur beïnvloed (Johnston & Bennet, 1996). In deze dissertatie draait het om een specifieke invloed van temperatuur, namelijk effecten van temperatuur op de seksuele ontwikkeling van dieren.

In veel koudbloedige diersoorten, zoals reptielen en vissen, wordt het geslacht van een individu bepaald door de invloed van temperatuur gedurende de vroege embryonale ontwikkeling, waarbij bepaalde temperaturen tot vrouwelijke en andere juist tot mannelijke ontwikkeling leiden. Een dergelijke vorm van geslachtsbepaling wordt ook wel temperatuurafhankelijke sexe determinatie genoemd (TSD). In schildpadden bijvoorbeeld, leiden hoge temperaturen tot vrouwelijke ontwikkeling en lage temperaturen tot mannelijke ontwikkeling. In krokodillen vindt juist het tegenovergestelde plaats, waarbij hoge temperaturen leiden tot mannelijke en lage temperaturen juist tot vrouwelijke ontwikkeling leiden. Bij de meeste andere organismen vindt echter een andere manier van geslachtsbepaling plaats, zoals bij vogels en zoogdieren, waar het genotype van het individu bepaalt of deze een man of een vrouw wordt. Een dergelijk systeem wordt genetische geslachtsbepaling genoemd en aangeduid met GSD (genetische sexe determinatie). Recent onderzoek naar de moleculaire mechanismen van geslachtsbepaling heeft aangetoond dat het verschil tussen deze beide vormen van geslachtsbepaling (GSD versus TSD) niet zo scherp is als voorheen werd aangenomen. De netwerken van genen die ten grondslag liggen aan de geslachtsbepaling verschillen slechts in geringe mate tussen TSD en GSD en bovendien blijken kleine genetische veranderingen al in staat te zijn om een overgang teweeg te brengen van TSD naar GSD en andersom. Phylogenetische analyses wijzen er bovendien sterk op dat overgangen tussen TSD en GSD meermaals en in beide richtingen hebben plaatsgevonden. Tenslotte blijkt ook dat de geografische verspreiding van geslachtsbepalings-mechanismen afhankelijk is van temperatuurgradiënten: TSD wordt voornamelijk gevonden in de warmere streken en ook sommige typen van GSD

zijn gebonden aan een geografisch verspreidingspatroon. Deze evolutionaire en geografische patronen worden op dit moment nog nauwelijks begrepen; het doel van deze dissertatie is dan ook om mechanismen die dergelijke patronen veroorzaken beter te leren begrijpen.

In het algemeen kunnen de effecten van temperatuur op geslachtsbepaling worden verdeeld in twee categorieën: aan de ene kant kan temperatuur gezien worden als een proximaat effect, een omgevingsvariabele die organismen direct beïnvloedt en daarom dus ook een rol speelt in hun geslachtsbepaling. Aan de andere kant kan temperatuur gezien worden als een ultieme, evolutionaire oorzaak van geslachtsbepaling, omdat temperatuur de selectieve voorwaarden voor de evolutie van - en de transitities tussen - de verschillende geslachtsbepalingsmechanismen biedt en daarmee dus ook een rol speelt in de geografische verspreiding van geslachtsbepalingssystemen.

In deze dissertatie wordt vooral de tweede, ultimate, categorie van effecten behandeld, waarbij de nadruk ligt op effecten van temperatuur die leiden tot veranderingen tussen geslachtsbepalingsmechanismen en hun verspreidingspatronen. Maar uiteindelijk kunnen dergelijke ultimate vraagstukken niet worden opgelost zonder daarin ook de proximate mechanismen te betrekken.

Deze dissertatie bestaat uit twee delen. In het eerste deel worden met behulp van modellen vragen behandeld die betrekking hebben op de evolutionare transities tussen TSD en GSD en de voorwaarden die kunnen leiden tot coëxistentie van verschillende geslachtsbepalingsmechanismen. Het gebruik van modellen staat echter niet los van de realiteit, omdat sommige modellen geparametriseerd zijn met empirische gegevens van de skink *Niveoscinus ocellatus*. Het tweede deel van deze dissertatie richt zich op empirisch onderzoek aan de huisvlieg, *Musca domestica.* De laatste is een cosmopolitische soort waarvan de frequenties van verschillende genetische geslachtsbepalingsmechanismen systematisch variëren volgens een geografisch patroon. Zowel experimenten als veldwerk zijn gebruikt om een reeks hypotheses te testen die betrekking hebben op de oorzaak van deze geografische verdeling.

## Deel 1: Theoretisch gedeelte

Er zijn verschillende hypothesen die de evolutie van TSD uit GSD proberen te verklaren (Ewert & Nelson, 1991; Shine 1999), maar verreweg de meest invloedrijke hypothese wordt omvat door het model van Charnov en Bull (1977). Het basisidee is dat de fitness van mannetjes en vrouwtjes verschillend wordt beïnvloed door variatie in de omgeving. Om preciezer te zijn, onder sommige omstandigheden hebben vrouwtjes een hogere verwachte fitness dan mannetjes, terwijl andere omstandigheden juist meer tot voordeel zijn van mannetjes. TSD vormt dan een flexibel mechanisme waarmee de seksratio kan worden aangepast in situaties waar oververtegenwoordiging van één van beide geslachten selectief voordelig is. Dergelijke flexibele seksratio aanpassingen zijn veel lastiger te bewerkstelligen met GSD, wat TSD een selectief voordeel geeft in zulke gevallen. Desondanks kan juist deze flexibiliteit van TSD ook weer een nadeel betekenen, wanneer fluctuaties in de temperatuur tussen de jaren groot genoeg zijn. In dat geval kunnen jaren met extreme temperaturen leiden tot overproductie van nakomelingen van een bepaalde sexe, wat weer tot gevolg heeft dat een populatie (bijna) kan uitsterven. Dit probleem is minder van toepassing op lang levende soorten, waarbij fluctuaties de neiging hebben elkaar op te heffen bezien over de volledige levensduur van een individu.

In **hoofdstuk 2** wordt een model gepresenteerd dat geen omgevingsafhankelijke en sex-specifieke effecten veronderstelt, maar dat is gebaseerd op selectie voor niet-facultatieve seksratios, die door lokale verwantencompetitie afwijken van 1:1. Seksratio theorie voorspelt dan een overproductie van het geslacht dat het meest onderhevig is aan dispersie, omdat het voor de hand ligt dat juist individuen van dit geslacht het minste concurreren met verwante individuen om voedsel en ruimte. Het basisidee is dat onder deze omstandigheden TSD makkelijker kan evolueren, omdat juist dit geslachtsbepalingsmechanisme facultatieve seksratios kan bewerkstelligen en daarmee een voordeel biedt ten opzichte van GSD. In dit model werden de sterkte van de omgevingsfluctuaties en de lengte van de levensduur systematisch gevarieerd om zo de interactie en het belang van de verschillende factoren te begrijpen. In een beginpopulatie die volledig uit GSD individuen bestond, werd onderzocht of en onder welke omstandigheden TSD zou kunnen evolueren. Er werden vijf verschillende

uitkomsten gevonden: (i) de beginpopulatie met GSD was stabiel tegen invasie van TSD; (ii) TSD evolueerde en verdreef GSD uit de populatie; (iii) een nieuw type GSD evolueerde op een tweede genlocus; (iv) stabiele coëxistentie van GSD en TSD; (v) verschillende coëxisterende GSD systemen evolueerden tezamen. In navolging van eerder onderzoek werd gevonden dat TSD gemakkelijker kon evolueren wanneer de omgevingsfluctuaties klein waren en de levensduur lang was. In de simulaties vonden snelle overgangen tussen GSD en TSD vaak plaats, zelfs zonder de aanname van temperatuur en sexe afhankelijke fitnessverschillen, hetgeen een nieuwe, aanvullende verklaring voor de evolutie van TSD biedt. Een ander belangrijk inzicht dat voortkomt uit dit model is dat verschillende geslachtsbepalingsmechanismen kunnen evolueren vanuit identieke begincondities, waarvoor wellicht toevalsprocessen zoals genetische drift verantwoordelijk zijn.

Hoewel conceptuele modellen zoals in hoofdstuk 2 belangrijk zijn om algemene inzichten over de evolutie van TSD te verschaffen, zijn specifieke modellen die zich toeleggen op bepaalde soorten nodig om deze inzichten daadwerkelijk te testen. De meeste soorten zijn ongeschikt om hypothesen over de evolutie van TSD mee te testen, omdat ze slechts één geslachtsbepalings-mechanisme bezitten. De skink *Niveoscincus ocellatus* is wat dit betreft een uitzondering, omdat er zowel GSD- als TSD-populaties van deze soort bestaan, verspreid over verschillende geografische regio's. Daarbij komt ook het feit dat er de afgelopen jaren een grote hoeveelheid gegevens over de levensloop van deze soort zijn verzameld. Vrouwtjes in de TSD-populatie die zijn geboren in een vroeg stadium gedurende het broedseizoen hebben een grotere kans om al een jaar eerder tot reproductie te komen dan vrouwtjes die later tijdens het broedseizoen worden geboren. Ook in de GSD-populatie verschilt deze kans om al eerder te reproduceren tussen vroeg- en laatgeboren vrouwtjes, maar in mindere mate. In beide populaties is de fitness van mannetjes onafhankelijk van hun geboortedatum. Terwijl vroeg- en laatgeboren vrouwtjes in de GSD populatie dus ook fitnessverschillen vertonen, kunnen juist grote temperatuursfluctuaties in hun verspreidingsgebied hebben geleid tot een selectief voordeel van GSD. In **hoofdstuk 3** zijn modellen, geparametriseerd zijn met gegevens van zowel de levensloop van deze skinkensoort als klimaatdata, gebruikt om te voorspellen welk geslachtsbepalingssysteem zou evolueren in de verschillende populaties. De resultaten laten zien dat het model

het geslachtsbepalingssysteem correct voorspelt voor iedere populatie, wanneer het model gebaseerd is op de lokale variatie in de temperatuur en de demographie van deze skink.

Drie belangrijke conclusies kunnen worden getrokken uit dit theoretische gedeelte van de dissertatie. Als eerste kan selectie voor niet-facultatieve seksratios al leiden tot overgangen tussen GSD en TSD, zelfs zonder de aanname van temperatuur en sexe afhankelijke fitnesseffecten. Deze resultaten geven een nieuwe verklaring voor de evolutie van TSD en kunnen wellicht verklaren waarom verschillende studies geen sex afhankelijke fitnesseffecten konden vinden bij bepaalde soorten. Ten tweede, laten simulaties zien dat naast snelle overgangen tussen GSD en TSD, evolutie van geslachtsbepalings-systemen ook kan uitmonden in een vorm van GSD welke bestaat uit verschillende onderliggende genetische factoren of in een stabiele coëxistentie tussen GSD en TSD. Hiermee is er dus een mogelijke verklaring gevonden voor de empirische waarneming van het bestaan van verschillende geslachtsbepalingsfactoren binnen één populatie. Als derde kan geconcludeerd worden dat het nuttig is om dergelijke evolutionaire modellen af te stemmen op een specifieke soort, omdat hiermee voorspellingen gemaakt kunnen worden over de evolutie van geslachtsbepalingsmechanismen binnen deze soort.

## Deel II: Empirische Benadering

Het hoofddoel van het tweede deel van het proefschrift was het bestuderen van het effect van variatie in temperatuur op de geografische verdeling van sexe-bepalende (SD) factoren in de huisvlieg, met behulp van een serie experimenten en statistische analyses. Het standaardgeslacht van een huisvlieg is vrouwelijk, aangezien alle individuen de vrouw-bepalende factor *F* in zich dragen. Individuen die hiernaast in het bezit zijn van een man-bepalende factor *M*, die de functie van *F* onderdrukt, in zich dragen worden mannetjes. In het zogenaamde standaard XY-systeem, wat het meeste op hoge breedtegraden en ver boven zeeniveau voorkomt, bevindt *M* zich op het Y-chromosoom. In populaties die zich dichter bij zeeniveau of op een lagere breedtegraad bevinden kan de *M* factor op een of meer van de vijf autosomen liggen. In populaties met autosomale *M*-factoren zijn vrouwtjes vaak in het bezit van een gemuteerde versie van *F*, de $F^D$ factor, die ongevoelig voor *M* is.

Wat verklaart echter deze clines? De meest voor de hand liggende factor die varieert met de breedtegraad is temperatuur. Dit leidde enkele auteurs er toe om te suggereren dat de ruimtelijke verdeling van SD-factoren op de een of andere manier door variatie in temperatuur veroorzaakt wordt (Franco *et al.*, 1982; Cakir & Kence, 1996); in hoeverre dit het geval is is waar dit deel van het proefschrift over gaat.

Een eenvoudige hypothese om de distributie van SD-factoren te verklaren is het feit dat de temperatuur de fitness van vliegen beinvloedt, op een manier die van hun SD-factoren afhangt. Het doel van **hoofdstuk 4** was daarom om middels experimenten te onderzoeken of huisvliegen met autosomaal M of $F^D$ in vergelijking met standaard XY-vliegen een hogere fitness hebben bij hogere temperaturen. Om te bepalen of een invasie van autosomale *M*-factoren in een standaard XY-populatie mogelijk zou zijn werden enkele mannetjes met de *M* factor op autosomen II en III in standaard XY-populaties geïntroduceerd, onder verschillende temperaturen. Hun frequentie werd daarna over meerdere generaties gevolgd. De resultaten waren echter minder eenduidig dan verwacht. Hoewel *M* op autosoom II het Y-chromosoom verving, nam *M* op autosoom III niet in frequentie toe, en er werd geen effect van de temperatuur gemeten. Om de fitness van vrouwtjes met en zonder $F^D$ te bepalen werden enkele fitness-parameters van vrouwtjesvliegen met beide mogelijke SD-factoren gemeten, bij verscheidene temperaturen. Er was een aanzienlijke variatie tussen de verschillende populaties, maar ook hier, net als in het mannetjes-experiment, werd hier geen effect van de temperatuur op de resultaten gevonden.

In het voorgaande hoofdstuk toonde geen van de onderzochte fitness-parameters een effect van de temperatuur aan. Het doel in **hoofdstuk 5** was om nog een fitness-component, namelijk de frequentie van interseksen (individuen met een lage fitness die zowel mannelijke als vrouwelijke eigenschappen hebben) in het nageslacht. Er is eerder gepubliceerd dat de frequentie van interseksen in de huisvlieg toeneemt bij koude temperaturen. Hierom werden er intra- en inter-populatie kruisingen gedaan voor populaties met standaard XY-systeem zowel als autosomaal-*M* populaties met verschillende frequenties van $F^D$, voor verschillende temperaturen. Voor iedere kruising werd het nageslacht onderzocht op intersekse-eigenschappen. Er werd geen effect van de

temperatuur op de frequentie van interseksen aangetroffen. De sekseratios van de populaties werden echter beinvloed door de temperatuur bij enkele van de experimenten. Dit suggereert dat seksuele ontwikkeling mogelijk beinvloed wordt door de temperatuur, maar meer experimenten zijn nodig om dit nader te onderzoeken.

Een andere manier om de dynamica van, en selectie over, SD-factoren in de huisvlieg te onderzoeken naast laboraturiumonderzoek is het onderzoeken van veranderingen over de lange termijn in de patronen van de distributies in het wild. Er werd voor het eerst zo'n 50 jaar geleden melding gemaakt van autosomale *M*-factoren, en de hypothese werd geopperd dat zij zich in noordelijke richting aan het verspreiden waren, en het standaard XY-systeem vervingen. **hoofdstuk 6** doet verslag van het verzamelen van huisvliegen uit populaties op een reeks plaatsen in Europa, die 25 jaar geleden ook bestudeerd werden (Franco *et al.* 1982), om zo te onderzoeken of, en op welke wijze, de verdeling in de loop der jaren veranderd is. In tegenstelling tot de vroeger gedane voorspellingen werd er echter geen duidelijke verandering gevonden in de verdeling van autosomale *M*-facoren in vergelijking met 25 jaar geleden. Dit leidt tot de conclusie dat autosomale *M*-factoren geen uniform voordeel over de standaard *M*-factor hebben, aangezien zij deze laatste anders geheel verdrongen zouden hebben. De ogenschijnlijke stabiliteit van de cline wekt de suggestie op dat het XY-systeem in het noorden een voordeel heeft, terwijl autosomale *M*-factoren in het zuiden in het voordeel lijken te zijn.

Het doel van **hoofdstuk 7** was het verder onderzoeken van het idee dat de geografische distributie van SD-factoren in de huisvlieg door temperatuurvariatie veroorzaakt wordt. Als dit inderdaad het geval is zou men verwachten vergelijkbare patronen in de verspreiding op het zuidelijk halfrond te vinden. Hierom werden steekproeven verzameld uit meerdere locaties in Zuid-Afrika en Tanzania. De resultaten tonen aan dat de huisvliegpopulaties op het zuidelijk halfrond hetzelfde patroon herhalen dat eerder op het noordelijk halfrond aangetroffen werd, er werden namelijk hogere frequenties van autosomale *M*- en $F^D$-factoren aangetroffen naarmate men dichter bij de evenaar kwam, of dichter bij zeeniveau.

Naast de temperatuur veranderen nog enkele andere klimaatsvariabelen mee met de breedtegraad en hoogte. Klimaatsdatabases zijn tegenwoordig

publiekelijk toegankelijk, wat het mogelijk maakt data te verkrijgen over een aantal klimaatsfactoren op wereldschaal. Met behulp van de nieuw-verzamelde data over frequenties van SD-factoren op het zuidelijk halfrond, samen met data uit eerder gepubliceerde onderzoeken werd een meta-analyse gedaan om te onderzoeken welke klimaatsvariabele de clinale verdeling van de SD-factoren het beste verklaart. De resultaten tonen aan dat de seizoensgebondenheid van de mate waarin de temperatuur varieert, en niet zozeer de gemiddelde temperatuur, de beste verklaring is voor de verdeling van de mannelijke SD-factor, in de zin dat de autosomale *M*-factoren meer voorkomen in plaatsen waar er minder temperatuurfluctuaties tussen de seizoenen zijn. Voor de verdeling van de vrouwelijke SD-factoren echter is de combinatie van vochtigheidsgraad en gemiddelde temperatuur (over het hele jaar gemeten) de beste verklarende factor. Een lage vochtigheidsgraad en hoge gemiddelde temperatuur zijn namelijk positief gecorreleerd met de frequentie van $F^D$.

Het hoofddoel van **hoofdstuk 8** was om de beschikbare moleculaire methoden ter bestudering van de huisvlieg uit te breiden door nieuwe microsateliet merkers te ontwikkelen en een “linkage map” of koppelingskaart te construeren. De merkers werden naar vijf koppelingsgroepen gesplitst die overeen kwamen met vijf autosomen van de huisvlieg, maar geen van de merkers lag op het X- of Y-chromosoom.

De theorieën over de evolutie van het geslachtschromosoom voorspellen dat op chromosomen die de functie van een geslachtschromosoom aannemen de mate van recombinatie langs het chromosoom zou moeten afnemen, beginnend bij de locatie van het geslachtsbepalende gen. Dit is omdat, bijvoorbeeld, op het Y-chromosoom zuch genen opstapelen die gunstig zijn voor mannetjes maar nadelig voor vrouwtjes. Een afname in de mate van recombinatie voorkomt dat deze genen in vrouwtjes terechtkomen. Hieruit volgt dat voor de huisvlieg de mate van recombinatie op de autosomen die de *M*- of $F^D$-factor bevatten ook lager zou moeten zijn dan op de autosomen zonder. Om deze voorspelling te toetsen werden de mate van recombinatie van autosomen met en zonder SD-factor vergeleken, door een van de merkers van het specifieke autosoom in kwestie te gebruiken die gevonden was in de koppelingsanalyse. Een statistische analyse toonde een significante afname aan in de mate van

recombinatie op autosomen met SD-factoren, wat overeenkomt met de verwachtingen die voortvloeien uit de theorie.

Kunnen wij nu, gezien onze resultaten uit huisvliegexperimenten, de uitspraak doen dat wij de geografische verdeling van de SD-factoren, en de rol die de temperatuur hierbij speelt, begrijpen? Enerzijds wijst het gevonden resultaat dat de verdeling van SD-factoren in het zuidelijk halfrond vergelijkbaar is met de patronen die op het noordeljk halfrond worden aangetroffen erop dat er een rol van de temperatuur is in de vorming van deze clines. Anderszijds hebben geen van de laboratoriumexperimenten een effect van de temperatuur op de fitness van huisvliegen met verschillende SD-factoren aan kunnen tonen. Dit laatste is wellicht echter niet zo verassend vanuit het perspectief van de meta-analyse die op de experimenten volgde, die suggereerde dat het niet zozeer de temperatuur is maar de seizoensgebonden temperatuurvariatie of de combinatie van vochtigheidsgraad en temperatuur is die verantwoordelijk is voor de clinale distributie van SD-factoren. Of de correlaties die in de meta-analyse gevonden werden daadwerkelijk een causaal effect weerspiegelen is nader te onderzoeken in toekomstige experimenten.

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Barbara

# Curriculum Vitae

Barbara V. Feldmeyer was born on 18th September 1978 in Schorndorf, Germany. After her secondary studies at the Werkgymnasium in Heidenheim she started her studies of Biology at Greifswald University in 1998. In 2000 she spent nine months on field courses in Queensland, Australia and East Kalimantan, Indonesia. After having encountered the tropics she decided to focus on tropical ecology and continued her studies at the University of Würzburg in 2001. Here she did her Master thesis on ant-plant interactions with the title "Differences in host usage of *Crematogaster* Msp. 4 and 10 on different *Macaranga* hosts". She obtained her Master's degree in 2004. The same year she started her PhD project in the Theoretical Biology and Evolutionary Genetics groups at Groningen University as part of a program funded by the Robert Bosch Stiftung. The project comprised both theoretical and empirical aspects and focused on the effects of temperature on sex determination. The results of this project are presented in this book. In March 2009 Barbara started her PostDoc position at the University of Frankfurt in cooperation with the Senckenberg Society to look for candidate genes of climatic adaptation in *Radix* and *Daphnia*.