Behavioural and genetic analysis of courtship song in *Drosophila melanogaster*

by

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SUMMARY

Courtship song of Drosophila melanogaster males has been investigated with ethological and genetic methods. The ethological part has produced the following results:

1. Simulated pulse song has a distinct influence on locomotor and sexual activity of males. Upon hearing pulse song they increase both activities significantly. Sine song does not have such an effect.
   a) The effect of pulse song is stronger with males which had been kept in isolation until the experiment.
   b) Once aroused the males stay active for at least 5 min.
   c) The arousal is dependent on the intensity of the song. Below 85 and above 120 dB the stimulation is not effective. Males without aristae respond only at intensities above 100 dB.
   d) The effect is not very ipi specific, because ipis from 30 - 100 ms are about equally effective in triggering a response in males.

2. Stimulation with simulated pulse song and sine song decreases locomotor activity of receptive females.

3. Female receptivity is significantly increased, if they are subjected to simulated sine song before being mixed with males. Pulse song applied under identical conditions does not have this effect. It is effective under the same conditions with reversed sex roles, when the males are prestimulated.

4. Simulated pulse song increases the receptivity of females if they are subjected to it while being courted by aristaless ('deaf') and wingless ('mute') males. This effect on females is ipi specific insofar as an ipi of 48 ms is less stimulatory to the females than one of 34 ms, which is the species specific value.

The experiments reported in the genetic part of the thesis have shown the following:

1. Male hybrids between Drosophila melanogaster and D. simulans have an ipi indistinguishable from that of D. simulans. Their wing beat frequency shows intermediate inheritance. In mating tests, hybrid males court and are accepted by D. simulans females just as hybrid females. D. melanogaster females reject hybrid males. Hybrid females accept D. melanogaster males readily, hybrids less readily, and D. simulans least.

2. A mutant - cacophony - has been isolated which has an aberrant courtship song. Ipi and pulse length are significantly increased. The pulses are polycyclic rather than monocyclic. Flight wing beat and sine song frequency remain unchanged. The mutation is sex linked and its approximate map position is 34.64. It was fate mapped, but the results were inconclusive as to whether the focus responsible for the song deviations maps to the head or thorax. It could only be demonstrated that the mutation is not thoracic and dominant. Fate mapping has revealed something about song production per se. To produce a normal song, wild type or mutant, as opposed to an abnormal one which cannot be classified into pulse song or sine song, a gynandromorph must

have some male head tissue, and certain thoracic structures which lie close to the thoracic cuticle on the blastula surface, must also be male. If the latter are female an entirely abnormal song results, for the production of which male head structures are necessary and sufficient.

3. Activity, reactivity and courtship behaviour of cacophony was investigated and a number of pleiotropic effects of the mutation on these behaviours have been revealed. Mutant males are equally active in a circular arena, but less reactive to conspecifics and a shadow stimulus than control males. Pair matings in all combinations between mutant and control flies revealed, that the males are responsible for the reduced mating success of cacophony, the lower percentage of vibration, the lower number of vibration bouts and the reduced number of licks. Both sexes play a significant role in the determination of the number of attempted copulations. None of the courtship parameters is determined to any significant extent by an interaction between the sexes.
Wingless cacophony males are also slower in achieving copulation, than wingless control males. Their relative successes in the winged and wingless situation indicate that the mutant song is an improvement on the wild type song. This seems possible because the mutants sing louder than control males.
The number of pulse song bouts is unchanged in the mutant, but the number of sine song bouts is reduced.
Mutant males frequently carry their wings in an abnormal way. This deviant wing posture is caused by the presence of females, because males kept in isolation or in unisexual groups hold their wings normally.

TABLE OF CONTENTS

INTRODUCTION

The broadest definition of behaviour genetics, which describes it as the study of the nature-nurture relationship in behavioural traits, encompasses practically the whole hierarchy of the behavioural sciences from sociopsychology to neurobiology. In practice, however, behaviour genetics is a rather more narrowly defined discipline, which has found a fertile niche in an area of overlap between ethology, psychology, neurobiology, genetics, developmental and evolutionary biology (Fuller and Thompson 1960, Hirsch 1967, McClearn and De-Fries 1973). Of the two at present most active battle grounds of the nature-nurture controversy, namely vertebrate electrophysiology (Horn et al. 1973, Kolata, G.B. 1975, Robertson 1975) and psychometrics (Jensen 1972, Hirsch 1970) only the latter one summoned up behaviour geneticists into the front rows of the two opposing sides.

Behaviour genetics is probably best defined by the methods of investigation it choses to study behaviour. These methods are those of conventional genetics. They all treat genetics as the independent variable and behaviour (or some intervening factor) as the dependent one. All of them therefore make use of genetic differences between existing individuals or populations, or they produce these differences by such genetic techniques as mutagenesis, selection, inbreeding, etc. The most important tools which genetics offers to the study of behaviour are listed in the following. In every case a more or less well defined genetic difference between individuals or populations and their respective controls exists, or is produced, and is then exploited by the behaviour geneticist. Such genetic deviations from a suitable control condition constitute:

1. morphological mutants (Wilcock 1969, Thiessen et al. 1970).
2. behavioural mutants (Benzer 1971, Brenner 1973).
3. carriers of different inversions in Drosophila (Spiess 1970).
4. inbred lines (Broadhurst et al. 1974).
5. more or less outbred strains, breeds or races which have been produced:
   a) by selection for behaviour (e.g. Tryon 1929, Hirsch 1961, Manning 1961).
   b) by selection for morphology (e.g. Ewing 1964)
   c) or which have been found in nature (e.g. Gwadz 1970)
6. species (Franck 1974).
7. individuals of known family descent (reviewed for man by Jensen 1972).

All these methods are employed to tackle problems which I shall discuss in the following pages. I will make some remarks about the suitability of the different methods for the task under discussion. The emphasis will be on a more or less personal evaluation of the scope of the different behaviour genetical tools, rather than on a literature survey. Behaviour genetics has been extensively reviewed every few years up to 1974 (Broadhurst et al. 1974) and the literature is very easily accessible.

1. One question which received much attention in the early days of behaviour genetics, is whether genetic differences play any causal role at all in the production of phenotypical, behavioural variation, (Caspari 1958, Fuller and Thompson 1960). This question, which probably only arose out of the "Zeitgeist" which was governed by behaviourism at the time of the birth of behaviour genetics, can be considered as solved. There seems

almost no behaviour which has been looked at with any of the methods listed above which does not involve a significant genetical variable. Most studies of this type have been made by selecting for a given behaviour and Tryon's (1940) work is an outstanding early example, where it was shown that maze learning ability in rats can be modified by genetic selection. In Drosophila, behaviours which have been successfully selected for and/or against, are photo-, geo-, and chemotaxis, oviposition and various aspects of mating behaviour, activity and reactivity.

2. Another question, which immediately follows, once the first one has been answered positively, is that of enquiring after the physiological or morphological correlates of genetic differences. This question has been called the direct approach (Manning 1975) and its aim is the unravelling of the causal chain between gene and behaviour. Except for the last method (No.7, individuals of known family descent), all can be employed to tackle this question. However, by far the most powerful method is that which takes advantage of behavioural mutants. To a lesser extent morphological mutants can also be employed. An interesting exploitation of biochemical mutants with behavioural effects is advocated by Burnet and Connolly (1975). They use phenocopying techniques to answer neurochemical questions (Burnet et al. 1973).

The other tools which provide genetic differences of a less well defined nature are full of pitfalls. The most important one being, that once a given behavioural difference has been established and correlated with a certain physiological difference,

it is uncertain whether the differences on the two levels have been brought about by the same genes (McClearn,1972). A very thorough physiological and behavioural analysis of Tryon's maze bright and dull rats has nicely demonstrated the intricacies of such an approach (Rosenzweig,1964). Besides this serious shortcoming, this approach also shares certain complications with the mutant research, where it is sometimes difficult to differentiate between cause and effect. A given physiological correlate does not have to be the cause of the behavioural deviation under study, it could also be its effect (Bulfield 1972). In most organisms this latter shortcoming can only be circumvented by very extensive and careful experimentation. In Drosophila, however, an epigenetic method (fate mapping) exists, which helps to pinpoint the primary cause of the behavioural deviation without the need to embark on elaborate physiological or anatomical investigations. The present question and the power of behavioural mutants in dealing with it will be treated in more detail later on in this introduction.

3. Another problem which has received much attention, is the genetic organization of behaviour. This question is approached primarily but not exclusively with two of the tools listed above, namely inbred lines and outbred individuals which are compared to some of their relatives. Quantitative genetics has produced certain generalizations about the genetic architecture of phenotypes in relation to their evolutionary past. For example, traits which have in the past been subjected to very high pressures of directional selection, should have very little

additive variance left. Consequently, they should have low heritabilities, and most of their genetic variance should be in the form of dominance variance, (Robertson 1955). Traits which have been subjected to strong stabilizing selection pressures, should display the converse genetic structure. These generalizations can be relied upon at least as far as nonbehavioural traits are concerned, (Falconer 1960). They are certainly very interesting for the behaviourist in that they permit him to assess the functional significance of a given behaviour on the basis of purely genetical information. As has been indicated above this work is most efficiently pursued with inbred lines or outbred individuals of known family descent. Inbred lines allow the use of a very powerful technique of analysis - the diallel cross - which extracts a large amount of information from the measurements of just two generations, (Broadhurst 1960). However a serious drawback with this kind of analysis is the fact that inbred lines do not exist in diploid, sexually reproducing organisms. The genetic parameters which the theory attempts to extract are highly idiosyncratic, in that they are very responsive to changes in the genetic and environmental background, (Falconer 1960). Furthermore they are themselves subjected to selection and at the present state of our knowledge, it is difficult to estimate what the effects of inbreeding and domestication are on the genetic structure of naturally integrated genomes. The information gained from inbred lines must therefore be regarded with the greatest caution. It should be mentioned, however, that due to different population structures, different species will be more or less suitable to an inbred strain analysis. For example, mice in the wild appear to have very

small effective population sizes (DeFries and McClearn 1972) and therefore the average degree of heterozygosity is relatively small, (Lewontin 1974). In Drosophila it is rather large (Lewontin 1974) and inbreeding is known to have serious detrimental effects on male mating ability (Pendlebury and Kidwell 1974) and fitness (Watanabe and Ohnishi 1975). Furthermore the diallel test involves the measurement of very large numbers of animals which should be accomplished in the shortest possible time. These restrictions usually mean that behaviours are chosen more for the sake of convenience in measurement than for their ethological significance. Consequently the quite large number of such studies which have been done (Broadhurst et al. 1974) are, at least for the ethologist, of limited value, (Manning 1975). The most promising investigations seem to be those which introduce several environmental variables into the experimental design, because here the link is made with developmental biology. An example of this work is that of Henderson on mice, who studied the effects of environmental enrichment on brain weight, food seeking behaviour and learning, and analysed these results genetically, (Henderson 1972).

The other method (No.7, comparison of relatives) which does not share the first very serious shortcoming, because outbred individuals are tested, is also very labour intensive (Robertson 1959). Except for the field of human intelligence (Jensen 1972), very few studies (e.g. Connolly 1966) have been done. I feel that a well designed study with Drosophila could produce important results.

It seems unfortunate that mice have such a predominance in this field, because for obvious reasons Drosophila is much more convenient in research which involves large numbers of animals. The emphasis of the quantitative genetic approach is on evolutionary mechanisms and therefore, if this work is to make a lasting contribution, it has to become more ethological in outlook. One very ambitious project, namely the identification of behavioural polygenes, falls into the present category. Hirsch and his co-workers (Hirsch 1967) have tried to map polygenes which play a role in Drosophila geotactic behaviour to individual chromosomes. Recently another such attempt has been undertaken with courtship behaviour, (Eastwood 1974). If carried a step further, to the actual identification of these genes on the chromosomes, according to the techniques advocated by Thoday (1961), this work could become very important. It would so to speak bridge the gap between Mendelian and quantitative genetics and it might become possible to apply the very powerful analytical tools of the former method to 'normal', continuous variation.

4. The last of the more reductionist questions, is addressed to the problems of the organization of behaviour. This type of work has been called 'parsing the phenotype' (Ginsburg 1958, Manning 1975). Results obtained from the use of practically any of the tools of behaviour genetics, except perhaps the last in our list, can be brought to bear on this question. The major problem of the organization of behaviour is the degree of autonomy of a given behavioural entity, say a fixed action pattern, which the observer believes to have discerned.

Ethologists would like to have some confidence that the behaviours they are studying represent true units in the sense of natural selection. If it were found that single genes control a given complex behaviour pattern, as for example in the case of nest cleaning behaviour in the honeybee (Rothenbuhler 1964), it could be concluded that such behaviour constitutes a rather good unit. Furthermore, if it is found that certain behaviours segregate independently or not, then conclusions can be drawn about their developmental dependence or independence, with obvious implications for the evolution of such traits. A lot of the evidence for unit arguments comes from species hybrids and selection experiments. The problem is discussed in great detail by Manning (1976) and needs no further mention here, except perhaps the indication that the hunt for behavioural units which correspond in a 1 : 1 relationship to genetic units might well be the hunt after a phantom. Clearly these problems will come much closer to their solution when significant advances in developmental biology will have been made, (Bateson 1976).

5. The next question is concerned with the changes behaviour has undergone during its evolution, as far as behaviour genetics has a contribution to make to this traditionally ethological field of enquiry. The type of behavioural changes which occur during selection, in species hybrids or through mutagenesis, permit inferences about general aspects of behavioural evolution. Generalizations which seem justified have been outlined by Manning (1965) and Ewing and Manning (1967). Behaviour appears to have changed in small steps involving

changes of frequency, intensity or speed of performance. All these alterations can be imagined to be the result of changes in performance thresholds of various components of behaviour patterns.

6. The last question has been the first of a series of functional, evolutionary ones which we shall treat now. Having discussed the effects of evolution on behaviour, we must now address ourselves to its reciprocal - the effects of behaviour on evolution. The most important behavioural mechanisms under study which influence the course and speed of evolution profoundly, are all aspects of selective mating. This research is helped by behaviour genetics mainly through the use of mutants, selected and inbred lines, different strains and species. Mechanisms of intra- and interspecific sexual selection are ubiquitous (Campbell 1972) and their investigation is perhaps one of the more successful of the behaviour genetical enterprises. Petit and Ehrman's discovery of frequency dependent mating success of male Drosophila (Petit and Ehrman 1969) and the work of many authors on sexual isolation has profoundly influenced our understanding of evolutionary processes. Selection for sexual isolation has been successfully performed several times (e.g. Dobzhansky and Pavlovsky 1971) and is perhaps the most far reaching instance of an "in vitro" imitation of macroevolutionary processes.
Other more subtle ways in which behaviour feeds back to evolution, are mainly discussed in relation to the evolution of man, (Washburn 1972). Attempts to synthesise our knowledge of ecological and behavioural genetics belong into the present

category, (Parsons 1973). The literature in this field is as voluminous as the results are remarkable.

7. The last problem, which I want to discuss, is that of the teleonomic (Pittendrigh 1958) aspects of behaviour. What is the function of a particular behaviour? What is its selective value? Behaviour geneticists can contribute to the answering of such questions by using genetic tools to change the behaviour under investigation and then look at the fitness of individuals which express such behavioural deviations in comparison to control animals whose behaviour has not been changed.
If the modification of behaviour has been achieved through the methods of selection or mutagenesis, which are the most obvious ones to use, then the results must be interpreted with caution. Correlated and pleiotropic effects are notorious in morphological traits (Falconer 1960, Caspari 1952) and can be expected to be as serious in behavioural characters. For these reasons it is very difficult to manipulate phenotypic variables by genetic means in a controlled fashion. In most cases other behaviours will also have been changed and this can confound the results considerably. These drawbacks need not be unsurmountable for every mutation, however, because it has been demonstrated that once the most obvious primary effect of a given mutation on, say mating behaviour, is removed, the carrier of this mutation can be equally fit as a wild type individual. For example, in one case the mating success of wingless mutant (vg) Drosophila males was compared to that of winged control males under conditions where the females could not receive any auditory stimulation from the winged males because they were

antennaless. Under these circumstances vg males were just as successful in mating as winged males, indicating no pleiotropic effects of the vg gene other than those directly stemming from the loss of wings, (Seegmiller and Hanks 1968). It must be added, however, that this study is not entirely satisfactory because it appears that the vg and control stocks were not coisogenic. In another case, where effects of the genetic background have been controlled for, it could be shown that genetically white eyed males of Drosophila which are known to be poor courters (Reed and Reed 1950) and which are visually handicapped (Burnet et al. 1968) are at no disadvantage compared to red eyed control males when competing with them for females in the dark, (Burnet and Connolly 1973). With some mutations such a straightforward investigation of pleiotropic effects is not possible because such a simple symptomatic 'cure' of the primary defect caused by the mutation is not available. In these cases phenocopying techniques must be resorted to (Burnet et al. 1968). These are less satisfactory because they are not acting at the level of the symptoms only and one might be erradicating pleiotropic effects along with the main effect.

From the treatment of practically all these questions, it becomes evident that the generalisations of behaviour genetics cannot reach very far as long as it treats developmental processes as taking place in an ominous black box. Once developmental biology has reached a stage where it can make direct contributions to the study of behaviour genetics, the power of the deductions in the latter field will increase considerably. The lack of developmental

knowledge has clearly been recognized by leading biologists (Crick and Lawrence 1975) and behaviourists (Bateson 1976) and we must hope that the time is near where we can start dismanteling the black box.

As has been indicated above, in the following I shall try to review the work which has so far been done with behavioural mutants. I will also try to outline the logic of this research enterprise, which occupies itself with problems of the kind which have been subsumed under question No.2 in the present classification of behaviour genetical problems. Attempts at the induction of behavioural mutants and their subsequent genetic dissection constitute a major part of the present thesis and more and more biologists embark on such an analysis. Therefore this research warrants closer examination.

The publication which triggered this upsurge of research (Benzer 1967), starts like thus: "Complex as it is, much of the vast network of cellular functions has been successfully dissected, on a microscopic scale, by the use of mutants in which one element is altered at a time. A similar approach may be fruitful in dealing with the complex structures and events underlying behaviour, using behavioural mutations to indicate modifications of the nervous system." The rationale of this ambitious attempt is to try an analogous attack on behaviour, which was so successful with cellular mechanisms. The originality of this approach stems less from this basic idea, which has been the motor of much reductionistic studies in behaviour genetics before, than from the attempt at producing these mutants in the first place. Earlier studies made use of existing morphological or behavioural mutants and did not try

to produce them specifically for the purpose. Furthermore this, call it Benzerian approach, is original as far as the production of behavioural variants is concerned only, in as much as it produces the variants through genetic means. Experimental psychology has a long history of a similar strategy which produces the variants through surgical means. The review of this work will concentrate on Drosophila as an experimental subject and will only touch briefly on the work that has been done with other organisms.

Drosophila has one invaluable advantage in that once a genetic lesion has been made in this organism one can use an epigenetic technique, fate mapping (Sturtevant 1929b), to localize the primary cause of the lesion as it is unfolded in morphogenesis. The focus of a gene's action can be uncovered with this rather straightforward method (Hotta and Benzer 1973), and does away with the necessity to embark on a laborious search for the structural defects which, if any are found, could still be due to secondary effects of the primary lesion rather than the primary lesion itself.

The first paper on behavioural mutagenesis mentioned above (Benzer 1967), dealt with the isolation of phototactic mutants. Other visual mutants have been isolated and/or studied by several authors (Heisenberg 1971a, Hotta and Benzer 1969, Cosens and Manning 1969, Pak et al. 1969, 1970). The aim of these studies is the stepwise dissection of the visual system. The group around Pak (Alawi et al. 1972) is primarily interested in the photo-transduction process. They have good evidence that at least one of their mutants (norp A^{p}) is defective in the photo transduction system. This mutant shows small structural abnormalities in the membrane of some retinula cells, but shows a clear cut difference in the protein composition of the eye (Ostroy and Pak 1973).

Clearly such differences could provide the key to the isolation of a protein responsible for the membrane processes accompanying photo transduction. From the kinetics of temperature sensitivity of the norp A^{p} mutant Deland and Pak (1973) have concluded that the change in the mutant protein is due to a single amino acid substitution. Ostroy et al. (1975) have established rhodopsin concentration differences between the mutant and wild type flies. But they consider it unlikely that these differences alone could be responsible for the ERG abnormalities of the mutant.

Other mutants show varying degrees of ERG abnormalities, from no response to an almost normal ERG (Heisenberg, 1972a). Most of them seem to be autonomous, in the sense that the developmental focus is in the eye itself (Hotta and Benzer, 1970). These different ERG's have been used to study the functional significance of some of the elements of the ERG (Heisenberg 1971b). Heisenberg (1972 b) has subjected mutants with varying ERG defects to a series of tests designed to define the exact behavioural insufficiencies of these mutants, none of which is completely blind. For example they differ in the phototaxis threshold intensities, the precise optical conditions under which they show an optomotor response, their visual acuity and their polarisation sensitivity. Of all behaviours, that dependent on the visual sense, has been most intensively studied by means of mutagenesis. This research is certainly well on the way towards making important discoveries.

Many other behaviours have been studied with these genetic techniques. Two groups of mutants stand out, besides the visual ones, in the amount of information that they have yielded. They are the paralytic mutants isolated and investigated by the group of Suzuki (Grigliatti 1973 et. al.) and the shaker mutants which

are being studied intensively by Kaplan (1972) and his co-workers. The latter are characterized by more or less intensive and continuous shaking of their legs during etherization. Some of them furthermore show a kinetogenic response, which makes these flies jump and fall on their back when an object is moved above a vial containing them. The leg shaking action is accompanied by endogenous rythmic bursts of motor nerve impulses (Ikeda and Kaplan 1970a) and fate mapping has shown that the expression of the gene is autonomous (Ikeda and Kaplan 1970b), and lies very close to the region of the blastoderm destined to become the ventral nervous system (Hotta and Benzer 1973). These mutants have also been subjected to a large variety of purely behavioural tests (Kaplan and Trout 1969, Burnet et al. 1974), and the different mutations have been studied in various combinations with each other (Trout and Kaplan 1973, Kaplan and Trout 1974), and one paralytic mutant (Williamson et al. 1974). It seems that these loci create an imbalance between excitatory and inhibitory neuronal systems when mutated (Kaplan 1972). The ultimate aim of these studies is to arrive at an understanding of the precise mechanisms of the genetic control over the characteristics of nerve cells. The shaker system might be one of the first to allow the integration of biochemical, neurophysiological and molecular developmental work, and thereby it could contribute immensely to our knowledge of the nervous system.

The other group of mutants mentioned above, are the paralytic ones. After the first temperature sensitive paralytic mutants (paralytic^{ts}) had been isolated (Suzuki et al. 1971) several more were recovered in a later experiment (Grigliatti et al. 1973). Adult paralytic^{ts} flies become immobilized within 5s at 29°C but

normal mobility is restored almost instantaneously when the flies are shifted back to 22°C. Fate mapping indicated that the mutation is autonomous and constitutes some defect of the nervous system (Grigliatelli et al. 1972). Another similar mutation, shibire^{ts}, is highly pleiotropic and seems to have set a lesion which affects a large number of bodily mechanisms (Poodry et al. 1973). Shibire^{ts} is also a mutation affecting the nervous system, because the mutant flies are resistant to tetrodotoxin, a toxin which blocks the regenerative sodium channel. This resistance is also temperature dependent, and it is hypothesized that the shibire locus is responsible for the coding of a protein which constitutes some part of the regenerative sodium channel (Kelly 1974).

One olfaction mutant (HPB-1) has been isolated (Kikuchi 1973). It is attracted to 18 chemicals which are repellent to wild type flies. Chemical analysis of these attractive odours indicates that the mutation causes some change in a receptor site. A mutation affecting the response to taste of sugar has been isolated (Isoho and Kikuchi 1974), and three to which a 1M NaCl solution is not repellent (Falk and Atidia 1975). The latter mutations, all at 1 locus, probably affect a perception center in the brain and not a receptor site because their focus is closer to the antennae than the proboscis.

A flightless mutant, stripe, has been investigated, and it was found that it produced short bursts of motor output at high frequencies which led the authors to suggest that the mutant either can only start flight but not maintain it, or that the excitation is so high that it leads to very quick fatigue which terminates the burst again (Levine and Wyman 1973).

Hotta and Benzer (1973) have found a drop dead mutant which dies precociously and which has a complex developmental focus lying in the head. Flies with a bilaterally mosaic head do not split up equally into drop-dead mosaics and survivors; many more flies belong to the latter group. It was concluded that there are 2 foci and that both of them have to be mutant for the fly to express mutant behaviour. A single mutant focus is submissive to the normal one. The converse case, where two foci interact in such a way that the mutant focus domineers the normal one, was found with a mutant which holds its wings up and has its focus in the thorax (Hotta and Benzer 1973). Microscopic examination of both these mutations revealed vast degenerations in the brain and wing musculature respectively.

Most other mutations which have been isolated, have so far only been described and little experimental work has been done to probe deeper into the structures and functions affected by the mutations. Such mutants include some affecting circadian rhythm (Konopka and Benzer 1971) and courtship behaviour (Rushton and Metcalfe 1971, Hall pers. comm.) At the moment a number of laboratories are trying to demonstrate learning in Drosophila (Quinn et al. 1974, Spatz et al. 1974, Hay 1975, Medioni 1975). Once an experimental set up has been found which shows learning perhaps on an individual basis and more strikingly than has so far been achieved, one can expect many attempts to induce learning mutants. This approach might hold the key to the solution of a problem which has occupied psychologists for generations.

Mutagenesis of complex phenotypes, such as learning or mating behaviour, is considerably more difficult than with simple behaviours. Very many genes will be able to upset highly

integrated systems, but most of these genes will be rather unspecific and will help little in the clarification of the problems. Poorly learning animals, for example, are not only those which are defective in their memory system, but also those with motivational, sensory, neuronal, muscular and metabolic abnormalities. This complication in the isolation of mutants has been well epitomized in the following sentence: "I fail to see how the study of phenylketonuric patients helps in the analysis of the development of intellectual functioning." (Manning 1976). This problem will be discussed in more detail in the introduction to chapter 4.

It is obvious that with the more complex behaviours the labour involved in isolating and characterizing the mutants becomes prohibitive in comparison to the relatively trivial ones. It seems that if one wants to investigate fundamental characteristics of the nervous system, one has to use simple behaviours which allow quick screening of large numbers of flies and a convenient way of reproducing the mutant behaviour. If one is interested in more complex behaviours (like courtship or learning) which usually make mechanisation of the scoring of the behaviour very difficult, one either has to make gigantic research enterprises or change ones aims slightly so as to adapt them to the means. All extremes of degree of complexity should be investigated simultaneously and naturally also the continuum in between. A proper understanding of any biological system can only result from an integrated and concerted attack at all levels of the hierarchy making up the system. This research strategy is in fact being employed, as is exemplified not only by the fact that drosophilists study membrane molecules of nervous tissue and learning, but also by the fact that

some Benzerian behaviour geneticists have chosen to work with bacteria (Adler 1969, Berg 1975), in vitro cell cultures (Minna et al. 1972) Paramecium (Kung et al. 1975) and algae (Bruce 1974), whereas others work with mice,(Laudis 1973, Bliss and Chung 1974, Deol 1974, Chung 1975) and chicken (Albuquerque and Warwick 1971, Kuenzl and Rubenstein 1974). Experimental subjects of intermediate complexity which have also been subjected to a neurogenetic analysis are crickets (Bentley 1975) and nematodes (Brenner 1974). The latter organism and Paramecium have perhaps drawn most attention, besides Drosophila. The nematode research centers around questions of the structural development of the nervous system and sensory orientation mechanisms (Ward 1973), whereas the Paramecium workers are primarily concerned with the physicochemical properties of excitable membranes (Kung et al. 1975).

The present thesis deals with one aspect of courtship behaviour, namely the song which the male flies produce during courtship. The elaborate displays performed by courting males of Drosophila melanogaster have been well described by Spieth (1952) and Bastock and Manning (1955). The males orientate towards a virgin female, extend the wing closest to the female's head, vibrate it, lick the female's genitalia with their proboscis and attempt to copulate. During vibration of the wing, the male produces a sound (Shorey 1962) which is the main subject of the present investigation. Courtship song is a rather complex behaviour in ethological terms and yet it can be scored rather conveniently. Mutations in this behaviour could yield answers on the reductionistic and compositionistic levels. Mutants might allow one to elucidate the neural and muscular physiology of the song, the precise mechanism of song

production, motivational systems underlying song production, its genetical organization, and the function of the song. The involvement of this behaviour in sexual selection allows one to use female coyness (i.e. the tendency of females to discriminate against abnormally courting males) as a screening instrument. Besides this mutagenetic research, this thesis approaches courtship song with the more conventional methods of experimental ethology.

In the study of evolution, all structures and behaviours concerned with sexual selection and sexual isolation are of paramount importance due to the enormous influence they have on direction and speed of evolution (Campbell 1972). It appeared important to investigate the song from several levels simultaneously in the hope that the different pieces of the jigsaw puzzle thus collected, might fit together at the end and with the knowledge that a prerequisite to any embracing understanding of any behaviour is the study of its mechanisms and functions.

Behaviour genetics, one of the most integrated of all biological disciplines, provides a highly adequate framework in which to make such an analysis of a particular behaviour.

Chapter 2

Function of courtship song

2.1 Introduction

The importance of wing vibration for successful courtship of male Drosophila has been recognized since Sturtevant's (1915) pioneering work. He demonstrated that wingless males of Drosophila melanogaster have a much reduced mating success. The nature of the stimulus produced during vibration was discussed in many papers up to (Mayr 1950, Spieth 1952, Bastock 1956, Petit 1958, Manning 1959) and beyond (Ewing and Manning 1963, Ewing 1964, Manning 1967, Narda 1966, Burnet et al. 1971). Shorey (1962) demonstrated that the males are producing sounds during vibration which can be recorded through a microphone. The sound consists of a series of pulses (pulse song) with a pulse repetition rate of 29.8/s, which corresponds to an ipi (inter pulse interval) of 33.56 ms. That the sound is indeed the element of wing vibration which stimulates the females was shown by Bennet-Clark and Ewing (1967). In these experiments an electronically produced simulation of the male courtship song was played to females which were being courted by wingless males. This increased their receptivity significantly over control females.

Other, very strong evidence in favour of the auditory nature of the vibration stimulus comes from experiments in which the female aristae, a major part of the sound reception apparatus of Drosophila (Petit 1958), have been amputated. Such females have a much reduced receptivity, whether the amputation has been performed surgically (Petit 1958, Manning 1967), or genetically (Burnet et al. 1971).

Waldron (1964) recorded the song of Drosophila pseudoobscura and

D. persimilis. She found considerable differences between these two sibling species in one parameter, namely ipi, and concluded that these differences might play a role in maintaining sexual isolation between these two species. Later it was shown that ipi is highly species specific for a number of other species as well, (Ewing and Bennet-Clark 1968, Ewing 1970). Bennet-Clark and Ewing (1969) then made the crucial experiment when they played back artificial songs with different ipi's to D. melanogaster females and showed that the females would respond maximally to their own species specific ipi and respond less well to ipi's of half or double that length or to an ipi characteristic of D. simulans males, a sibling species of D. melanogaster. The other song parameter which was investigated, pulse length, was found to be of no importance.

More recently the function of the song has been investigated with experiments designed to test the hypothesis that the females are summating the song over a certain period of time, thereby gradually increasing their receptivity. Such a priming function of pulse song could indeed be demonstrated. Females when hearing continuous simulated song in a 5 min period before they have access to males will copulate faster than those females which have not received such a prestimulation. This effect is detectable up to about 5 min after the sexes have been mixed. If an interval of 2.5 or 5 min is introduced between the end of the prestimulation and the mixing of the sexes then there is no effect. Furthermore, there is no increase in mating speed if ipi's are used which are either half or double the species specific length, (Bennet-Clark et al. 1973).

All song recordings described so far (Shorey 1962, Waldrom 1964, Bennet-Clark and Ewing 1967) had been made with pressure sensitive microphones. Due to the nature of the sound producing organ, namely the wings, it is better to use particle velocity sensitive microphones, (Bennet-Clark 1971). With such a microphone (Bennet-Clark 1973) another component of the courtship song, besides pulse song, is revealed. It constitutes a buzzing noise rather like the flight sound only lower in frequency (160 Hz) and was termed sine song, because of its sinusoidal nature on an oscillogram, (Schilcher 1976).

The present chapter attempts to probe deeper into the functions of both pulse song and sine song and describes the effects simulated songs have on the behaviour of males and females. In the first part a more detailed description of the songs is given.

2.2 Maintenance of the flies

All stocks of flies employed in the experiments reported in this thesis were kept on Drosophila medium prepared in the Department of Genetics, Edinburgh University, which has been described in the UFAW handbook (3rd ed. 1967, p.907). This medium was contained either in half pint milk bottles or 2.5 x 6 cm vials. Shortly before the flies were introduced into bottles or vials, the food was seeded with a few pellets of dried active baker's yeast. For stock maintenance the flies were kept in bottles and each new generation was started with at least 50 pairs, but usually many more. In bottles which were intended to yield experimental subjects, the parent females were

allowed to lay eggs for 1 or 2 days only, to prevent larval overcrowding. The temperature control in the stock room was rather poor. The temperature went from a minimum of 22°C to a maximum of 29°C. However, the extreme temperatures never occured at the time of the experiments described in this thesis. The temperatures prevailing in the stock room in the time leading up to and during the experiments will be indicated for each experiment separately. The light regime was LD 12:12. Whenever flies had to be used for experiments the bottles from which they were to be collected were cleared around "light on" and virgin flies were collected 5-8 hours later. For collection the flies were etherized in such a way, that as soon as the last fly stopped moving, the flies were removed from the etherizer and sexing started. From etherization up to the time of the experiment they were always kept in vials, singly or in groups. If the flies had to be transferred to particular containers for any of the experiments, this was always done without further etherization.

2.3 Recording of the song

The courtship song of the male flies was recorded with a ribbon microphone, (Reslo RBT, 30/50 Ohm). The output signal of this microphone was, after appropriate preamplification (200 x), fed into a Tandberg 3041 X tape recorder. Ribbon microphones are particle velocity sensitive (Borucki 1973) and if one brings a vibrating male within 2 - 5 mm of the ribbon no elaborate sound insulation is necessary to obtain a very good record. The particle

velocity intensity produced by a vibrating male is about 95 dB at a distance of 2.5 mm from the wing and therefore well above the background noise level, (Bennet-Clark 1971).

The flies were placed into small wiremesh cells (10 x 15 x 5 mm) with a perspex top. The floor of these cells contained a small circular hole through which the flies could be introduced, in the following way. A funnel with a stop cock near its narrow end was fitted over an open vial containing the fly. A finger was placed over the opening at the narrow end of the funnel and the stop cock was opened. The fly was shaken into the space between the finger and the gate and the latter was closed, thereby confining the fly to the space in between the finger and the gate. Then the narrow opening of the funnel was placed over the opening of the recording cell and the fly was shaken into the cell. After the second fly had been introduced in the same manner, the cell was brought into position on the microphone. The whole process lasted 10 - 30 s per fly and is therefore considerably faster than methods which rely on the flies' geo - and phototactical biases to lead them into such a small cell, through a rather narrow (2 mm) opening.

To bring the courting male as close as possible to the ribbon of the microphone, the outer protective shielding of the latter was removed and the cell was placed on the grid directly above the ribbon. The floor of the cell was approximately 2 mm above the ribbon.

After the male had begun to court, the tape recorder was switched on and an adequate amount of song, no less than 10 long bouts, was recorded. These recordings could then be displayed on the screen of a Telequipment oscilloscope and filmed with a continuous recording camera (Nihon Kohden PC-1B). Whenever song

records were filmed, a calibration signal (50 Hz square wave from the oscilloscope amplitude callibration output) was displayed on the second beam, providing a time reference independent of film speed. All song records described in this thesis were obtained in this manner.

The temperature during the recordings was always carefully controlled at 25 $\pm$ 0.5°C, because of the known temperature sensitivity of song parameters such as ipi, (Shorey 1962).

The measurements of the different song parameters were taken from the film records. The following definitions and procedures were employed.

ipi was defined as the distance between the beginning of one pulse to the beginning of the next one. Ipi's occur in bouts, and sometimes it is difficult to decide whether a given interval between pulses constitutes a true ipi, or an interval between bouts. There is no other objective criterion available in such a case but an arbitrary limitation of the length of an ipi. Therefore, whenever ipi was measured in any of the experiments reported in this thesis a cut off point was introduced beyond which no ipi's were measured. This cut off point will be indicated for each experiment separately. Usually ipi's were measured only if they came from bouts containing more than 6 pulses.

Sine song was defined as any sinusoidal train of waves which was clearly above background intensity and longer than 5 cycles. The frequency measurements were taken from several long bouts of a single male. Each bout's mean was derived by making three measurements of 10 cycles length within the bout. Several such bouts gave the mean of the male. Several males of one group gave the mean and variance of the sine song frequency of this group of males. Different groups were compared by t-tests.

Table 2.1 Some results from the recording of 76 courtships of attached -X males

Mean ipi (ms)	34.94 ± 0.26 (SE)
% time pulse song	17.79 ± 0.95 "
% time sine song	9.75 ± 0.69 "
% time vibration	30.75 ± 12.38 "
Mean pulse song bout length (s)	0.32 ± 0.01 "
" sine song " " (s)	0.42 ± 0.02 "
" vibration " " (s)+	1.18 ± 0.07 "
No. of pulse song bouts+	532
" " sine song bouts+	187
" " vibration bouts+	167

+ from 16 of the 76 courtships

2.4 General description of the courtship song

2.4.1 Methods and Results

2.4.1.1 Some song parameters

The data presented in this section (2.4.1.1) were mostly collected and analysed in colaboration with Mr. M. Dow. We recorded 76 matings of 3 d old males and females acoustically and visually. The flies came from an attached-X stock (att-X) whose genetic characteristics will be described in more detail in section 4.2.1.1. Males from this stock are wild type and the females are homozygous for y, w and f. The stock was obtained from the Institute of Animal Genetics, Edinburgh. They were kept at 25 ± 2°C and the experiments were performed at 25 ± 0.5°C. The flies had been kept in isolation up to the time of the experiment. All sounds emitted during the whole courtship were recorded on a tape recorder and the visually monitored behaviours on an event recorder. Vibration, a visually recorded behaviour, is defined as the extension of one wing at a 90° angle from the main body axis. This behaviour was recorded on the event recorder and simultaneously on the second channel of the tape recorder with the help of a square wave generator. The latter recording permitted a precise investigation of the relationship between the visual and acoustical components of vibration.

Each ipi of all males, with no regard to the length of the bout it was a part of, was measured with a cut off point at 100 ms. Equally all sine song bouts were measured, but it was not possible to get an estimate of the frequency of this song component. The results as they are pertinent to this thesis are displayed in Table 2.1.

It can be seen that the males spend slightly more time in vibration than they do in either pulse song or sine song. The differences are however not significant due to the enormous variability of the percent time a male spends in vibration. The bout length of vibration is considerably longer than that of pulse - or sine song. Sine song has a longer bout length than pulse song. The number of pulse song bouts is vastly larger than the number of vibration - or sine song bouts. Looking more closely at these figures, we find that out of 532 pulse song bouts, 182 occurred when the wing was not fully extended. Of 187 sine song bouts only 10 occurred outside vibration. On the average each bout of vibration consists of 2.1 bouts of pulse song and 1.06 bouts of sine song. The large number of pulse song bouts which are emitted when the wing is not fully extended serve to show that the visually recorded vibration is only a very rough guide to the underlying acoustical phenomena. In other words, it is not a precise measure of the auditory stimulation a male provides for the female. What has been called 'curtailed' forms of scissoring in D. melanogaster (Manning 1959) is not analogous to the scissoring of D. simulans. In the latter species scissoring is completely silent (Schilcher, unpublished) whereas these curtailed scissoring movements of D. melanogaster which are rather quick, occur frequently, remind one of wing flicking and during which the vibration of the wings is very difficult to see, are always accompanied by sound output.

Seventy five out of seventy six of the recorded matings ended with a bout of pulse song shortly before the final successful attempted copulation. Only one copulation was preceded by a bout of sine song.

able 2.2 Analysis of variance and repeatability of ipi.

source	SS	df	MS	F	p
between males	25.426	39	0.6519	10.4576	0.001
within males	46.195	741	0.0623		

variance components:

source	component	percent
between males	0.0303	32.67 (repeatability)
within males	0.0623	67.33

2.4.1.2 Repeatability of ipi

An attempt was made to get an estimate of the repeatability of ipi. The repeatability of a given trait gives a quick estimate of the upper limit of its heritability, (Falconer 1960). The latter measurement provides useful information for evolutionary arguments, as has been discussed in the introduction. Even such an admittedly rough estimate of the heritability might allow some conclusions about the evolutionary past of ipi. Forty att-X males were recorded while courting one day old virgin att-X females. The males were from 4 age classes, 2, 3, 4 and 5 days of age. As there were no differences between the ages the results were pooled. At least 10, maximally 29 ipi's, all from one bout, of each male were measured with a cut off point at 50 ms. The resulting mean ipi was 34.57 $\pm$ 0.37 (SE) ms. The within and between male variances were calculated (Model II ANOVA, Sokal and Rohlf 1969) and the repeatability derived from them, (Table 2.2). It can be seen that the between group component adds a significant amount to the within group component. The repeatability is 32.67%. It follows that the heritability of ipi would have to be expected to be rather low. Therefore it can be concluded that ipi has been subjected to rather intensive selection in the past, and has lost much of its additive variance, (Robertson 1955). A list of morphological traits in Falconer (1960) includes no repeatability lower than 40%. Such traits as activity in an arena in Drosophila have repeatabilities of 75 or 87% depending on the sex, (Ewing 1963). Activity in a circular arena has a heritability of 51% (Connolly 1966).

2.4.1.3 Coefficient of variation of ipi

Another measure which would allow one to compare the variability of a given behaviour to that of other characteristics, behavioural or morphological, is the coefficient of variation (CV) (Sokal and Rohlf 1969). Under the hypothesis that ipi is a species isolation signal one would expect it to have a rather low CV. The interindividual CV of ipi, as obtained from the data of preceding section, is 6.78%. The intraindividual values range from 5 to 15%. Ipi is considerably more variable than, for example, the time which elapses between the two snaps which are part of the strut behaviour of sage grouse, (Wiley 1973). It is however well in the range of the degree of stereotype of many morphological characters (cited in Wiley 1973), and also that of certain behaviours of an Anolis lizard, which are thought to be highly stereotyped, (Stamps and Barlow 1973).

2.5 Effect of song on males

2.5.1 Methods

The flies used in these experiments were from the att-X^B stock, (see section 4.3.2.). They were 3 days old, kept at 25 $\pm$ 1°C and the experiments were conducted at the same temperature. The males were allowed to hatch for 20 hours before being collected and were kept in groups of 6 in vials up to the time of the experiments.

For the behavioural observations the insects were introduced into cylindrical perspex cells, 2.3 cm high and 2.6 cm in diameter, with a wiremesh floor and a gauze top. The introduction procedure was the following. A funnel was placed over an open vial containing

the flies. The latter were then shaken into the narrow end of the funnel which was closed with a finger at the narrow end. The vial was removed and a cotton plug on a wireholder was inserted into the funnel. The cotton plug closed the narrow part of the funnel, thereby confining the flies to the space between it and the finger. Then the narrow end of the funnel was placed over the opening of the observation cell and the flies were gently pushed through it by slowly moving the plug forward. The cells were placed on top of a loudspeaker through which simulated song could be played. The simulation system has been described before (Bennet-Clark and Ewing 1969), and only minor modifications were made; i.e. an unmodified commercial loudspeaker was used and the sound absorbing column was dispensed with. Unless indicated otherwise, the sound intensity in the experiments was 105 dB as measured with a Bruel & Kjaer type 4117 microphone, at the level of the floor of the observation cell. The intensity measurements throughout these experiments have been made with the type 4117 microphone. A ribbon microphone (Reslo, Bennet-Clark 1973) was calibrated by comparison with the type 4117 microphone at a distance of 1.5 m in the 'farfield' condition and then the difference between the two microphones was measured in the 'nearfield' condition, at the level of the floor of the observation cell. In the nearfield condition the sound is highly divergent and particle velocity, measured by the ribbon microphone, and pressure, measured by the type 4117 microphone are not in phase, particle velocity being relatively higher, (Olson 1957). It was found by these means that in the acoustic condition and with the simulated courtship song, the particle velocity was 10 dB louder than the pressure measurement indicated. All sound levels

Figure 2.1 Oscilloscope recordings of the wave form produced by the simulator. (a) recorded through a ribbon microphone; (b) recorded directly from the simulator.

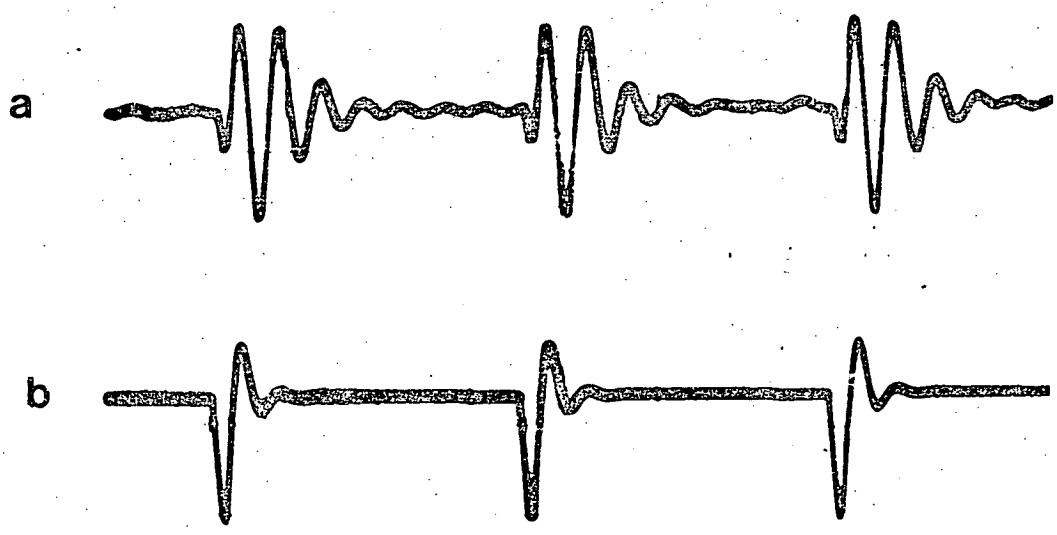
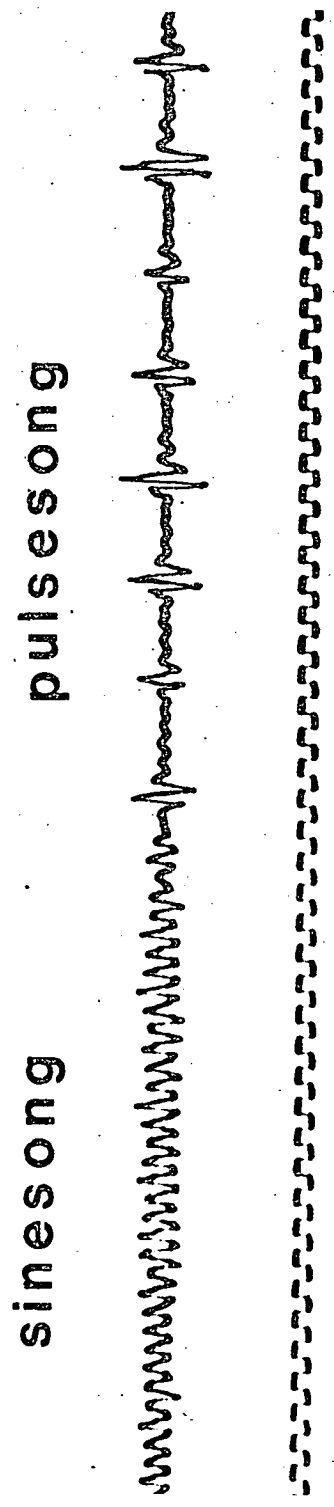


Figure 2.2 Oscilloscope traces of the courtship song of *D. melanogaster*.

Calibration signal: 100 Hz



considered here are corrected for this difference and are quoted in terms of particle velocity with reference to a threshold of 0.5×10^{-7} m/s which in the plane wave condition is equivalent to the threshold of 10^{-12} W/m^2. This correction is made because the antennae of a Drosophila are particle velocity receptors (Bennet-Clark 1971). The ipi is, if not otherwise indicated, 34 ms which is approximately the natural ipi of D. melanogaster (Shorey 1962). Fig.2.1.b shows the waveform which the simulator was producing and which was employed throughout these experiments. Fig.2.1.a shows the same waveform after it has been played through a loudspeaker, picked up by a ribbon microphone, and recorded on a tape recorder. This should be compared with Fig.2.2 which gives a typical example of waveform as produced by a male D. melanogaster recorded via a ribbon microphone.

Sine song was produced with the same simulator and constituted a sine wave with a frequency of 160 Hz. The intensity was the same as for pulse song.

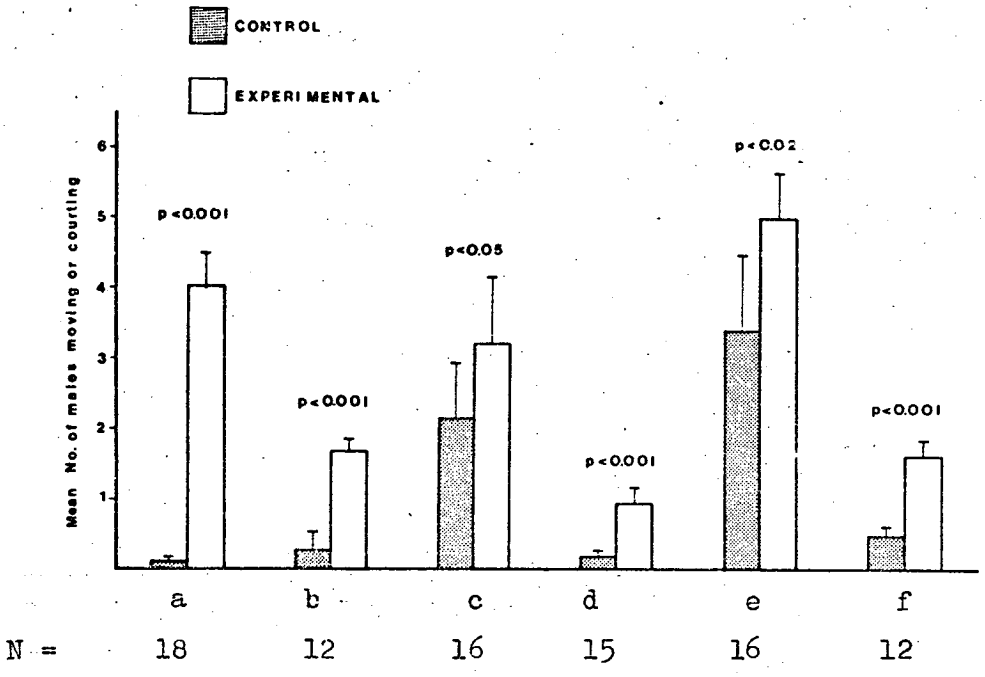
The majority of the data was collected in the following way: two observation cells, containing six flies each were placed, 1 cm apart, on the loudspeaker, approximately 8 cm above the cone, and given 1 minute to settle down. From then on every 10 s the number of individuals moving or the number of courtship interactions which were in progress at this instant in time were recorded in the left cell first and then in the right one. One courtship interaction was scored whenever two animals were orientated towards each other. This definition includes all other sexual behaviour which might have occurred, like tapping, chasing, vibrating, licking and attempted copulation, since orientation always accompanies these activities. When several males were courting each other in chains or crosses of

interaction, the number scored was N-1, where N is the number of males involved. The first 2 minutes were without any sound and served as an internal control for the next 2 experimental minutes where sounds of varying properties were played to the animals. The mean number of flies in movement, or the mean number of courtship interactions was calculated for the control and experimental situation of each experiment and a mean and a variance were obtained for the means of the different repetitions of the same type of experiment. Comparisons were made between different types of experiments by subtracting the control mean of each single replicate from the corresponding experimental mean. Mean and variance of the differences were calculated and different experiments were compared on the basis of these corrected data with Student's t-test. t-tests were also used to compare control and experimental scores within and between experiments.

In certain tests the wings of the males had to be amputated; this was achieved with a scalpel at the time of sexing. Practically the whole wing was thus removed. This operation meant that the animals had to be kept etherized for a longer period than that necessary for sexing alone, therefore all winged control animals were held etherized for the same length of time.

One test involved aristaless males. This amputation was carried out with watchmaker's forceps in such a way that the aristae only were clipped off. The other antennal segments did not appear to be harmed. Again the control flies were kept under the influence of ether for an equivalent length of time. With good forceps this operation can be performed so reliably that errors of surgery do not occur.

Figure 2.3 Means (± 95% CL) and significance levels of male locomotor and courtship activity in the silent control situation and the experimental situation where courtship song is played.



a) activity, group housed wingless males
b) courtship, " " " "
c) activity, " " winged "
d) courtship, " " " "
e) activity, single " " "
f) courtship, " " " "

2.5.2 Results

2.5.2.1 Wingless and winged males

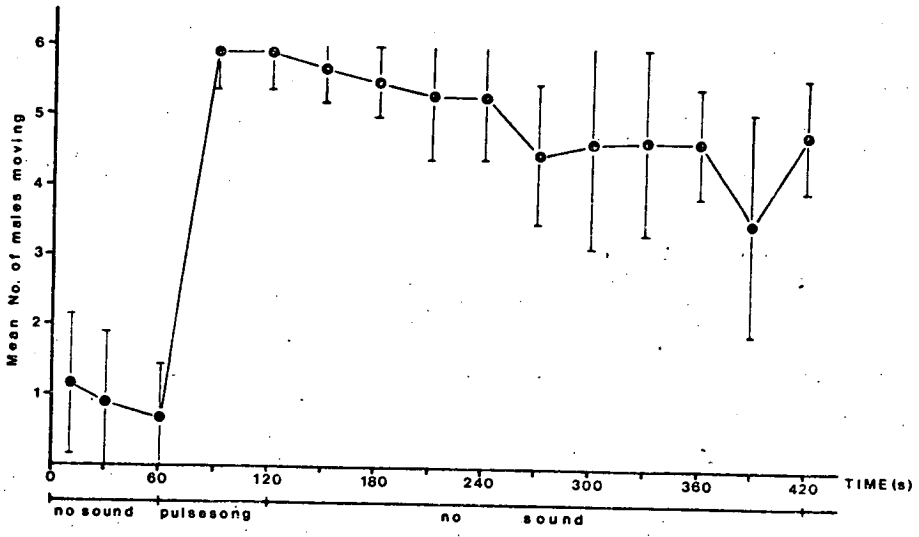
Figures 2.3a and c show the non-sexual locomotor activity of wingless and winged males in the control and experimental situations. Figures 2.3b and d indicate the numbers of orientations observed in wingless and winged males. As can be seen the differences between the control and experimental situation are significant in every case. For locomotor activity this difference is however much larger in the wingless males than it is in the winged ones.

Looking at courtship activity (Figures 2.3b, d) we find that the controls are very similar to each other but that the experimental winged males score lower than the wingless ones, ($p < 0.01$). This is probably due to the fact that wingless males lack their major rejection stimulus, namely flicking of the wings and therefore any courtship interaction is likely to last longer in the wingless situation, with the effect of a higher score at the same frequency of interactions.

2.5.2.2 Effect of preexperimental housing

Figures 2.3e and f show the scores of winged males which had been kept in isolation from the time of sexing up to the time of testing. As can be seen the measures are considerably higher in this case as compared to c and d. The differences between control and experimental are not significant between e and c, but they are between d and f, ($p < 0.02$). Although courtship activity did

Figure 2.4 Retention of the effect courtship song has on the locomotor activity of males. Means and 95% CL are plotted.



increase in the no sound condition, it increased even more with sound. This result confirms the finding of Ellis & Kessler (1973) that males housed in isolation show an increase in their mating speed.

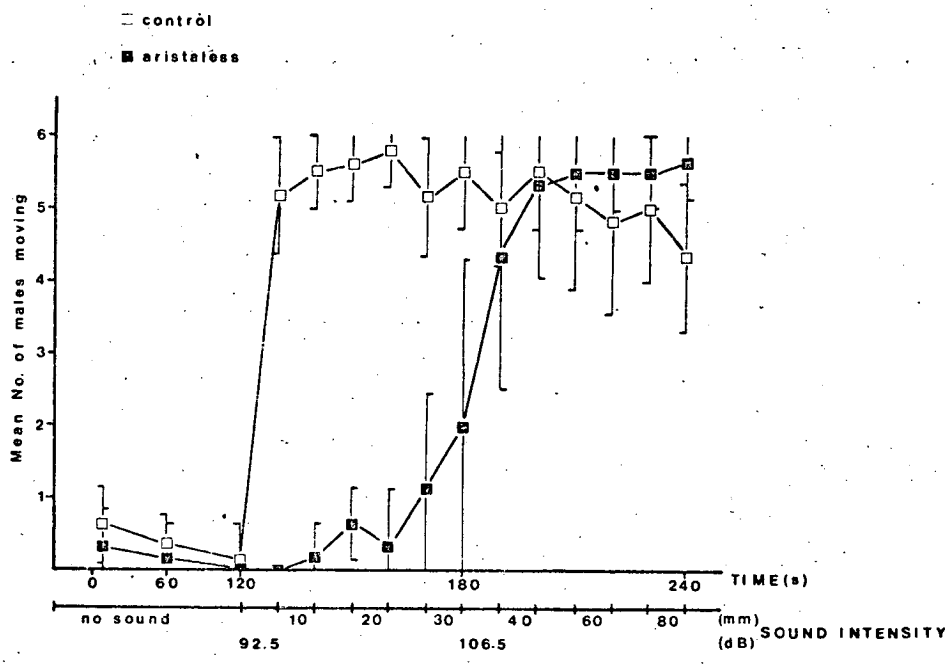
2.5.2.3 Retention of the effect of song on male locomotor activity

Two groups of wingless males were placed on the loudspeaker, given one minute to settle down and then for one minute courtship song was played to them. After this they were observed for another 5 minutes. During both periods the number of males moving was counted every 30 seconds. As Figure 2.3a shows, locomotor activity is the most striking and therefore most accurate and convenient measure of the effect of the song on males. Therefore in this and the following experiments locomotor activity rather than courtship activity is taken as a measure of response. Figure 2.4 gives the means and 95% confidence limits for 6 replications of this experiment. It is evident that even after 5 minutes the flies are still aroused.

2.5.2.4 Sensory basis of the effect

Wingless males without aristae and control flies with their aristae intact, were allowed a 1 minute period to settle down, then they were subjected to a 2 minute period without sound during which their activity was scored after 10,60 and 120 seconds followed by a

Figure 2.5 Effect of aristae amputation on the sensitivity of males to courtship song of increasing intensity. Means and 95% CL are plotted.



2 min period with sound during which their activity was scored every 10 seconds. In each consecutive 10 second period the intensity of the sound was different, increasing by 5 mm peak to peak until 40 mm, and then in steps of 10 until 80mm. As the intensity was measured directly on the oscilloscope, as the amplitude of the pulses in mm without a microphone, the values plotted in Fig. 2.5 can not be directly converted into dB; the corresponding values were measured with a microphone and 2 such values are shown. The means and 95%CL are based on 6 replications. The loss of the aristae clearly results in the flies responding at the higher intensities only.

Waldron (1964) has suggested that D. pseudoobscura and D. persimilis females perceive the song of the male via the substrate through their chordotonal organs. This suggestion was based on the observation that wingless males could be recorded through the microphone nearly as well as winged ones. I have tried to record wingless males repeatedly and always found them completely mute. Waldron was recording through a pressure-sensitive crystal microphone, whereas I recorded through a ribbon microphone (Bennet-Clark 1973), which, like the female's aristae, is a particle velocity receptor (Borucki 1971). At a distance of 5 mm from a male D. melanogaster the sound level is 72 dB, at 2.5 mm it is 92 dB, (Bennet-Clark 1971). These theoretically derived values agree well with my direct measurements. An average courtship distance in D. melanogaster will be around 3 mm between the male's wing and the female's aristae. In other words, only rarely will the female encounter intensities above 100 dB. Aristaless males hear the simulated song from about 106 dB onwards. If females have comparable alternative mechanoreceptors and if the

Figure 2.6 Lower sound intensity threshold of the effect of courtship song on the locomotor activity of males. Mean and 95% CL are plotted. The intensity increases in steps of 3 dB.

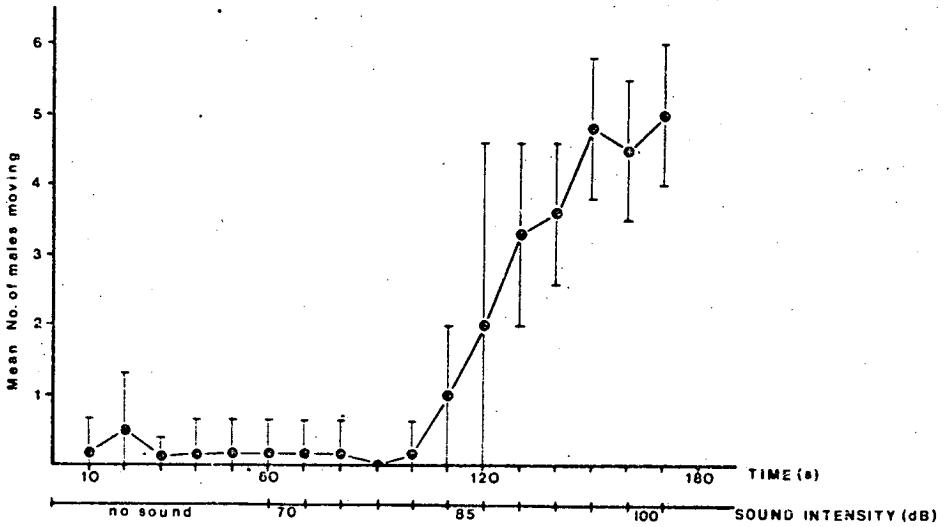
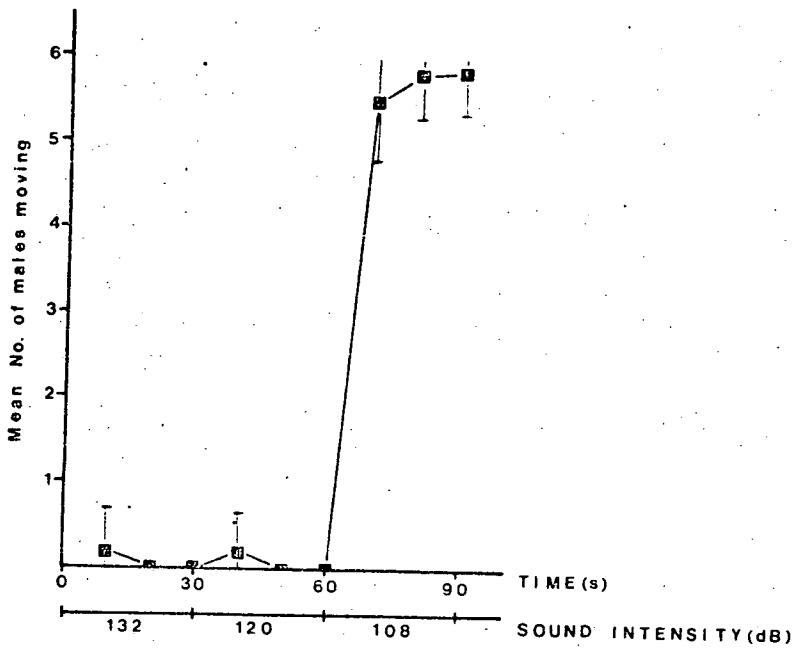


Figure 2.7 Upper sound intensity threshold of the effect of courtship song on the locomotor activity of males. Means and 95% CL are plotted.



acoustic properties of the substrate can be compared in the 2 situations, then they would not hear most of the male's singing. It therefore seems likely that the song is perceived through the aristae. This hypothesis gains further support from the well known observation that wingless males have a considerable mating disadvantage (e.g. Ewing 1964); also females discriminate against wingless males in the dark in the same way as they do in light, (Bastock 1956) which indicates that the wing vibration does not serve as a visual stimulus and aristaless females are much less receptive than control females, (Manning 1967, Burnet et al. 1971).

The threshold intensity for the response of normal wingless males to sound was determined in a separate experiment. Wingless males were given 1 minute of no sound during which their activity was scored every 10 seconds. Then they were subjected to 110 seconds of sound which increased by 3 dB after each 10 second period. For this stepwise increase in sound intensity a fixed 3 DB interval potentiometer was used as an attenuator. Again the number of active males was counted every 10 seconds. It can be seen from Fig. 2.6 that the males start to respond clearly at about 88 dB, (N = 6).

It has been observed that the sensory system responsible for the reception of the sound can be overloaded or overridden with very high intensities. Fig. 2.7 gives the results of an experiments designed to show this. Male wingless flies were observed for 1.5 minutes and the number of active insects was counted every 10 seconds. For every period of 30 seconds song of a different intensity was played decreasing by 12 dB each period. It is apparent that sounds with a particle velocity of 120 dB or more are not effective.

Figure 2.8 Means and 95% CL of the effect of courtship songs with different ipi's on the locomotor activity of males.

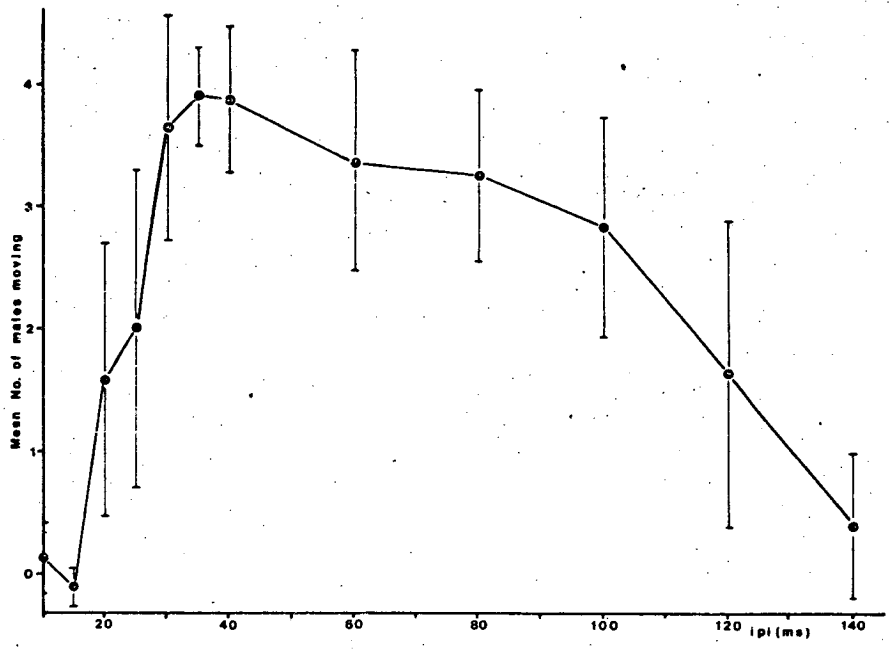


Table 2.3 Effect of sine song on the locomotor activity of males. Number of males moving. (N = 8).

	Mean	SE
no sound	0.1978	0.0953
sine song	0.2187	0.0830

2.5.2.5 The effective ipi range

Activity measurements were made on wingless males. The scores of the experimental and control period were subtracted and these adjusted scores are given in Fig. 2.8 for varying ipi's. Each mean is based on at least 6 replications. It appears that ipi's between 30 and 80 ms are about equally effective in initiating movement in wingless males. This rather wide range seems to contradict the hypothesis that ipi functions as a species identification signal, but it must be understood that we are dealing with males, and that they may be expected to be less critical than females, (Trivers 1972).

2.5.2.6 The effect of sine song on male locomotor activity

Table 2.3 shows that if sine song is played to wingless males during the experimental period, they do not increase their activity. There are no signs that would suggest that they perceive the song at all. At the end of all these experiments pulse song was played back to the experimental subjects which did not respond to sine song. All groups of males reacted instantaneously in the typical manner.

2.5.3 Discussion

This striking influence of pulse song on the behaviour of unisexual groups of males is not a general unspecific response which would need no further attention. Several lines of evidence indicate

that it is a specific and genuinely sexual behaviour. Very high intensity courtship interactions, like attempted copulations have been frequently observed. These are rarely, if ever, seen in groups of males which have not been sexually stimulated. Moreover it was found that the normally quite motionless flies can be induced to move by sudden and intensive light flashes, but this activity dies down rapidly and no sexual behaviours occur. The different degrees of response to different ipi's, the fact that sine song does not act as a stimulus and the sensory specificity of the effect, as evidenced by the arista ablation experiment, serve as further support for the specific and sexual nature of the response.

Accepting this hypothesis, there appear to be two possible explanations for this song induced increase in male sexual activity: (1) it is of advantage for a male to be instantly aroused as soon as it hears another male singing, because this indicates that there is in all likelihood a virgin female nearby, and it is a good strategy to look for her immediately, or (2) a male is stimulated by its own song. A positive feedback loop of this kind might be necessary to increase the sexual excitation up to a level where copulation can take place (Ewing, pers. comm.). This latter point is made rather unlikely by the observation that wingless males are very energetic courters, (Bastock 1956, Cook 1973). The first conjecture is supported by the observation that in the wild Drosophila seem to congregate on feeding sites at certain hours of the day and copulations seem to take place there under rather crowded conditions, (Spieth 1974). Such conditions are a necessary prerequisite for the evolution of the proposed response, because the sound does not carry very far, (Bennet-Clark 1971). Under the very high

population densities often found in laboratory cultures this arousal phenomenon would obviously be very advantageous.

There have been reports in the literature which would allow an interpretation on the basis of this male response to sound. For example, in one such case wingless males were found to be at a lesser disadvantage when together with winged ones, than they are when by themselves, (Sturtevant 1915). In another case it was reported that wingless males (this time genetically wingless) in a competitive mating situation can even be more successful than winged ones, (Burnet and Connolly 1974). Dow (1975) has demonstrated that the mating success of yellow males increases with the number of males being allowed to court one female. This increase ceases however when more than 4 males per female are present. Clearly these results can also be explained with an alternative hypothesis which interprets the result from the female's, rather than the male's, point of view. In every case, as the mating success of the males increases, so does the amount of stimulation which the females receive. A distinction between these two alternative explanations can only be made with mating competition experiments where the auditory sense(s) of the males or females has been extirpated in order to enable one to distinguish effects on the one sex from those on the other.

That the male arousal response has evolved specifically for pulse song and not for sine song can be explained by the fact that the sound intensity of sine song is considerably lower than that of pulse song and that the former is emitted much less frequently than the latter. A male would therefore practically always hear the pulse song emitted by another male before it would perceive its sine song. In order to exclude the possibility that the behaviour

described is a strain idiosyncrasy, preliminary tests were made on a stock (Haren) which had not been in the laboratory for a very long time and which is therefore more representative of a natural population. This stock was found to exhibit the same response to simulated pulse song.

2.6 Effect of song on females

2.6.1 Introduction

The demonstration in the last section that simulated courtship song triggers a specific response in males, makes it necessary to take a fresh look at the interpretation of the earlier playback results, which have been mentioned in the introduction to this chapter. In all these experiments the males were present during the stimulation period and could therefore also perceive the song, the effect of which on the females was under investigation. It is possible that the results reported were obtained because of the effect of the simulated song on males, rather than on females, as has been assumed. For example, the data of the earliest report (Bennet-Clark and Ewing 1967) could very well be interpreted on a male basis only, because all the results are in accord with those from the male stimulation tests. The second publication (Bennet-Clark and Ewing 1969) is not so easily amenable to an interpretation from the males' stand point, because of an ipi specificity which has not been found with the males alone. Comparing Fig. 2.8 of this chapter to Fig. 6 in Bennet-Clark and Ewing (1969) one can see that their finding with an ipi of 17 ms is in agreement with those of the

present investigation of the males, where ipi's of 10 and 15 ms have no effect and one of 20 ms results in a weak reaction by the males. However in the ipi range of double the species specific value, there is some discrepancy. Males seem to respond almost as well to 60 and 80 ms as they do to 34 ms; whereas 68 ms in the Bennet-Clark and Ewing (1969) paper results in a distinctly smaller increase in female receptivity than 34 ms. Nevertheless 68 ms is better than 17 and therefore the differences in the results of the two investigations are of degree rather than quality.

In the prestimulation report (Bennet-Clark et al. 1973) an ipi specificity has also been demonstrated. Here, however, the magnitude of the effect is so small that differences are difficult to assess, and it might well be that they are of the same order of magnitude as those found with the male sex by itself.

Although there are strong indications that the early playback results are not 'artefacts' brought about by the males, it seems nevertheless no more than prudent to have a renewed look at the effects of simulated song on females, under conditions where the complication with the males can be controlled. This repetition might be especially called for in view of the notorious strain differences in Drosophila which by themselves might account for the discrepancies just discussed.

The following experiments will investigate the effect of simulated song on female locomotor activity and receptivity. The latter tests will be of two kinds: stimulation, where the males are present while the females are perceiving the stimulus (Bennet-Clark and Ewing 1967, 1969) and prestimulation, where the males are introduced to the females after these have been stimulated and the sound has been switched off, (Bennet-Clark et al. 1973).

2.6.2 Methods

2.6.2.1 Effect on female locomotor activity

These experiments were exactly analogous and performed under the same conditions as the ones which have been reported in the last section for males. Virgin att-X^B (see section 4.3.2) females were subjected to a 2 min control period and then to a 2 min period where various sounds were played back to them. The number of flies moving was counted every 10 s in both the control and experimental periods. Several experimental conditions were employed, pulse song (34 ms), sine song (160 Hz), no sound and white noise. The latter sound condition was derived in this and all later experiments from the output of the vertical amplifiers of a Telequipment oscilloscope, (Bennet-Clark, pers. comm.).

2.6.2.2 Effect on female receptivity

In these investigations flies from the Haren and an al;th stock were used. The former was obtained from Mr. M. Dow and is a wild type stock which has been collected in the Netherlands in 1972. The latter stock was obtained from Dr. B. Burnet and is characterized by extremely vestigial aristae, (Burnet et al. 1971). They were kept at 25 $\pm$ 2°C and the tests were carried out at 25 $\pm$ 0.5°C with 3 d old flies. The insects stayed in unisexual groups of 5 up to the time of the experiment. Wing amputations, where necessary, were carried out in the same way as has been described before. The simulation system was also the same. Pulse song with an ipi of 34 or 48 ms was used, it had a pulse length of 5 ms

and an intensity of 105 dB. Sine song had the same intensity. Both songs were however patterned. A sound period of 2 s alternated with a silent period of 3 s. This modification was made to render the stimulus more like the natural one, which is given in a patterned fashion by the males. Furthermore there are actual indications that patterned song is more effective than a continuous one in stimulating females, (Bennet-Clark, pers. comm.). In the control tests the females were subjected to patterned white noise of the same intensity.

2.6.2.2.1 Prestimulation

Haren females were subjected to 1 min of patterned stimulation before they were mixed with Haren males. For this purpose rectangular perspex cells were built which fitted tightly on top of each other. The lower one contained the females and was suspended in a clamp 5 cm above the loudspeaker; it had a gauze floor and a ceiling of the same material which could be pulled out. The upper cell contained the males and had a removable floor. When the two cells were placed on top of each other it was possible to pull the ceiling of the lower one out together with the floor of the upper one, thus producing a single mating chamber 2.2 x 2.3 x 2.4 cm. Five males and five females were introduced into their respective cells through a whole in the sidewall with the same technique as has been described in section 2.5.1. The females, after their cell had been suspended over the loudspeaker, were allowed 1 min to recover from the transfer and then they were

subjected to 1 min of patterned auditory stimulation. As the sound was switched off the males' cell was placed on top of the female one and the partitions were removed, allowing the sexes to intermingle and courtship to begin. This process took from 5 - 10 s. During the prestimulation period the males were kept away from the loudspeaker under sound intensities far below their threshold of response.

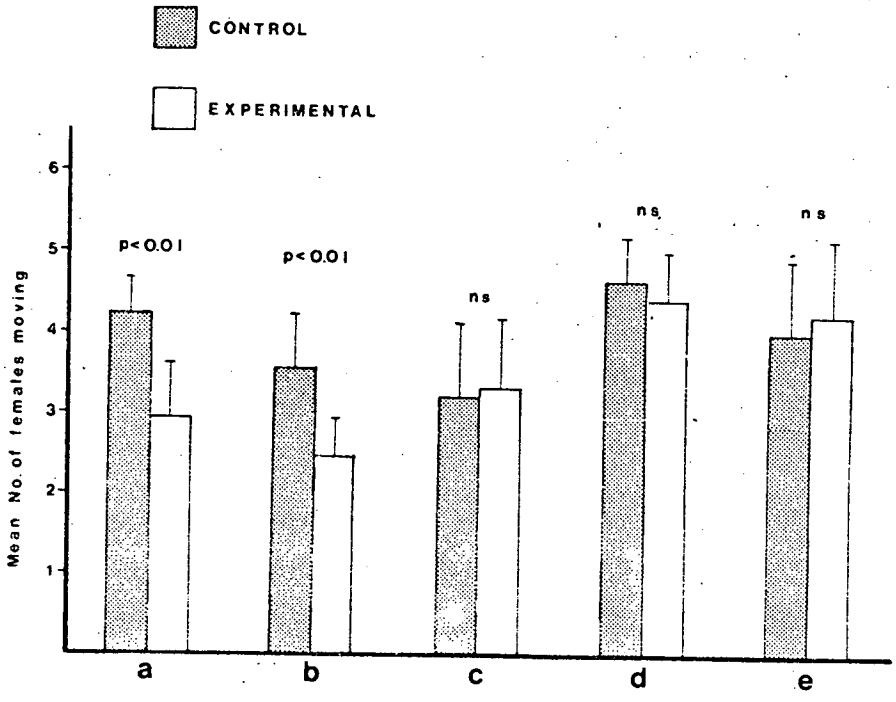
In one test the roles of the sexes were reversed. The males received the prestimulation (5 min continuous pulse song) and the females were kept away from the sound source and introduced to the males after the sound had been switched off.

2.6.2.2.2
Stimulation

For these experiments Haren females were introduced into a cylindrical perspex cell (2.6 cm in diameter, 2.3 cm high) with a gauze top and a wire mesh floor. This cell was suspended over the loudspeaker and after 1 min of recovery wingless males carrying the two arista mutations were introduced through a funnel. As the last male entered the cell the sound was switched on and left on until the end of the experiment. Introduction of the males was accomplished in 5 - 10 s. As the rationale of this experiment required the males to be deaf, it was first established in pilot experiments, that at the sound intensities employed here they do not respond to the song. These males start reacting only at intensities around 120 dB. Therefore in the context of the present investigations these males will be considered deaf; they are also mute due to the wing amputation.

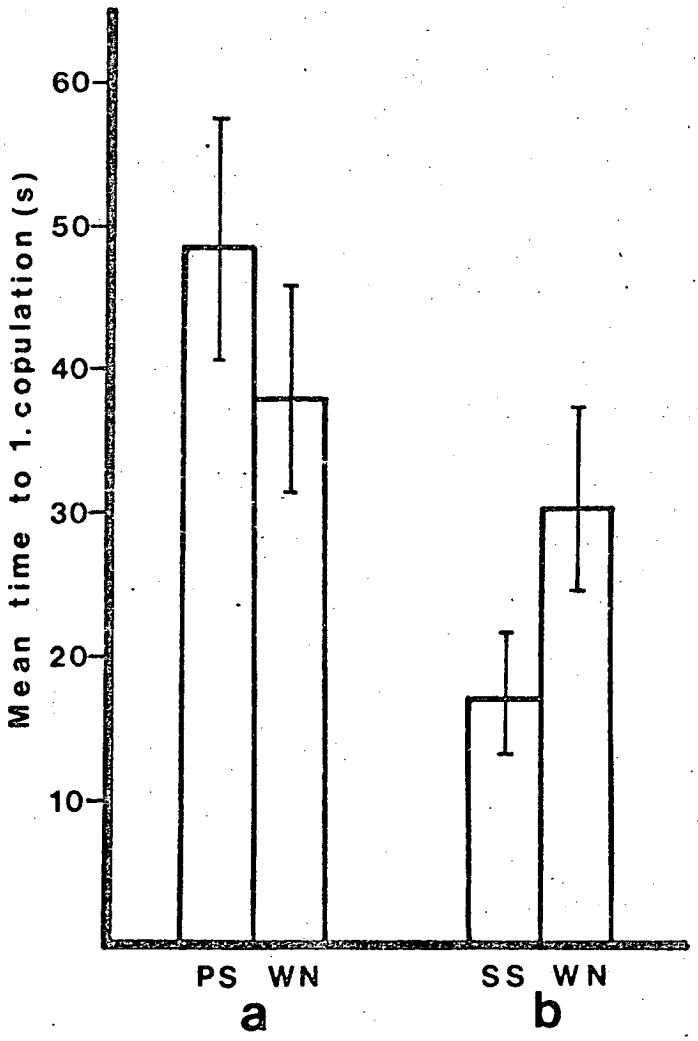
In both types of experimental set ups the time to the first copulation was scored. These data were log normally distributed and t-tests could be used to compare the log means of the control

Figure 2.9 Means and 95% CL of female locomotor activity and significance levels for the difference between each experimental and its control situation.



a) pulse song, 3 day old virgin females
b) sine song " " " " "
c) pulse song 1 " " " "
d) no sound 3 " " " "
e) white noise " " " " "

Figure 2.10 Means and 95% CL of the effect of pulse song (PS, N = 30) and sine song (SS, N = 20) on female receptivity (time to first copulation). Both types of song are applied as prestimulators and compared to white noise (WN).



and experimental scores. The relevant Figures or Tables show the antilogs of these results and the confidence limits, are therefore not symmetrical around the mean. Control and experimental tests were always performed on the same day in an alternating sequence.

2.6.3 Results

2.6.3.1 Locomotor activity

Fig. 2.9 shows the influence pulse song, sine song, no sound and white noise have on the activity of 3 day old females. Furthermore, the effect of pulse song on 1 day old females is plotted. All histograms are based on ten replicates. It is evident that pulse song and sine song significantly reduce female activity. White noise and no sound in the experimental 2 min period have no such effect. One day old females which are not yet receptive (Manning 1967) do not decrease their activity if subjected to pulse song.

2.6.3.2 Receptivity

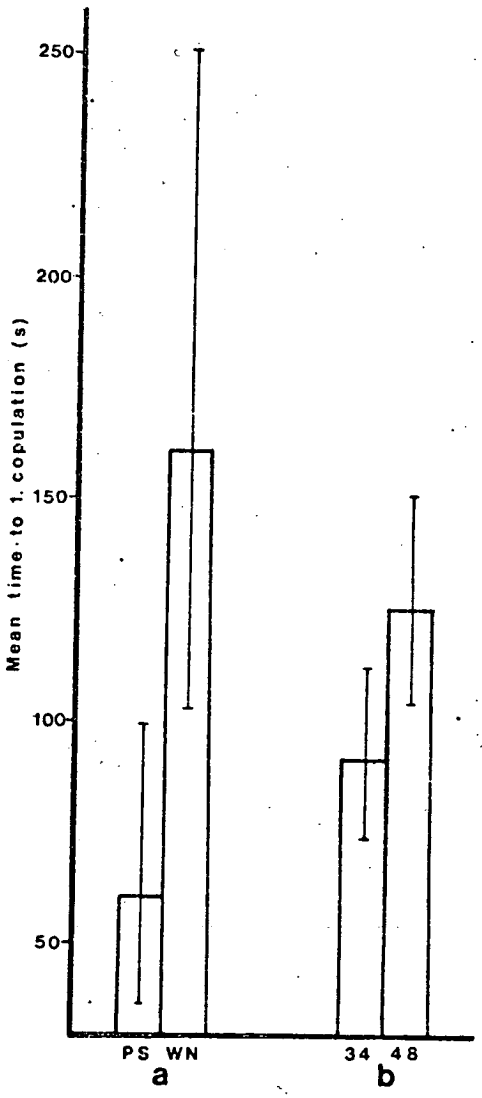
2.6.3.2.1 Prestimulation

Fig. 2.10a indicates that if the females are prestimulated with pulse song (ipi = 34 ms) the time to the first copulation does not change in comparison to the prestimulation with white noise (N = 30). When they are subjected to sine song, the time to the first

Table 2.4 Effect of pulse song on mating speed when the males receive the prestimulation

	Mean (s)	95% CL
pulse song	24.08	31.92 18.16
white noise	49.43	60.69 40.26

Figure 2.11 Means and 95% CL of the effect of pulse song (PS, N = 18) compared to white noise (WN) and of pulse song with an ipi of 34 ms (34, N = 55) compared to one of 48 ms (48) on female receptivity, (time to first copulation). All sounds are applied as stimulators.



copulation is significantly reduced in comparison to the control, ($p < 0.001$, $N = 20$) (Fig. 2.10b).

In Table 2.4 the results from the experiment with reversed sex roles are displayed. The differences between the control and experimental tests are highly significant ($p < 0.001$, $N = 18$).

2.6.3.2.2 Stimulation

Fig. 2.11a gives the results of stimulation experiments with 'deaf' and 'mute' males. It is clear that pulse song reduces the time to the first copulation considerably, ($p < 0.01$, $N = 18$).

In Fig. 2.11b the effects of pulse songs with different ipi's are investigated. An ipi of 34 ms which is characteristic of D. melanogaster significantly reduces the time to the first copulation in comparison to an ipi of 48 ms, which is that typical for D. simulans, a sympatric sibling species of D. melanogaster, ($p < 0.05$, $N = 55$).

2.6.4 Discussion

The results of the experiments on female locomotor activity suggest that the male's song does indeed slow down the female, as has been proposed by Bennet-Clark and Ewing (1967), although they attributed it to the tonic component of the song which was not involved in the present experiments. Cook's (1973) finding that lowered activity of the female is correlated with the successful

outcome of courtship, is also corroborated by these results.

The receptivity investigations indicate that sine song, which increases receptivity if the females are subjected to it before being mixed with males, acts like a pump rather than a trigger in the sense of Adler (1974). It could be summated by the female over a certain period of time thereby steadily increasing her receptivity. The same does not, however, hold for pulse song, which increases female receptivity only if the females are hearing it in the presence of males which court them silently. Therefore one can conclude that pulse song acts more like a trigger. This would be expected of a species identification stimulus, because such a signal should be so unambiguous that it takes no time to get the message. Furthermore it would be maladaptive if such a stimulus were summated or only memorized over a short period of time, because this would mean that a heterospecific male could inseminate a female after she has received a certain amount of stimulation from a conspecific male. The fact that 34 ms is a better stimulus than 48 ms even if one controls for the male effect, lends further support to the hypothesis which ascribes a species identification function to pulse song. Further evidence for the trigger like function of pulse song comes from the observation that out of 76 courtships, 75 ended in a bout of pulse song shortly before copulation. These results confirm the earlier stimulation experiments, where the males had not been isolated from the auditory stimulus (Bennet-Clark and Ewing 1967, 1969) but they are at variance with the experiment which ascribed a prestimulation function to pulse song, (Bennet-Clark et al. 1973). It must be assumed that the males are responsible for this discrepancy. This explanation finds special support in the

finding that the prestimulation of males does decrease the time to the first copulation significantly. It is however still possible that the slight but perhaps important difference in method are the reason for the different outcomes of the prestimulation experiments. It seems that in the early report the introduction of the males to the females might have been less disturbing for the females than in my experiments. Also, the time required for the introduction of the males was probably shorter in the former investigations.

2.7 General discussion

The results which have been communicated in the present chapter can be summarized in the following way: (1) Pulse song has a dramatic effect on males. This effect is rather unspecific with regard to ipi. (2) Sine song does not trigger any response in males. (3) Females are definitely able to memorize sine song, whereas under the same conditions they are unable to remember pulse song. (4) Nevertheless pulse song does result in an increased receptivity when played back to the females while they are being courted by wingless males. This female response to pulse song is ipi specific.

These results warrant the tentative conclusion that pulse song is a species identifier and sine song a stimulus which subserves the purposes of intraspecific sexual selection. What is the degree of 'uncertainty' of this conclusion?.

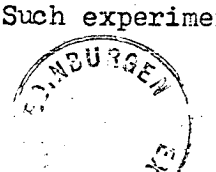
If we speak of the function of a given structure, it is implied

that the selective forces shaping this structure have had their origin in the need to fulfill exactly this function that we are concerned with. We do not mean a side effect (Otte 1974) which can be associated with any structure and which can very easily be confused with a function in the proper sense.

For example, say ipi is a signal which has evolved entirely for the purposes of intraspecific sexual selection. These purposes, for unknown reasons, have imposed such restrictions on the evolution of the signal that it emerged with the properties that we observe today. If this were so, then ipi would automatically appear to act as a species identifier if we would test this hypothesis with the kind of experiments described in this chapter. To complicate matters further, the reverse also holds. If the true function of ipi is species isolation, it would also fulfill an intraspecific sexual selection task, or rather we would find in our experiments that it has this effect. In other words, the observation that females discriminate against deviant ipi's tells us very little more than that it is involved in sexual selection (inter- and intra specific) in general. To separate the two hypotheses it would be necessary to design experiments mimicking evolutionary processes. A large outbred population, living under conditions where no mechanisms of sexual isolation are required, should, given enough time, show a deterioration of the traits which are involved with the prevention of interspecific hybridization. These characteristics should drift away from the values where selection has kept them, or the coefficients of variation should increase. These are long term experiments, but at least with Drosophila they are not prohibitively long term.

What about the other pieces of evidence which would implicate

ipi as a species identifier? Its species specificity, for example, clearly constitutes a necessary prerequisite for it to be discussed as a potential isolating mechanism. However this is no more than a necessary prerequisite, it certainly is not a sufficient one. Different ipi's of D. melanogaster and D. simulans could just as well be a by-product of divergent evolution. Indeed in the D. paulistorum group of incipient species, Ewing (1970) has found no signs that ipi is involved as a causal factor in the early stages of evolutionary divergence. Other, probably pheromonal mechanisms appear to do the task. The observation that ipi works in a trigger like fashion, of course is also only very weak evidence of its isolating function, and this mainly only in conjunction with the ideas about sine song and the assumption that one structure is enough to deal with one function. This assumption could well be wrong and pulse song and sine song might be doing the same job. Indeed it is possible that sexual behaviour is organized in such a way, that it always serves both aims, the intra- and interspecific ones. This hypothesis would also be testable with the kind of long term experiments outlined above. One would however have to be able to make predictions as to the speed and degree of deterioration of a given trait which has suddenly been rendered non functional. Such a prediction seems impossible at the present state of our knowledge. The other main type of experiment which can be designed to test the species isolation function of ipi is that in which the actual degree of reproductive isolation is measured under conditions in which the stimulus under investigation cannot contribute to the degree of isolation, because the relevant input and/or output organs have been extirpated. If such a manipulation has no effect on the degree of isolation, then the stimulus in question is not involved in ethological isolation. Such experiments have been performed by



other authors and they will be discussed in the next chapter. Suffice it to say here, that the same limitations apply to them as to playback experiments and that they have additional drawbacks stemming from the difficulty of properly controlling for certain sensory systems in insects and the side effects which can be the result of the amputation of sensory or output structures.

Such measures as repeatability and coefficient of variation could be good indicators of the function or rather nonfunction of certain characters, if a large body of data from diverse organisms and behaviours were available to compare them with. Unfortunately these data are missing and comparisons are rather unfruitful. Finally a more general evolutionary consideration in relation to isolating mechanisms. It seems that most authors take it for granted, at least implicitly, that there is always a very high premium on the female (in the case where this sex has the higher parental investment, which it usually has, (Trivers 1972)), to distinguish conspecific from heterospecific males and that there is no need for males to differentiate. Although it is true that the sex with the higher parental investment will be under higher pressure to be critical when selecting a mate, it is not true that the other sex is under no pressure at all. Indeed after two incipient species have lived in sympatry for a long enough time one would expect the males to be most active in sexual isolation. The males should evolve means to recognize heterospecific females at a distance so that they do not waste time and energy courting these females, (Schilcher and Dow 1976). It seems indeed that in Drosophila interspecific courtships the males are responsible for the maintenance of sexual isolation, because most such courtships, once initiated, are interrupted by the male, (Spieth 1974, Schilcher

and Dow 1976). Therefore there is an a priori argument against ipi as an isolating mechanism. However, it would be difficult to conclude that for these reasons alone it could not serve an interspecific purpose, because one would have to make too many unfounded speculations about the history of the species involved.

As concerns the function of sine song, it must be noted that a priming or summating action of sine song has not strictly been demonstrated. It has only been shown that it is memorized over at least a period of 10 - 20 s. Its summation function could only be proved (as far as positive proof goes) by showing that the degree of female receptivity rises with the amount of pre-stimulation. However, as such a summation mechanism is rather wide spread in the sexual behaviour of several species (Adler 1974) and as sine song is memorized it seems that summation remains a reasonable hypothesis which awaits to be subjected to the crucial test. Returning to the second part of the present chapter, namely the male reaction to pulse song, it should be added perhaps, that this response can be thought of as having evolved in two ways. Firstly it could be that it is entirely a male issue. Males could just have evolved the means to make use of this information which is being provided accidentally by other courting males. In this case one might expect the expression of pulse song to become eliminated, as males find other means of stimulating the females, ways which do not have this disadvantageous side effect. The other possibility is that the females insist on the males to sing precisely for the reason that this stimulates other males. What is a side effect under the first hypothesis has now become the function. The females might assess the male's fitness by taking a measure of the handicap the male can impose on himself, (Zahavi 1975). The handicap obviously would be the calling in of mating competitors,

rather like in red deer. In this case one would expect the males to sing as loud and often as can be tolerated by them. The handicap theory has, however, been severely criticized, (Smith 1975). Unfortunately all evolutionary speculations of the kind we have just indulged in, are interlarded with if's and but's to such a degree that one should interrupt them as soon as they lose touch with empirical knowledge altogether and become untestable. I cannot think of any experiments which might be designed such that they could differentiate with any degree of certainty between the two hypothesis outlined above.

Chapter 3

Sexual isolation and the behaviour of Hybrids between D. melanogaster and D. simulans

3.1 Introduction

This chapter attempts to elucidate questions of function and genetic organization of the song, through the study of two sibling species and their hybrids.

An enormous amount of mainly genetically oriented work has been done on species isolation. Some of this work allows one to make inferences as to the function of the different male displays and female sensory organs alleged to be involved in species isolation. Therefore this introduction will start with a discussion of the isolation work which is relevant to our problem. The emphasis will be on D. melanogaster and D. simulans, other species will only be mentioned where they provide additional information. Later on the major objectives, which have been pursued with this work on hybrids, shall be discussed.

Since the recognition and description of D. simulans as a separate species (Sturtevant 1919), a number of experiments have been described, which produced and studied hybrids between these two sibling species. The first such study was made by Sturtevant (1920), who found that the cross D. melanogaster x D. simulans results in hybrid females only. The converse cross gives hybrid males and a few females. All hybrids were found to be completely sterile. Sturtevant (1929a) was also the first to compare the success rates of the two reciprocal crosses. Hybrids from the cross D. melanogaster x D. simulans are much easier to obtain than those from the reciprocal cross. This has been confirmed by many workers since (Morgan 1929, Biddle 1931, Uphoff 1948, Manning 1959,

Parsons 1972, Eoff 1973). Barker (1962) has reported an apparent exception to this rule, but it could be ascribed to differences in the methods employed for the measurement of the degree of sexual isolation (Barker 1967). These laboratory findings were confirmed by Sperlich's (1962) field collection where, in an area where both species are sympatric, he found 5% of the females produced only sterile female offspring, making it likely that they were D. melanogaster females which had been inseminated by D. simulans males. No females producing only sterile males were found. Sturtevant (1929) states that the "males of each species court females of either species indiscriminately, but neither type of female will accept a foreign male as readily as she will a male of her own species". Due to the different success rates of the two reciprocal crosses between the two species, one must conclude that Surtevant implies that D. melanogaster females are less discriminatory than D. simulans females. Manning (1959) in a much more detailed study of the ethological mechanisms involved in the isolation between the two species, has found that although Sturtevant's conclusion is right, the premise from which he derives it, needs revision. D. simulans males are more discriminatory than D. melanogaster males in that a much lower percentage of D. simulans males show full courtship to the heterospecific females. Despite this fact, the D. melanogaster x D. simulans cross is more successful. In the same study Manning (1959) found that the male fore tarsi play a significant role in the male discrimination against heterospecific females. Removal of the antennae had no detrimental effect on the ability of males to differentiate between females. This clearly indicates that contact chemical stimuli play a major role in species isolation. This had been found before with a strain of

D. virilis, (Spieth 1952). Removal of the female antennae had no effect on species isolation in Manning's (1959) study, which led the author to suggest that the females use no airborne chemical stimuli for species isolation. We may conclude furthermore that this makes it also unlikely that they use auditory stimuli for this purpose, because the major receptor for these (Johnston's organ) is also part of the antennae (Petit 1958). This conclusion must be regarded with caution, however, because it is not clear to what degree antennaless females are really rendered 'deaf' by this operation. As has been shown in section 2.5.2.4 aristaless males still respond to the song at high intensities. It is not known whether this response is based on alternative mechanoreceptors or on residual stimulation which Johnston's organ could still receive from movements of the funinculus which might be reacting to the particle displacements caused by the sound. If it is the former, then females presumably also have these receptors and might still get enough information to identify the species of the male courting her.

Results different from the ones just discussed were obtained by Mayr (1950) working with D. pseudoobscura and D. persimilis. The removal of the antennae reduced sexual isolation. Therefore in these species airborne chemical and mechanical factors cannot be excluded as possible candidates which contribute to species isolation. With the same species, however, it had also been demonstrated that removal of the male's wings had no effect on reproductive isolation and this was interpreted to mean auditory stimuli do not contain any species specific information for the female, (Mayr and Dobzhansky 1945). As has been known for a long time (McEwen 1918) and as we have seen in the last chapter,

wingless males became very inactive due to the wing amputation and it could well be that this was a confounding variable in the experiment of Mayr and Dobzhansky. Indeed it seems possible that wing amputation raises the male part of the isolation which could even result in an increased overall isolation, which has in fact been found. Therefore we must conclude that auditory information provided by the males, is unlikely to be an important component of the mechanisms of reproductive isolation between D. melanogaster and D. simulans, but it remains a viable hypothesis for the reproductive barrier between D. pseudoobscura and D. persimilis.

The degree of sexual isolation between D. melanogaster and D. simulans is amenable to environmental modification. For example rearing males of one species with females of the other increases heterospecific copulations when these males are tested in a male choice experiment, where they are confronted with one hetero- and one conspecific female, (Le Moli and Mainardi 1972). Culturing flies together has a similar effect, (Eoff 1973). These experiments can be taken as further evidence of the important role which the males play in the prevention of interspecific copulations, (Schilcher and Dow 1976).

One objective of the work described in the present chapter was to try to test an idea on the genetic organization of communication systems between sexes. Alexander (1962) has proposed that the evolution of such systems is most easily understood if the reception and emission of the specific informational content which is to be transmitted, through whatever medium, is based on an identical coding template in the two sexes, and that the characteristics of this template are determined by the same genes in both sexes.

This is a very elegant hypothesis which would explain any instances of rapid evolution of parameters involved in communication. Thus, once the sender has changed his message through mutation, he does not have to wait for a complementary mutation to take place in the receiver. The latter would automatically understand the new message, once he has also received the mutated gene. This hypothesis has been restated by Ewing (1969), but nevertheless the evidence in its support is rather sparse. One report on hybrid crickets (Hoy and Paul 1973) goes some way to support it, but results from hybrid grasshoppers (Perdeck 1957, Helversen and Helversen 1976) seem to falsify it. If one would find that hybrid females mate faster with hybrid males than males from either of their parent species, one would have furnished circumstantial evidence in favour of the hypothesis. It is clear, however, that such a result could also be explained on a number of other hypotheses with less specific characteristics. Such a result could, for example, also be brought about by a change in one sex only (hybrid males are more attracted to hybrid females, or the latter are not attractive to males from the two parent species).

The main objective of the hybrid experiments described in this chapter was to get some idea about the distribution of song genes in relation to the X-chromosome. For mutagenesis studies it is most convenient to use a technique which screens only the X-chromosome for recessive mutations. Such mutations on other chromosomes will be overlooked with this technique, which will be described in more detail in the next chapter, and mutations have to be dominant to be discovered. For these reasons it was important to try and get some information on the location of song determining genes on the D. melanogaster chromosomes. Crosses between species

which differ in their song, as D. melanogaster and D. simulans do for ipi, could provide evidence on this point. If hybrid males, which receive their X-chromosome from their D. simulans mother, sing like the maternal species, two mechanisms of inheritance could be responsible for this result: (1) the genes (or gene) responsible for ipi are located on the X-chromosome or, (2) the genes (gene) are on one or more of the autosomes, but the D. simulans alleles are dominant over the homologous ones derived from D. melanogaster. Unfortunately with the two species employed in the present study we have no way of distinguishing between these two alternative hypothesis, because the hybrids are sterile and yield males from only one cross between the parental species. However the X-linkage hypothesis could be falsified, namely by intermediate inheritance in the hybrid males and therefore it was considered worthwhile to undertake the study.

Ewing (1969), working with D. pseudoobscura and D. persimilis could locate the control of certain qualitative features of the song (persimilis type vs. pseudoobscura type) to the X-chromosome. His results with ipi, have been interpreted by him to indicate that ipi was controlled by dispersed genes. This interpretation is however uncertain because it is not quite clear what the variance indications in Ewing's Table II are. It would seem that the F 1 s are significantly different from each other if the measures of variability given in Table II are really standard deviations, (O.v. Helversen, personal communication). Futch (1973) found that the mating success of hybrids between D. pallidosa and D. ananassae depends on the origin of the males' X-chromosome. Hybrid males were more successful with females from their mother's species, whose X-chromosome they received.

It seems therefore that there is some good evidence for X-linked inheritance of song or other courtship characteristics. In the following we shall see what contribution hybrids between D. melanogaster and D. simulans can make to the clarification of the questions discussed above. Experiments will be described which investigate mating speed and song parameters of D. melanogaster, D. simulans and their hybrids.

3.2 Methods

The data reported in this chapter have been collected in collaboration with Professor A. Manning. Flies used in these experiments were from the att-X and att-X^B stocks, which have been described in the last chapter. Furthermore a stock of wild type, outbred D. simulans was used. This stock was obtained from M. Dow who had collected it in Rome in 1972 as a large population. Crossing att-X females to D. simulans males results in hybrid males which carry the X-chromosome of their father (see section 4.2.1.1) whereas crossing females of the att-X^B stock to D.simulans males results in hybrid females. By using this alternative strategy to the reciprocal crosses between the two species the much more difficult cross between D. simulans ♀ and D. melanogaster ♂ was avoided. At the same time the simulans X-chromosome in the hybrid males was combined with D. melanogaster cytoplasm. Therefore this cross avoids complications which might arise in the interpretation of the results due to maternal effects, which could simulate sex-linkage.

The interspecific crosses were set up with 5 1-day old females and 10 2-day old males in vials. After one day the contents of 4-5 such vials were transferred to a stock bottle for egg laying and development. Since the cultures of hybrids produced only one sex they were allowed to hatch for 24 hours before being sexed. Mating speed tests were performed at 25 ± 1°C with 25 pairs of flies in 250 ml flasks. These were held horizontally in a clamp and indirectly illuminated by a 60 W lamp at their base, which was slightly pointed upwards, so that the flies tended to aggregate at this end and could be sucked out with an aspirator. The time for each copulation was recorded and all tests were concluded after 30 min.

The percentage of flies which copulated within the observation period was calculated for each test, subjected to an arcsine transformation and the different crosses (at least four replicates each) were compared with t-tests. The D. melanogaster females used in these experiments came from the att-X^B stock. The song records were produced by the methods described in the last chapter, with a cut off point for ipi at 70 ms for D. simulans and hybrids and 50 ms for D. melanogaster. The D. melanogaster data are the same as those reported in section 2.4.1.2.

The flight records were obtained by glueing the flies with their scutum to entomological pins under light etherization and suspending them over the ribbon microphone. Usually they start flying within 1-3 min. All song and flight tone tests were carried out with the temperature controlled at 25 ± 0.5°C because of the high temperature dependance of both courtship song and flight wing beat frequency, (Shorey 1962, Reed et. al. 1942).

Table 3.1 Mating speeds (% mated out of 25 in 30 min) from the crosses between the genotypes indicated

		males		
		simulans	melanogaster	hybrid
♀	simulans	83.4 (N = 5)	-	62.4 (N = 4)
	melanogaster	-	83.5 (N = 5)	5.6 (N = 4)
	hybrid	10.5 (N = 9)	64.0 (N = 10)	27.1 (N = 4)

Table 3.2 Results from t-tests between the different crosses displayed in Table 3.1

1. cross		2.cross		value of t	p
♀	♂	♀	♂		
sim	hyb	hyb	hyb	2.426	ns
mel	hyb	hyb	hyb	5.168	< 0.01
hyb	sim	hyb	hyb	3.582	< 0.01
hyb	mel	hyb	hyb	4.916	< 0.001
mel	mel	hyb	mel	3.012	< 0.01
sim	sim	sim	hyb	1.730	ns

3.3 Results

3.3.1 Mating success

Mating speed tests were made using all combinations between the three genotypes, except for the interspecific crosses themselves, which are so slow in mating as to preclude measurements. The results are presented in Tables 3.1 and 3.2.

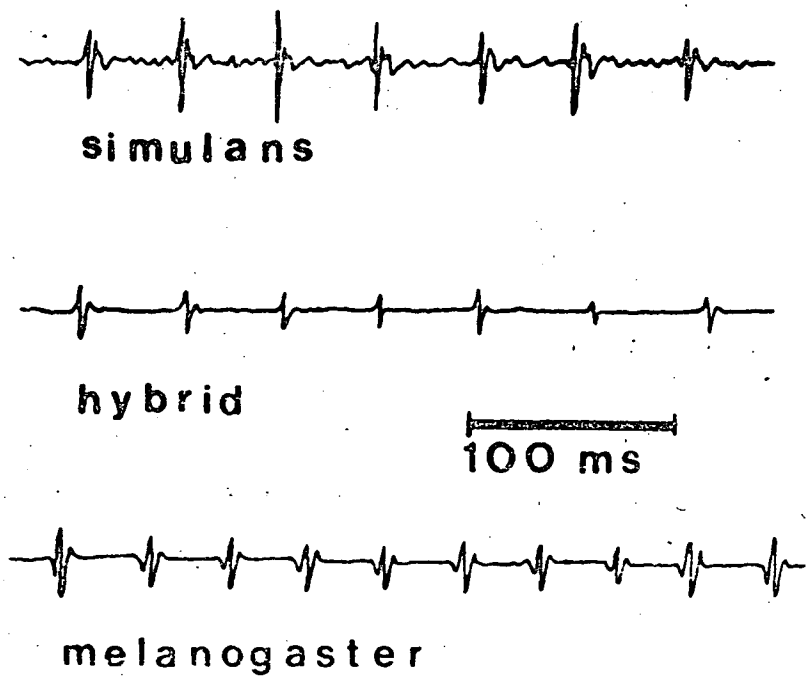
It can be seen that the two parent species have very similar mating success. Hybrid males are equally acceptable to and/or stimulated by *D. simulans* females than hybrid females. With *D. melanogaster* females they have very low mating success indeed. All together in the four replicates only 6 copulations occurred. It has been observed during these tests that the males were extremely sluggish courters in this combination, so that one cannot ascribe this result to female coyness alone.

Hybrid females copulate reluctantly with *D. simulans* males, are intermediate with hybrids, and accept *D. melanogaster* males most frequently. The difference between hybrid and *D. melanogaster* males accepted being significant at the 1% level. Hybrid females appear less acceptable to *D. melanogaster* males, as their own females. As has been identified above, *D. simulans* males mate very slowly with hybrid females. In this case qualititative observations suggest again that the males were at least partly responsible for the result because they were extremely inactive in this combination. There is no significant difference between *D. simulans* and hybrid males with *D. simulans* females.

Table 3.3 ipi and wing beat frequency of males of *D. melanogaster*, *D. simulans* and their hybrid

	ipi ± SE	wingbeat frequency (Hz) ± SE
D. melanogaster	34.6 ± 0.4	218.3 ± 2.4
D. simulans	48.7 ± 1.2	252.7 ± 2.9
hybrid	47.7 ± 1.2	240.6 ± 2.6

Figure 3.1 Oscilloscope records of the songs of males of *D. simulans*, *D. melanogaster*, and their hybrid. Redrawn from a photograph.



3.3.2 Courtship song and wing-beat frequency

The ipis of D. simulans and hybrid males are almost identical (Table 3.3, Figure 3.1). The ipi of D. melanogaster is significantly different from both of them ($p<0.001$). Wingbeat frequencies of hybrid and D. simulans males are significantly different from each other ($p<0.01$) and the hybrid is indistinguishable from the mid-parent value for this trait.

3.4 Discussion

At first some general points which emerge from the mating speed tests shall be discussed. It is quite remarkable that D. melanogaster and D. simulans which have very different courtships - D. simulans males being very sluggish courters (Manning 1959) - end up having the same mating speed. This means that D. simulans females are more receptive than the females of their sibling species. In the interspecific matings however, where, as we have already noted previously, D. simulans males are again the less energetic courters, the cross that involves them is the more successful one. Can this be taken as evidence that D. simulans females which are less critical in the intraspecific context, are more discriminatory in the interspecific context? This is exactly what one would expect. Due to the different male courtship intensities, D. simulans females would be expected to be subjected to the heterospecific courtship more often than D. melanogaster females.

The results furthermore allow us to make some remarks about the intricacies of communication systems which function for sexual

selection. As we have seen, particular males/females can be very fast copulators with one female/male, but very slow ones with another. Many studies have been trying to determine which sex is the more important in the determination of mating speed (Kessler 1968, Elens et. al. 1973, Kaul and Parsons 1965). It seems that this question should not be approached through the study of strains or species which have not had the chance of coadapting their communication systems, because in such a case the result seems rather arbitrary. It will depend on effects which have no functional significance at all, because the functions make sense only in an inter individual context. From such an investigation almost every result can emerge and this is indeed what has been found (e.g. Kessler 1968, Elens et. al. 1973). The decision on this question, which is of great theoretical importance (Trivers 1972) must be based on a study of long established, outbred populations or sympatric species, depending on whether one is interested in intra- or interspecific aspects of the problem.

The observation that hybrid males are most successful with D. simulans females could well imply that ipi is a major variable in species isolation. Hybrid males have the same ipi as D. simulans males are equally successful with D. simulans females. But this result could of course also mean that hybrid males have inherited dominant or sex linked genes from their D. simulans fathers which code for the sensory and/or neuronal wiring responsible for the reception and recognition of stimuli functioning as releasers of sexual behaviour. In other words, hybrid males could be more stimulated by D. simulans females. Unfortunately the present investigation was not designed to test this relationship between male arousal caused by females and its converse. That

females do influence male arousal is strongly supported by the well known role of pheromones in Drosophila courtship. Although it is not quite clear whether pheromones do actually stimulate males sexually (Shorey and Bartell 1970, Sloane and Spiess 1971) it is certain that they play a major role in intraspecific sexual selection (Ehrman 1969).

With reference to the hypothesis put forward by Alexander (1962) that the structures responsible for song emission and reception could be determined by the same genes, it can be concluded that the present investigation does not lend any support to it at least as far as ipi is concerned. As we have seen, hybrid females have the fastest mating speed with D. melanogaster males. Hybrid males copulate fastest with D. simulans females. It seems, however, that Drosophila is a rather unsuitable organism to test this hypothesis because there are a number of different sensory channels (chemical, visual, auditory) involved in its courtship. Possibly all communication systems, on all channels, would have to obey the rules set out in the hypothesis, for the realization of the expected outcome. Those Orthopteran courtships, which, in the early stages at least, involve only the transmission of auditory information, are better suited to test the hypothesis. But, as we have said earlier, the results from crickets and grasshoppers are ambiguous. The fact that D. simulans and hybrid males have nearly identical ipis could be explained by two different modes of inheritance, as has already been pointed out in the introduction to this chapter. The alternatives were sex linkage or autosomal dominance. Our results have certainly not falsified the sex linkage hypothesis and this can be taken as information which makes the mutagenesis study, to be described in

the next chapter, more promising than it would otherwise be. It should however be noted that dominance seems to be the rule rather than the exception for the morphology of the male genitals, (Tsacas et al. 1971).

The interesting difference in inheritance between ipi and wing beat frequency proves that the similarity between hybrid and D. simulans ipi is not simply due to a similarity in their thoracic structures. Clearly ipi has had an evolutionary past distinct from that of wing beat. This is not surprising, if one adheres to the hypothesis that ipi acts or acted as a species recognition signal and was subjected to the probably very specific and strong selection pressures characteristic of such traits.

CHAPTER 4

Genetic alteration of courtship song by means of chemical mutagenesis

4.1 Introduction

In the general introduction to this thesis, the opinion has been enunciated that among all the different behaviour genetical techniques, one stands out in the potential it holds for the future. This is the paradigm for reductionist neurobiology as it has been outlined by Benzer (1967). It constitutes an ambitious attempt to analyse the structure and function of the nervous system on all levels of organization, through the study of lesions, set by genetical mutations in the nervous system. These mutations are generated through chemical mutagenesis and isolated by mass screening procedures which allow the experimenter to spot abnormally behaving individuals with minimum expenditure of time and energy. The faster one can generate large numbers of specific mutations, the sooner one can start with the interesting part of the work, namely their structural and functional characterization, which can be expected to permit inferences about the action of the normal structures and functions. It is clear that the screening procedure is a rather critical step in the whole process, therefore it will be discussed in more detail now.

If one wants to study, say the phototransduction mechanism, one first has to generate mutants defective in their visual sense. One cannot immediately screen on the level of the phototransduction process itself because this would be unacceptably labour intensive. The first technique which has been used to isolate such or other visual mutants (Benzer 1967) can serve as an example of the difficulties involved in such an enterprise. To screen for non-

phototactic flies, Benzer started a number of individuals at the bottom of a long and thin double-tube, which was illuminated from above. After a given time interval the flies had distributed themselves throughout the tube and the lower and upper halves of the tube were separated and each joined with a new, empty half. The flies were shaken to the lower end and the process repeated. After several such steps, flies which never moved towards the light were still in the original lower half of the starting tube. It is clear, however, that besides visual mutants there will also be a number of other flies in this vial. For example, those with low reactivity to conspecifics under high density, with defects in their locomotor system, those which are strongly positively geotactic etc. In fact in Benzer's case 2 nonphototactic mutants have been isolated, none of which has, to my knowledge, shown up in the later vision mutant publications, by the same (Hotta and Benzer 1969) or other groups. Other workers have, however, been more successful with the same technique, (Heisenberg 1971a, Pak et al. 1969). A modification of the system was designed by Götz (1970). It dispenses with the rather rough treatment the flies receive at the start of each new round and might be less vulnerable to picking up reactivity mutants. It is, however, more time consuming.

The kind of complication just outlined almost invariably arises in mutant screening tests. Only the simplest organisms and behaviours are relatively immune to such draw backs and in most cases the ingenuity of the researcher will be stressed to its maximum if he is to develop efficient screening procedures for individuals defective in more complex behaviours. In most cases several screens will have to be employed.

For reasons which have already been pointed out, a mutagenetic

analysis of courtship song could yield very valuable results. Some of these reasons have not been made very explicit and warrant closer examination especially in view of the results which we have discussed in the last two chapters.

In both chapters we have been rather pessimistic about the chances of finding the precise functions of pulse- and sine song. Mutants affecting one or both of these behaviours, in a qualitative or a quantitative way, could certainly be used to probe deeper into these teleonomic problems. For the reasons which have been described in connection with the playback experiments, mutants can also not be expected to yield final answers on the function of ipi, but they might still help clarify several issues. For example if sine song mutants could be generated, say with a different frequency, it could be discovered in mating speed tests whether sine song frequency contains any information for the female. If mutants without any sine song or pulse song could be obtained one clearly would have a tool to add some information to the playback experiments described in section 2.6. All these potential uses are of course subject to the restrictions which have been discussed earlier, namely those arising through possible pleiotropic actions of genes. But as we have seen this is not an obligatory obstacle and can furthermore be controlled for with song mutants. All it takes to discover pleiotropic action on mating speed is to compare wingless mutant males to wingless control flies. In this case song cannot be made responsible for any differences which are found, so that they have to be a result of some pleiotropic effect.

A point which has not been raised in the general introduction is the fact that song mutants could also be used to test the hypothesis on the genetic determination of song emission and

reception, (Alexander 1962). Analogously to the case with the hybrids discussed in the last chapter, male song mutants should be preferentially accepted by females who also carry this mutation, if the female neuronal ipi recognizing structures are coded for by the same genes as the male output structures which control ipi.

If a song mutant turns out to carry a high pleiotropic load, then this could be studied in detail and the results might become useful for the understanding of courtship behaviour in a more general sense. Depending on what the pleiotropic effects are, one can check the function of the structures which have been changed through the mutation, even if they do not concern courtship song.

Besides these mainly evolutionary problems, there are all the other questions of a more reductionist nature which have been listed in the general introduction, for the solution of which song mutants might be profitably employed. The most important such problem is perhaps the identification of the neuronal driver of the song and the subsequent study of its mechanism. It is obvious, however, that this will be an extremely difficult undertaking. However as we have seen in the elegant work on shaker mutants by Ikeda and Kaplan (1970 a, b) such an enterprise need not be impossible, depending on what this structure is and where it is located. Ideally the course of a mutagenetic behavioural analysis in D. melanogaster would consist of the following stages:

isolation of mutants - genetic mapping - epigenetic mapping - high resolution anatomical characterization of the area circumscribed by the fate mapping results - if no structural defects are found, physiological characterization of the same area - precise description of the behaviour of the mutant - repetition of the last

three steps with two or more mutations acting in the same individual - anatomical, physiological and behavioural comparison of several mutants acting separately and together.

As we have seen, this rather strenuous path has so far only been gone more or less to its end with the shaker and visual mutants. This is not surprising, because after fate mapping has been concluded then dead lock can easily follow. Anatomical or physiological micro-manipulations are both extremely difficult with Drosophila. The former is far less difficult with an organism like Caenorhabditis elegans where serial sections of the nervous system which consists of only 250 neurons (Brenner 1973) can be obtained and analysed electron microscopically, (Ward et al. 1975). Drosophila with its approximately 10^5 neurons (Benzer 1971) would not easily be accessible to such an approach, in particular because of the formidable structural complexity of insect ganglia (Hoyle 1970). Certainly for reasons of size, physiological, in particular electrophysiological, work is very much easier with crickets (Bentley 1973) than with Drosophila. However it must be added that Drosophila is amenable to both techniques, even if it is not ideally suited for any of them, and that it has furthermore the advantages which have made it the geneticist's favourite organism, and those advantages which arise from this fact.

We may conclude that it seems desirable and not impossible to produce song mutants in D. melanogaster and exploit them successfully.

The present chapter will describe the isolation of a song mutant and its genetic, epigenetic and behavioural characteristics.

Table 4.1 Genetic characteristics of attached-X stocks

		male gametes	
		X	Y
female gametes	XX	XX X non viable	XX Y attached-X female homozygous for y, w, f.
	Y	X Y normal male	YY non viable

4.2 Isolation and genetic and epigenetic characterization of a song mutant.

4.2.1 Methods

4.2.1.1 Mutagenesis

The stocks were kept at 25 ± 2°C. One day old males from the att-X stock were treated with ethyl methane sulfonate (EMS) according to the method of Lewis and Bacher (1968). The method was modified occasionally by depriving the males of food and water for 12 hours before they were exposed to EMS in a sugar solution. This ensures that all males will take up large quantities of the sugar solution and therefore also of EMS. After having fed for 24 hours on 10 ml of a 1% sucrose solution containing 0.024 ml of EMS they were mated to females of the same stock. These females carry two fused X-chromosomes, and are homozygous for the markers y, w, and f. The genetic characteristics of att-X stocks are demonstrated in Table 4.1. The fused X-chromosome will always produce females and the Y chromosome carried by att-X females, together with the X-chromosome of a male will result in males. In other words, the inheritance of sex-linked genes is patroclinal, sons always receive their X-chromosome from their father rather than from their mother, as is normally the case. For this reason it is possible to recover sex-linked, recessive mutations in the first generation after treatment. This system is generally employed in behavioural mutagenesis, because it takes only one generation rather than two, to isolate sex-linked mutants. As we have seen, there is some circumstantial evidence that song genes are sex linked and this, together with the fact that the X-chromosome contains about 20% of all genes of D. melanogaster, makes it reasonable to screen for sex linked recessive mutations only.

Dominant mutations, which are generally very rare, can of course be discovered on each chromosome.

The effectiveness of the EMS treatment can be verified by determining the sex ratios in the normal stock and in the offspring of treated males. Recessive and dominant lethal mutations on the X-chromosome will only be transferred to males and therefore the first generation after treatment should contain fewer males in relation to the number of females present. Under the conditions employed by the author to isolate song mutants the male/female ratio dropped from 1.49 in the att-X stock to 0.34 in the generation after treatment. We can conclude that about 77% of the X-chromosomes receive at least one lethal mutation.

Another advantage of the att-X system is the ease with which mutations, once recovered, can be maintained. Simply crossing the male in question with virgin att-X females results in a stock in which all males are identical - as far as the X-chromosome is concerned - to their original father who carried the mutation.

4.2.1.2 Screening for song mutants

Female readiness to mate is at least partly determined by the auditory stimulation which the courting male provides, (see Chapter 2). It was therefore decided to employ female coyness as a first sieve which would screen for any abnormally singing males.

Male offspring of EMS treated fathers and att-X mothers were subjected to single pair mating speed tests with wild type females. To reduce the variability of the latter, F1 females from a cross

between two inbred lines were used. Both, females and males, were transferred singly to vials at the time of sexing and after three days they were put in single pairs into one vial. The time to copulation was scored. Males which did not achieve copulation within 15 min were observed more closely for their courtship vigor. The other males were discarded. If the former courted intensively for more than 80% of the time (sampled over 2-5 min) they were considered putative mutants, separated from the females, and transferred to a fresh vial. This strategy was employed in order to discriminate against nonspecific mutants, which would of course have to be expected in rather large numbers among the unsuccessful courters. The following day the putative mutants thus collected were made to court virgin 1 day old females on top of a microphone. Several of their song characteristics were monitored through earphones and on the oscilloscope screen. If they showed no abnormalities during this second screening process, they were discarded, but abnormally sounding males were mated to virgin att-X females. These females have been shown to be more receptive than outbred wild type females (Schilcher, unpublished) and can be expected to accept song defective males, in particular when given enough time. This increased receptivity is not astonishing when one recalls that these females carry 'y' homozygously. This mutation is known to make females less critical in their choice of a mating partner, (Dow 1976). In the next generation 25 male offspring from each clone were paired with 25 F1 females in a mass mating test as described in the last chapter. If their mating speed (Number of pairs copulated in 30 min.) was significantly slower (X^2 test) than that of a simultaneously tested control (males from the original att-X stock) they were discarded.

If their mating speed was slower than that of the control males in two such tests, the song of 10 individual males of this mutation was recorded and filmed. From the film, measurements of all relevant song parameters were made, as has been described in Chapter 2.

4.2.1.3 Mapping

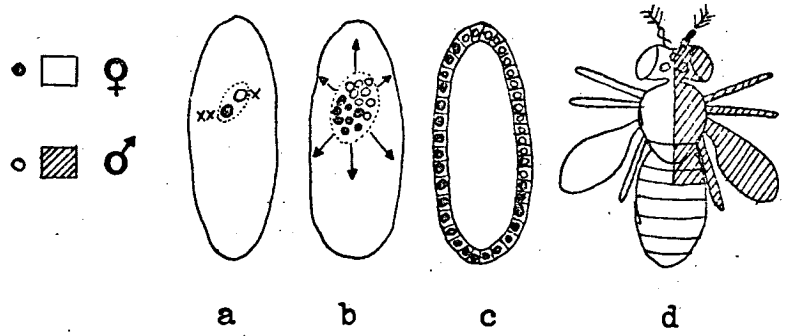
Males carrying the song mutation were mated to females homozygous for the markers sc, v, f and car (Lindsley and Gruell 1967). After preliminary tests had revealed two markers, one on each side of the song mutation, recombinants between these markers were tested for the presence or absence of abnormal song. The proportion of particular recombinants carrying the song mutation was calculated and from these values the map position of the behavioural mutations could be calculated in relation to the position of the two markers.

4.2.1.4 Fate mapping

It has been said in the general introduction that Drosophila offers the great advantage of fate mapping techniques, which can be used to uncover the primary site of action of a mutant gene, (Hotta and Benzer 1973). The embryonic development of D. melanogaster is highly deterministic in the sense that certain parts of the blastula more or less invariably produce certain parts of

Figure 4.1 Schematic drawing of the events leading up to the formation of a gynandromorph.

(a) In the first nuclear division one daughter cell receives 2 and the other one 1 X-chromosome. (b) The nuclei go on dividing and finally migrate to the surface of the egg, cell membranes are laid down and the blastoderm is formed (c). The distribution of XX (full circles) and X (open circles) cells faithfully reflects the plane of division (the orientation of the centromeres) of the first division. (d) The adult fly consists of male and female tissues the distribution of which reflects the orientation of first nuclear division. (modified from Hotta and Benzer, 1970).



the adult organism. Furthermore, the orientation of the spindle in the first nuclear division is arbitrary in relation to the main axes of the egg and the spatial coordinates of the egg are rather rigidly carried over to the blastula stage, such that with appropriate methods one can conclude from the blastula, and ultimately the adult organism, what the dividing plane in the first nuclear division was (Fig. 4.1). Methods permitting such a conclusion are genetic conditions which induce chromosome loss in an early nuclear division. If a female zygote which is heterozygous for certain recessive cuticle markers, undergoes its first division and one X-chromosome is lost during the process then this zygote will develop into a gynandromorph which consists of half female (X/X) and half male (X/O) cells. The male parts will be recognizable because they express the recessive cuticle markers. This technique permits the investigator to determine, in the case of a recessive, sex-linked behavioural mutation, which parts of the fly have to be mutant for it to express mutant behaviour. If a certain structure, say the eye, mostly has to be mutant for the gynandromorph to behave in the mutant manner, then it is most probable that the defect causing the deviant behaviour resides in the eye, because only very few if any blastular dividing lines (or spindle division planes) cut in between the eye and the site which causes mutant behaviour. To know, in the case of a behavioural mutant, which parts are mutant and which wild type, one has to align the behavioural mutation with suitable cuticle markers on the same X-chromosome. In this way the morphological markers label the parts which are hemizygous for the behavioural mutation. With this method, it becomes possible to erect fate maps which indicate the distance between different structures in terms of the surface

of the blastula. Structures which are far apart will frequently be discordant for the recessive markers and those close together will be mostly concordant. The logic of this technique is precisely analogous to that of conventional chromosome mapping, with the only difference that it is performed in two dimensions rather than one. This technique allows one to fate map internal structures, if one has some independent means of establishing their identity. Such means exist in the form of recessive biochemical mutations which, when hemizygous in certain parts of a mosaic, allow one to differentiate these parts histochemically from those parts where they are heterozygous. With suitable biochemical markers which are expressed in the whole insect one can determine the precise site of a given gene's action in gynandromorphs. The method is the same as has been described for cuticle markers, except that it permits the experimenter to look - so to say - directly inside the gynandromorph, for the distribution of male and female tissues or even cells. Suitable biochemical markers are being developed by a number of researchers, (Hall et al. 1975) and they can be expected to boost the efficiency of fate mapping considerably. Several fate maps have been erected with different techniques for the induction of chromosome loss and the results seem to agree very well with each other. This is a strong indication of the validity of the assumptions underlying the procedures of fate mapping, (Hall et al. 1975).

The method most frequently used, is that of ring chromosome elimination. Certain ring shaped X-chromosomes tend to be eliminated during early nuclear divisions. This, of course, results in gynandromorphs. With suitable recessive markers on the normal rod X-chromosome, say one or two cuticle markers in addition to the behavioural one which is under investigation, one can determine

the primary site of action of the mutant gene. Several behavioural mutations have already been fate mapped in this way, (Hotta and Benzer 1970, Ikeda and Kaplan 1970b, Suzuki et al. 1971, Hotta and Benzer 1973, Falk and Atidia 1975). Such maps can be preliminary in that they do not attempt to locate the site in quantitative terms but only in relation to major anatomical landmarks such as the head, thorax or abdomen. Others determine whether a given, for example visual mutation, has its defect in the eye or somewhere else, by looking whether gynandromorphs with a mutant eye always display mutant behaviour or not. If they do, the mutation is said to be autonomous; the primary site of gene action resides in the eye itself.

Fate mapping through ring chromosome loss is only possible with recessive genes. The mutant gene will be present in all parts of the gynandromorph - heterozygously in the female ones and hemizygously in the male ones. Therefore dominant mutations cannot be mapped in this way. For these another method must be employed which allows the elimination of the mutation carrying X-chromosome itself. Such a method exists in the form of a mutation on the third chromosome, claret non-disjunction ($\underline{ca^{nd}}$), which, induces the loss of maternal chromosomes in offspring of females homozygous for this mutation. Therefore one can obtain gynandromorphs which in their male parts do not contain the mutant gene because it has been introduced from the mother and has been lost in an early division. The female parts contain the mutation heterozygously and therefore express it. With this technique one has to cross a female homozygous for the behavioural mutation and $\underline{ca^{nd}}$ to a male hemizygous for certain sex-linked cuticle markers. The resulting female zygote will tend to lose the maternal X-chromosome and therefore will give rise to the required gynandromorph whose male parts are

recognizable, due to the recessive markers on the paternal X-chromosome.

In the present thesis both techniques have been employed and the details of both are described below. It is obvious that fate mapping can be done in a straightforward manner only with qualitative mutations, where the mutant phenotype can be recognized easily and unambiguously. Mutations which cause small quantitative changes of a given behaviour would be very difficult to fate map, because with the same expenditure of time and energy only very many fewer gynandromorphs could be scored. Taking into account the possibility of errors in the classification of the behavioural phenotype of such minor mutations, one would need many more gynandromorphs to reach the same accuracy as with a qualitative mutation. It follows, that we can foresee that considerably more work will be done in the future on mutations causing large behavioural deviations. These are in any case already favoured in the screening procedures. It must be hoped that this does not cause any serious bias in the type of answers which will emerge from this research, which could be the case if such mutations were not representative of behavioural genes in general.

4.2.1.4.1 Ring chromosome elimination

Females heterozygous for the ring shaped X-chromosome (In(1)w^{vc}, obtained from the stock centre at Cal. Tech.) were mated to males carrying the song mutation under investigation and the morphological markers y and w on their X-chromosome. These males were obtained by crossing y, w, f, females (obtained from the stock centre at

Bowling Green) to song mutant males. The F2 male offspring were screened for males which were yellow, white and sang abnormally. A few such males were mated to att-X females to establish the y, w, song mutation stock.

The offspring of the above cross were screened for mosaic individuals, which occurred at a frequency of 5-10% of all flies. With the markers employed in this investigation gynandromorphs were recognizable because parts of their cuticle were yellow and/or because parts of their eyes were white. The sexual mosaics which were found, were then subjected to a test of their song qualities on the microphone. Flies which sang with one wing only during the 5 min testing period (8.3%), were eliminated from the analysis. The song tests were made with two virgin att-X or $att\text{-}X^{B}$ females and the gynandromorph. This strategy was employed because sexual mosaics are rather poor courters and two females provide more stimulation than one. The mosaics were classified according to their song and the distribution of male and female cuticle areas was indicated on a preprinted schematic outline drawing of a Drosophila (Figure 4.1). It was carefully monitored which type of song was produced with which wing, right or left. For the type of analysis attempted here it is very important to establish the song performance of mosaics not carrying the song mutation. It could be that mosaics generally sing abnormally which would prevent analysis. Such mosaics were obtained through a cross of heterozygous $In(1)w^{vc}$ females to y, w, f, males. The resulting gynandromorphs did not sing at all unless some patches of their head cuticle were male. Gynandromorphs which were entirely male, save for their head which was completely female, never sang. It is therefore necessary and sufficient to have

some male head tissue for mosaics to vibrate their wings and produce song. This confirms the observations of other authors, (J. Hall, pers. comm.).

With one exception, all types of mosaics sang, if they sang at all, in a perfectly normal way. The exception were some of those (6 out of 9) which had an all female body but a partly or completely male head. Although these clearly vibrated, they produced a sound which could not be classified as pulse- or sine song.

No clear predictions can be made from the maleness of a particular cuticle site about the maleness of underlying tissues. It seems possible that the mosaics which sang normally did in fact have some crucial male thoracic structure, even though their whole thorax cuticle was female, which the ones which sang abnormally lacked. In other words, it could be that to vibrate the wings, male head structures are necessary and sufficient but to produce a proper song they are only necessary but not sufficient. This hypothesis postulates a thoracic structure which, if it is female gives rise to an abnormal song, and if it is male to a proper one.

This structure is probably an epigenetically complex one, which exhibits a domineering effect (Hotta and Benzer 1973), because the bilaterally symmetric gynandromorphs never sang abnormally with the wing on the female half of their thorax. This problem of thoracic structures involved in song production will be discussed again in section 4.2.3.

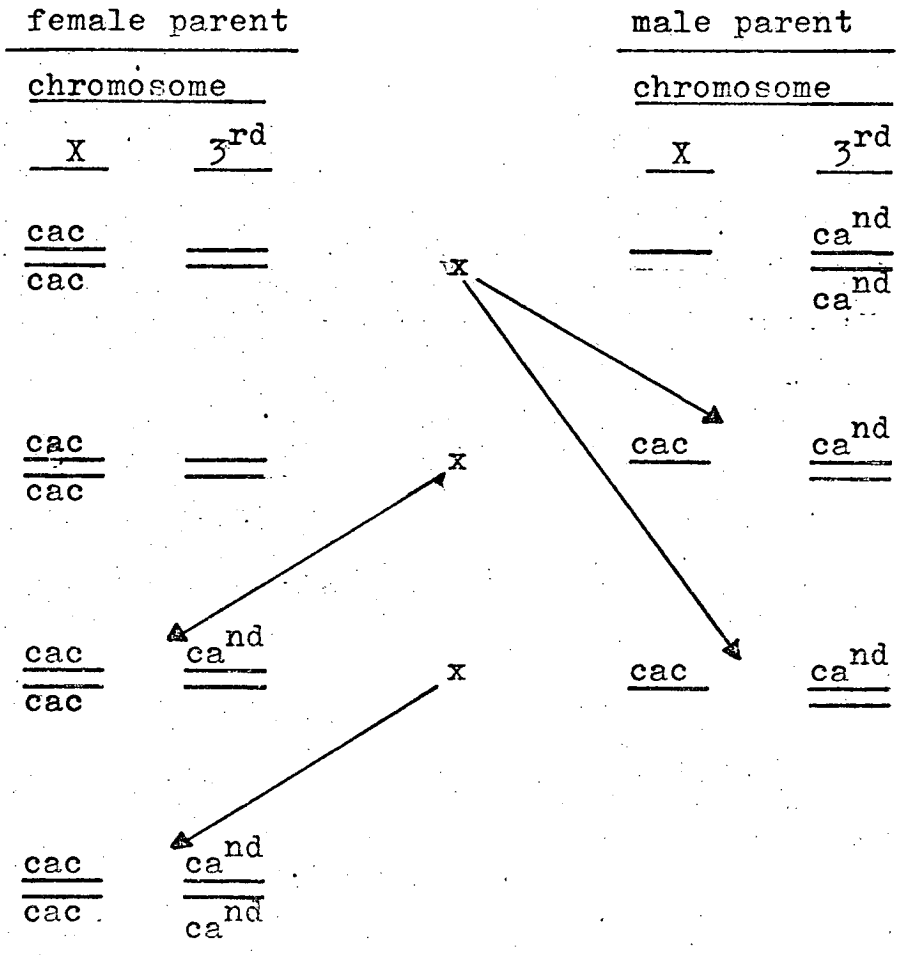
With these limitations in mind it is still possible to study the effects of the song mutations acting at various sites in the flies' body. The fact that only flies with at least some male tissue in the head are available for the collection of fate mapping

data, means that only preliminary fate maps can be erected for song behaviour, because not all classes of gynandromorphs can be scored. Gynandromorphs with a female head do not sing and no information about the site of action of the mutant gene can be gained from them. This limitation could be circumvented with another genetic condition which induces the loss of Y-chromosomes. Paternal loss (pal) is a mutation which causes such Y-chromosome eliminations, (Baker 1975). If one had a Y-chromosome which carries a translocated bit of the X-chromosome, including the wild type allele of the song mutation, one could recover mosaics which have lost the Y-chromosome in some parts of their body and which therefore express the recessive song mutation on their X/0 parts. These mosaics are not gynandromorphs and therefore are not affected by the complications arising through sex limited expression of traits, such as courtship song, (J. Hall, pers. comm.).

4.2.1.4.2. Claret non-disjunction

cand (Lindsley and Guell 1967) was received from the stock centre at Bowling Green. Because of the complications arising with sex limited traits, such as courtship song, which do not allow one to decide whether females carry the mutation or not, a rather complicated crossing scheme had to be used to generate the desired genotypes. A further complication is the known poor fertility of homozygous cand females. Females homozygous for the song mutation were crossed to homozygous cand males (homozygous cand flies are recognizable through their claret eye colour). The resulting males, hemizygous for the song mutation and hetero-

igure 4.2 Mating scheme for the production of females homozygous for $\underline{ca^{nd}}$ and a song mutation.



zygous for $\underline{ca^{nd}}$, were again mated to females homozygous for the song mutation. The resulting females, homozygous for the song mutation, heterozygous for $\underline{ca^{nd}}$, were crossed with males of the type of their fathers. This produced the required females, homozygous for $\underline{ca^{nd}}$ and the song mutation, which could now be mated to y, w, f males to produce gynandromorphs, (Figure 4.2).

Unfortunately it turned out that the frequency of mosaics produced by these females was very low (approximately 0.1%) in comparison to the values reported in the literature, (5%, Hall et al. 1975). Furthermore homozygous females were not as infertile as they are said to be. Clearly some selection in the background must have rendered these females rather inefficient as non disjunction inducers and must have increased their fertility. Nevertheless a number of gynandromorphs were recovered and they were sufficient in numbers to yield the necessary answers. Clearly they were not spontaneously occurring gynandromorphs, because these are expected at frequencies of only 0.01%, (Hall et al. 1975).

4.2.1.5 Recording of the song and flight tone

Both procedures have been described before, (sections 2.3 and 3.2). ipis were measured only up to 100 ms. Control males came from the original untreated att-X stock, which is isogenic to the mutant stock except for spontaneous mutations which could have arisen in both stocks since the isolation of the mutant. Effects from such mutations are however considered negligible because the present experiments were all carried out within 18 months of the

Table 4.2 Number of males out of 147 displaying the indicated song abnormalities and the number of sterile males in each group

	sine song	pulse song	sine song +pulse song	no sound	total
No. of males	20	9	21	5	55
No. of steriles	8	4	14	4	30

isolation of the mutant.

The measurement of the amplitude of the pulses was achieved in the following way. Five wild type and five mutant males were recorded under identical gain settings of the amplifiers. Several bouts of each male were filmed and the highest pulse in each bout was measured peak to peak, giving the mean of that male. The two types of males were compared with t-tests, which were also used for all other comparisons of song and flight parameters.

4.2.2 Results

4.2.2.1 Mutagenesis

2668 male offspring of EMS treated fathers were subjected to the first screening process - the mating speed test. 147 putative mutants emerged from this first sieve and were tested for their song behaviour. 55 sang in an abnormal way and were bred. These 55 males have been categorized according to their song deviations. It must be remembered that this classification was done in a subjective manner, because no precise measurements were made of the song. Sine song was monitored acoustically and pulse song acoustically and visually on the oscilloscope screen. After one has heard the song of hundreds of males, this screening procedure becomes very efficient and one can have considerable confidence in the results. As Table 4.2 shows 20 of these 55 abnormally singing males were putative sine song mutants, 9 pulse song, 21 were affected in both songs and 5 did not sing at all even though they clearly courted and extended their wings. 8 of the sine song defective flies were sterile, 4 of the pulse song suspects, 14 of the ones

Table 4.3 Mating speeds (No. of copulations out of 25 in 30 min) of two mutants

	1st Test	2nd Test
control	25	18
1st mutant	6	3
control	20	21
2nd mutant	4	7

Table 4.4 Means (± SE) of song parameters and flight wing beat frequency of cacophony and wild type males

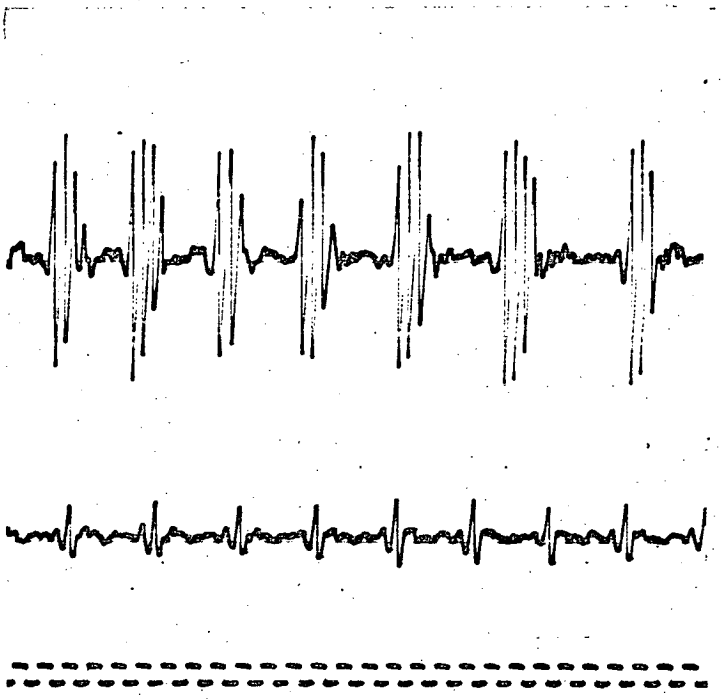
parameter	cacophony	wild type	p
ipi (ms)	44.23 ± 0.55	34.57 ± 0.37	< 0.001
pulse length (ms)	11.85 ± 1.49	3+	
amplitude (cm)	1.49 ± 0.09	0.83 ± 0.03	< 0.001
sine song frequency(Hz)	151.3 ± 6.06	159.9 ± 4.90	ns
wingbeat frequency(Hz)	212.8 ± 2.48	218.3 ± 2.40	ns

+ from Ewing and Bennet-Clark (1968)

Table 4.5 Distribution of the number of cycles per pulse for cacophony (N = 71) and wild type (N = 79) males.

No. of cycles per pulse	percentage of pulses with a given No. of cycles	
	cacophony	wild type
1	8	97.2
2	24	2.8
3	41	0
4	10	0
5	14	0
6	4	0

Figure 4.3 Oscilloscope traces of the pulse song of a cacophony (upper trace) and wild type (lower trace) male. Calibration signal: 100 Hz.



which showed deviations in both types of song, and 4 of the 5 males which did not sing at all. Of the remaining 25 males which were bred successfully, two turned out to have significantly lower mating speeds than control males, (Table 4.3). One was a semilethal, as evidenced by the drastic change in sex ratio, which sang normally and was discarded. The other one had an altered courtship song and no apparent morphological abnormalities, except that with increasing age a certain proportion of the males held their wings in an abnormal position.

4.2.2.2 Song of the mutant

The mutation increases ipi, pulse length, the amplitude of the song and the number of cycles per pulse. The frequency of the mutants wing beat and sine song remain unaffected, (Tables 4.4 and 4.5, Fig. 4.3).

Occasionally the mutants produced a veritable cacophony of song which gave them their name - cacophony (cac). All these changes in song qualities which cacophony causes in its carriers make it very easy to distinguish mutant from wild type song. Just a few seconds of vibration are enough for the experienced listener, to know what kind of male - mutant or control - is singing. Therefore it was possible to undertake mapping and fate mapping experiments with cacophony.

4.2.2.3 Mapping

Preliminary tests with a variety of F2 males revealed that the mutation must lie somewhere in between v (1-33.0) and f (1-56.7). 86 recombinants between these two markers were tested for their song behaviour and it was found that 6 recombinations had taken place between v and cac and 80 between cac and f. Therefore the approximate map position of cac is 34.64.

4.2.2.4 Fate mapping

4.2.2.4.1 paternal loss

Due to the known limitations of fate mapping with sex limited traits, which furthermore give rise to ambiguous results in one type of gynandromorph (section 4.2.1.4), it was attempted to use the Y-chromosome elimination technique, which theoretically is capable of overcoming the shortcomings of the more conventional techniques.

A pal stock was obtained from J. Hall and a stock carrying a translocated part of the X-chromosome on the Y-chromosome (y^+Yv^+) was brought to the authors attention by J. Hall and was obtained from Tobler, (Tobler et al. 1971). This Y chromosome might well have contained the wild type allele of cac, because cac and v are rather close to each other.

Unfortunately males carrying this composite Y-chromosome and a cac X-chromosome sing in a manner typical for the mutant. Therefore this approach had to be abandoned, because this meant, that the composite Y-chromosome does not cover cac, or that cac is dominant. In both instances the method would not be applicable.

Table 4.6 Number and types of gynandromorphs - derived through a given method - which sang in a given way. The percentage of given gynandromorphs which sang at all is also indicated.

type of gynandromorph	method of production	% singing	type of song*		
			cac*	wt*	nc*
	w^{vC}	70	36	0	0
	ca^{nd}	75	0	18	0
	w^{vC}	50	8	0	6

* cac - cacophony, wt - wild type, nc - not classifiable

Other such Y-chromosomes which are available were not tried because there was no indication at all that they might cover cac. The possibility of synthesizing an adequate Y-chromosome especially for the purpose was considered but rejected in view of the potential which the conventional methods still hold even though they have certain limitations. It would for example still be possible to obtain information about the question of the relationship of the cac focus to head, thorax or abdomen. Furthermore it was considered important to discover whether cac is a dominent mutation or not.

4.2.2.4.2 Ring chromosome elimination

As can be seen in Table 4.6, of 51 gynandromorphs which were produced through ring chromosome loss and which had at least half of their head cuticle male, their thorax cuticle half male, half female (the boundary running along the longitudinal midline) and an abdomen of unknown mosaic status, 36 sang in the 5 min observation period with both wings. All of them sang like the mutant when vibrating either of their wings. In other words, they produced mutant song with a wild type wing on the wild type half of their thorax.

28 gynandromorphs with a different constitution, at least half of their head cuticle male and no male cuticle on the rest of their bodies, were also tested. 14 of them vibrated, 8 sounded like the mutant and the rest sang in an unclassifiable way, (Table 4.6) as it has been described before (section 4.2.1.4.1) for the same type of gynandromorphs which carried no song mutation. Making certain

assumptions (see section 4.2.3) one would expect the same frequency of unclassifiable singers in this class of gynandromorphs as we found classifiable ones in the former class. If 100% of the ones with a half male thorax sing normally, than 100% of the ones with a female thorax should sing abnormally. The observed frequencies are 100% and 43%. A Fisher Exact test of the original numbers reveals that this discrepancy is significant, ($p < 0.0002$).

4.2.2.4.3 Claret non-disjunction

Of 24 gynandromorphs obtained through the action of the ca^{nd} gene and with a constitution identical to the first ring chromosome mosaics which have been discussed, (head: at least half male, thorax: half male, half female, abdomen: not scored) 18 vibrated both wings and this time they all sang like wild type flies with both wings, (Table 4.6).

4.2.3 Discussion

The abnormal song of cacophony could be the result of alterations in neuronal, muscular or even sensory structures. Changes to the resonant properties of the thoracic box could also be responsible, but since the flight wing beat frequency of mutant flies is normal, this seems less likely. Muscular structures could be ruled out if it was found that the primary focus of the defect maps to the head. Unfortunately the fate mapping data are ambiguous on this point. The finding that all mosaics with

heads derived through ring chromosome loss sing like cacophony with both wings could be interpreted in three ways: (1) the mutation is recessive and the focus is in the head, identical with or very close to the primary neuronal driver of song, (2) the mutation is recessive but the focus is in the thorax and is a complex one exhibiting a domineering effect, (3) the mutation is dominant and in the thorax. The last of these interpretations is excluded with the gynandromorphs derived from ca^{nd} females, which sing like the wild type in every instance. If the mutation were dominant and the focus in the thorax, these individuals should express a mutant song with the wing on the female half of their thorax. To decide which of the other two hypotheses holds true one has to look at mosaics which are all female save for their head. If these have been produced through ring chromosome elimination and sang largely a cac type song, clearly the first hypothesis would be correct. Unfortunately these mosaics sing like the analogous ones without the song mutation and cannot all be classified. Therefore, the question of the focus of cac cannot at the moment be resolved. It seems possible that all the mosaics of this class which sang like cac have certain internal male structures even though their thoracic cuticle is female. The others, which if the focus were in the thorax would sing wild type, are prevented from doing so because they cannot sing properly at all. Therefore we cannot exclude the possibility that the focus of cac is in fact identical to, or very close to the thoracic structure, which has been conjectured as being necessary for a proper song, (section 4.2.1.4.1)

The nature of this hypothetical thoracic structure can be illuminated with the fate mapping data just discussed. As we have seen, the gynandromorphs with a bilaterally symmetrical mosaic thorax split up very clearly into either mutant or wild type singers - depending on the method of derivation. None of them sang in an unclassifiable way. This must be taken to mean that the postulated thoracic structure, which has to be male if the fly is to sing a proper song, lies very close to the thoracic cuticle. In 36 of the ring chromosome gynandromorphs and 18 of the $\underline{ca^{nd}}$ ones, the blastular dividing line never fell in between the cuticle and this hypothetical thoracic element. This seems to indicate that the thoracic element in question is the cuticle itself. This cannot be the case, however, because mosaics with an all female thorax sang mutant-like in several instances. This is rather bewildering because it means that there is a considerable asymmetry in the probabilities of discordance of cuticle and hypothesized thoracic structure, depending on what the gynander looks like. Male cuticle on one half of the thorax means that with very high probability (100% as we have seen) the thoracic element is also male, female cuticle on the entire thorax, however, does not produce a similar probability that the element will be female. In this case it is male rather more often than expected. This discrepancy again indicates that we are dealing with a complex focus exhibiting a domineering effect (Hotta and Benzer 1973). In such a case the probabilities of discordances in maleness between two structures can become asymmetrical. To illustrate the point, imagine a complex focus which is made up of several subfoci any one of which, if it is male, causes the gynander to sing like a normal male. If one starts off with an all male thoracic cuticle, then the probability

of a male song phenotype is very high indeed if cuticle and thoracic structure are closely linked because all the subfoci would have to be discordant to make the fly sing abnormally. If one starts off with an all female thorax, however, then the probability of a male song phenotype is not equally low as it has been high in the former case, because now the probability of maleness of the phenotype is equal to the sum of the probabilities of discordance in maleness between cuticle and hypothetical structure for each single subfocus. Just one subfocus which is discordant for example, results in a normal song.

Hotta and Benzer (1973) have erected a rather detailed fate map of Drosophila and report that the probability of discordance between the coxa of leg 1 and the ipsilateral internal structure which causes this leg to shake under ether in shaker mutants is 0.13. This structure is almost certainly part of the thoracic ganglion and is repeated six times because each leg's shaking action is independent from that of the other legs, (Ikeda and Kaplan 1970b, Hotta and Benzer 1973). If in the present case we hypothesise that the thoracic structure which has to be male if a gynander is to sing properly (1) is part of the thoracic ganglion, (2) is also repeated six times (three times on each side), (3) that anyone of these units, if it is male, causes the mosaic to sing normally, and (4) that these units each have a fate map distance to the cuticle of 0.13, then we can calculate the expected number of discordant cases, between cuticle and song, we should observe, starting with a gynander of a given type. With these assumptions, which are not unreasonable if one remembers that the thoracic ganglion is a compound structure which arose out of the three thoracic and all the abdominal ganglia of more primitive insects, it can be calculated that the probability

Figure 4.4 Calculation of the probability of finding a given gynandromorph exhibiting the indicated song behaviour under the assumption of 6 subfoci each with a domineering effect, such that a single male subfocus suffices to make the gynander sing in a normal way. The distance in sturts of each subfocus from the cuticle is 13. (p of discordance = 0.13).

p that this gynandromorph has a normal song = 1 - p that all subfoci are female =

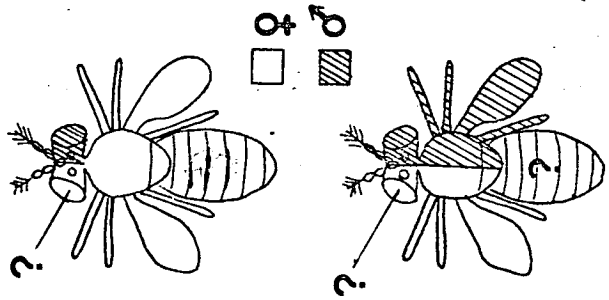
$= 1 - (0.87)^6 =$

$= 0.57$

p that this gynandromorph has an abnormal song = p that 3 subfoci on the right are discordant x p that 3 subfoci on the left are concordant =

$= (0.13)^3 \times (0.87)^3 =$

$= 0.0014$



that a gynander with an all female thorax will sing normally is 0.57, whereas the probability that a gynander whose thorax is half male and half female will sing abnormally is only 0.0014, (Figure 4.4). These numbers clearly agree very well with the observed values of 0.57 and 0.00. But in view of the sparsity of the data and the multitude of possible alternative models which would fit the data equally well, it must remain a very tentative hypothesis which awaits further testing.

An alternative explanation for the asymmetry discussed above, is of a more mundane kind. It could be that with the second kind of gynandromorphs (head only male), more errors of classification were committed. It seems, however, very unlikely that this is the reason because yellow is a very good marker for the thorax. But the possibility that very small male patches are occasionally overlooked cannot be ruled out entirely.

To decide unambiguously where the focus of cacophony resides, one either has to refine the fate mapping techniques, or start neurophysiological or anatomical studies. The former, as we have seen (section 4.2.1.4.1), would be possible if adequate Y-chromosomes were available. The latter, especially neurophysiological studies, are promising, because A. Ewing (pers. comm.) has shown that tethered males, with electrodes inserted into their thorax, will court and vibrate their wings to females, which are brought close to them. Anatomical studies are probably only worth considering after one has an approximate indication of the area afflicted by the mutation.

4.3 The behaviour of cacophony; activity, reactivity and mating behaviour

4.3.1 Introduction

In the introduction to this chapter the potential values of song mutants for functional studies have been emphasized. Truly promising mutations for functional studies would be ones with little or no pleiotropic action. These are the only ones which permit the experimenter to manipulate a single independent variable in a controlled manner. The following experiments will try to clarify (1) whether cacophony is a specific song mutation which does not affect the fly in other behavioural aspects, (it has already been demonstrated that it does not influence flight wing beat frequency). These aspects will be activity, reactivity and courtship behaviour. All are rather highly integrated behavioural systems which are very well suited to bring pleiotropic effects into the open. Courtship behaviour in particular involves an enormous number of bodily functions and if any of them is defective the end result, mating speed may be influenced. To exclude the song itself as a variable which influences mating speed, one just has to amputate the wings of the flies, as been indicated earlier. (2) It will be attempted to find the cause for the increased incidence of abnormal wing posture in mutant males. (3) The "Alexander hypothesis" (Alexander 1962) will be tested.

4.3.2 Methods

All experiments were carried out at 25 ± 1°C. The temperature in the culture room was 25 ± 2°C in time leading up to the experiments. All male mutant flies were taken from the att-X stock in which they had been kept since their isolation. The control males were taken from the original untreated att-X stock. The females came from two isogenic stocks, one of which (control, att-X^B) had been derived from the original att-X stock and the other one (homozygous cacophony) was identical to it except for the cacophony gene which was introduced into this stock in the following way. The fused metracentric attached-X chromosomes of att-X females are known to break occasionally, (Ch. Auerbach, pers. comm.). Such a break occurred in the stock used in the present investigation, as evidenced by the sudden occurrence of wild type females and y, w, f males in the att-X stock bottles. These y, w, f males are proof that the wild type females could not have arisen through contamination. If contamination were the cause then the only contaminators could have been y, w, f females which, however, were not kept in the stock room at the time in question. 13 of these wild type females were collected as virgins and mated to att-X males. All F1 offspring were wild type, which indicates either that the females were at least from the second generation after the break had occurred, or that the attached-X chromosome had somehow undergone recombination with the wild type X-chromosome, such that it still was an attached-X chromosome but carried the markers heterozygously and did not express them anymore. The latter possibility was excluded by crossing 15 virgin att-X^B females to y males. In no case have any y males been recovered in the F1, which would have had to be expected if the females still carried an attached-X

chromosome. Virgin females from the stock thus synthesized were mated to att-X males for another two generations to establish the att-X^B stock. This stock is therefore genetically identical to the att-X stock, except that both males and females carry the X-chromosome which was limited to the males in the att-X stock.

To obtain homozygous cacophony females, cacophony males from the att-X stock in which they were maintained and from which they had originated, were mated to att-X^B females, which, as we have just seen, were derived from the same att-X stock. The resulting F1 females were again mated to cacophony males and the F2 females, 50% of which should be homozygous for cacophony, were mated singly to cacophony males. Three females which produced only male cacophony offspring (10 male offspring were tested for each female) were taken as the foundation of a homozygous cacophony stock, which according to its derivation must be isogenic with the att-X^B and att-X stocks.

4.3.2.1 Activity, reactivity and wing posture

Activity measurements were made on single flies in a circular arena, marked off at regular intervals, (Connolly 1966). The number of lines crossed during 0.5 min was counted, after the fly had had 1 min to recover from the transfer procedure. 10 flies of each sex and stock (cacophony and control) were scored in this way. Comparisons were made with t-tests.

In the first type of reactivity measurement twenty, 2 day old flies (10 females and 10 males) were introduced into a cylindrical perspex cell (2.6 cm in diameter, 2.3 cm high) with a wiremesh

floor and a gauze top. This cell was suspended over a ribbon microphone and after 1 min of recovery the number of flight buzzes occurring was counted over a 5 min period, (Kaplan and Trout 1969). t-tests were employed to compare mutant and control scores of several such tests.

A second measure of reactivity was made on single flies which were subjected to a shadow stimulus, (Kaplan and Trout 1969, Angus 1974). A fly was introduced into an empty vial which was illuminated from above (30 cm) by a single light bulb (100 W). As soon as the fly settled down to preen, a piece of cardboard (20 cm long, 5 cm wide) was passed 7.5 cm above the vial, throwing a shadow over it. As soon as the fly became visible again, it was scored as standing still or moving. Each fly was subjected to one test only. The cardboard was moved by an electric motor at a constant speed. Comparisons were made with Chi-square tests on the data pooled between sexes, because there were no differences between them.

As has been noted before, a substantial number of male mutant flies in the stock bottles held their wings in a more or less opened, raised and twisted position. This was never observed in flies which were kept singly in vials, therefore an attempt was made to identify the factors responsible for this abnormal wing posture. Fifty, 1 day old mutant males were introduced either singly or in groups of ten into vials containing food. Fifty mutant and control males were introduced in groups of five into vials which also contained five females. After three days the flies were etherized and the number of individuals holding their wings in an abnormal way was counted. To be scored abnormal, the wings had to be either raised and/or twisted and/or opened in such a way that the two wings would not overlap.

4.3.2.2 Courtship behaviour

Virgin three day old pairs of flies from all combinations of mutant and control flies were introduced into cylindrical perspex cells (2 cm in diameter, 0.7 cm high) with a coverslip top. The ensuing courtship was observed through a binocular microscope and the male's courtship displays were recorded continuously on an event recorder. The start of courtship was defined as the first bout of wing vibration, which was only scored if the wing was fully extended. Orientation was defined as by Manning (1959), it starts with the first vibration and is included with all other male courtship behaviours. Licking and attempted copulation was scored whenever the proboscis was fully extended or the abdomen curled under, with no regard as to whether contact was made with the female's genitalia or not. The angular transformation was applied to all percentage scores and after the variances were found sufficiently homogenous analysis of variance was used to analyse the results statistically, (Sokal and Rohlf 1969).

Mating speed was measured in a mass mating set up as described before, (Section 3.2). The percentage of flies which copulated out of 25 in 30 min was scored, and the arcsine transformed data were subjected to an analysis of variance. In one mass mating test 20 wingless males courted 10 winged control females for 40 min. The wings of these males had been amputated at the time of sexing with a scalpel. The percentage of copulations occurring within the observation period out of a possible 10, was arcsine transformed and t-tests were used to compare mutant and control males, which had been tested in parallel experiments.

Table 4.7 Means (± SE) of the activity of single mutant and control flies. Number of lines crossed in 30 s. N = 10 in each group.

	females	males
cacophony	10.6 ± 1.72	17.3 ± 1.82
control	20.4 ± 2.68	18.6 ± 2.97

Table 4.8 Number of flies, out of 15, reacting to the shadow stimulus

	females	males
cacophony	3	5
control	14	13

Table 4.9 Number of flies, out of 50, with abnormal wing posture.

	cacophony		control	
	females	males	females	males
1 fly / vial	-	2	-	-
10 flies / vial	-	4	-	-
5 flies / vial + 5 flies of opposite sex	1	31	2	3

A comparison of the relative mating success of mutant males with and without wings was made by calculating the percentage of mutant males which copulated within the observation period; where 100% is the number of control males which had copulated in a parallel test. The resulting data were again subjected to the angular transformation and t-tests furnished the probability levels of the differences.

The number of pulse song and sine song bouts was counted through earphones from mutant and control males courting one day old virgin control females on top of a ribbon microphone. t-tests were employed to compare the results.

4.3.3 Results

4.3.3.1 Activity, reactivity and wing posture

Table 4.7 shows that whilst mutant females are less active than either mutant males ($p < 0.02$) or wild type females ($p < 0.01$), the mutant males are just as active as wild type males.

As concerns reactivity to other flies (No. of flight buzzes in 5 min), it was found that mutant flies are much less reactive (6.6 ± 1.81 SE) than control flies (26.8 ± 4.47 SE), ($p < 0.01$). Furthermore they are also less disturbed by a shadow stimulus as is shown in Table 4.8, ($p < 0.005$).

Table 4.9 gives the numbers of flies out of 50 which held their wings in an abnormal way, after having been kept for three days under the conditions indicated. It can be seen that in no case is this number higher than 4, except when five mutant males are kept together with five mutant females. In this case 31

Table 4.10 Means of the different courtship behaviors in the four crosses between mutant and control flies. N = 9 in each group

females	males: cacophony	males: control	behavior
cacophony	47.00	64.50	percentage copulated out of 25 in 30 min
control	40.50	75.60	
cacophony	63.78	69.54	percentage orientation
control	68.24	77.81	
cacophony	19.91	27.15	percentage vibration
control	17.99	25.16	
cacophony	1.50	2.47	No. of vibration bouts in 8 s of orientation
control	1.28	2.36	
cacophony	1.14	0.96	Mean bout length of vibration (s)
control	1.16	0.92	
cacophony	0.70	1.64	No. of licks in 8 s of orientation
control	0.42	1.11	
cacophony	0.53	1.50	No. of attempted copulations in 8 s of orientation
control	0.16	0.71	

Table 4.11 Analysis of variance of the data in Table 4.

source	df	MS	F	p	behavior
females	1	22.77	0.290	ns	percentage copulated
males	1	2157.45	27.790	< 0.005	
interaction	1	256.20	3.420	ns	
error	32	77.64			
females	1	146.41	0.924	ns	percentage orientation
males	1	208.70	1.317	ns	
interaction	1	15.32	0.097	ns	
error	32	158.54			
females	1	12.70	0.540	ns	percentage vibration
males	1	235.36	10.010	< 0.01	
interaction	1	0.38	0.016	ns	
error	32	23.51			
females	1	0.25	0.635	ns	No. of vibration bouts
males	1	9.36	23.579	< 0.005	
interaction	1	0.12	0.310	ns	
error	32	0.40			
females	1	0.28	0.005	ns	Vibration bout length
males	1	256.01	4.745	ns	
interaction	1	4.83	0.090	ns	
error	32	53.95			
females	1	1.51	4.84	ns	No. of licks
males	1	5.98	19.179	< 0.005	
interaction	1	0.14	0.452	ns	
error	32	0.31			
females	1	3.01	8.016	< 0.025	No. of attempted copulations
males	1	5.23	13.904	< 0.005	
interaction	1	0.38	1.02	ns	
error	32	0.38			

Table 4.12 Mean ($\pm$ SE) of the number of pulse song and sine song bouts emitted by cacophony and control males in 8 s of orientation. N = 5 in each group.

	pulse song	sine song
cacophony	5.93 $\pm$ 0.79	0.42 $\pm$ 0.16
control	4.99 $\pm$ 0.50	1.63 $\pm$ 0.22

males, but only one female, held their wings in an abnormal way. This clearly shows that the presence of females is responsible for the high incidence of abnormal wing posture in mutant males.

4.3.3.2 Courtship behaviour

Tables 4.10 and 4.11 summarize all visually monitored components of the courtship. It can be seen from the magnitude of the F values in Table 4.11 that males are responsible for the reduced mating speed of cacophony, the lower percentage of vibration while being orientated, the lower number of vibration bouts and the decreased number of licks. Both sexes play a significant role in the determination of the number of attempted copulations. The mean bout length of vibration and the percentage orientation are unchanged in the mutant. None of the results is determined to any significant degree by an interaction between the sexes.

The mating success (percentage copulated) of wingless cacophony males with control females is also significantly lower (21.14 $\pm$ 3.32 SE) than that of control males (50.58 $\pm$ 3.40 SE), ($p < 0.01$). If one expresses the mating success of wingless and winged mutant males as a percentage of the success of control males subjected to the same treatment and tested in parallel experiments, one finds that mutant males fare relatively better with wings (48.28 $\pm$ 4.14 SE) than they do without (28.52 $\pm$ 5.38 SE), ($p < 0.05$).

Table 4.12 gives the results of the acoustically monitored behaviours. The number of pulse song bouts is unchanged, whereas the number of sine song bouts is considerably lower in the mutant, ($p < 0.01$).

4.3.4 Discussion

Alexander's hypothesis (Alexander 1962) would predict that males which had undergone genetic alteration of the template which they use as a comparator for efferent signals would be most readily accepted by females homozygous for the same mutation, because these females have undergone the same changes in their template which they use as a comparator for afferent signals. In other words in the results presented in Tables 4.11 and 4.12 there should be a strong interaction effect for the data on mating speed. Such an effect is not found, although it should perhaps be noted that the differences are in the right direction.

The change in ipi of the mutant is largely accounted for by the increase in pulse length. If the females measure ipi from the end of one pulse to the beginning of the next one, unlike the experimenter, then the ipi change of cacophony cannot be used to test the hypothesis. In this case ipi could also not be made responsible for the reduced mating success of mutant males with their own and control females. Song must be excluded as a factor which affects the mating speed of cacophony males detrimentally anyway, because wingless mutant males are still at a disadvantage when compared with wingless control males. Other factors must be made responsible for the low mating success of cacophony males. Possible candidates are the lowered reactivity of the mutants and their decreased number of licks and attempted copulations. The former seems to be an unlikely explanation, because mutant males court as persistently as control males, and they show no reduction in the percentage of orientation during courtship. The latter two behaviours are a stronger feasibility because it has been shown that the more of these behaviours a male displays the faster he will copulate, (Dow and

Schilcher, unpublished).

The fact that cacophony males fare relatively better with their wings than they do without, is further evidence that the mutant song is not responsible for the low number of cacophony males which copulate in the test period. On the contrary, these results suggest that the mutant song constitutes an improvement on the wild type song, or alternatively that the presence of wings somehow improves the performance of mutant males in an unspecific way. The former possibility must be seriously considered, because cacophony males sing louder than wild type males, and it is possible that they thereby stimulate the females more effectively. Although the improvement of a system subjected to the forces of natural selection seems to be an extremely unlikely result of mutagenesis, it is conceivable that this has been achieved at a cost, which under normal conditions would more than offset the improvement. This cost might be the competition which a male would create for himself if he sang too loud. Pulse song has been shown to stimulate males strongly (section 2.5) and as far as the intensity of pulse song is concerned, a compromise between the necessity to stimulate the female and the adverse effects of arousing other males might have evolved.

Cacophony and control males give equal numbers of pulse song bouts, but the former give fewer sine song bouts. If sine song is an important sexual stimulus for the females, as the experiments in section 2.6 seem to imply, then this disadvantage must also be compensated for by the increase in sound intensity.

The fact that there is only one significant female effect in the visually recorded courtship behaviours is probably just a consequence of the much more active role that males play in courtship. However, females do influence the number of attempted

copulations which can be taken as another confirmation of the close relationship between female activity and receptivity, (Cook 1973). Mutant females receive more attempted copulations and are less active than wild type females. Such a correlation has been found before in a study of female rejection responses, (Connolly and Cook 1973). It is somewhat surprising that this female effect on attempted copulations does not carry through to mating speed, where the females have no significant influence. It must be concluded that other factors which also contribute to mating speed counteract and annihilate this influence, or that the number of attempted copulations is not an important courtship stimulus, at least not for cacophony females.

The rather striking influence the presence of females has on male wing posture indicates that mutant males either have defective muscles or that neurological changes make these males hold their wings in constant threat or wing flick position (Dow and Schilcher 1975), when they court and compete for females. Qualitative observations suggest that this might indeed be the case although it is of course hard to understand why they should maintain their wings in this position when etherized. It has been observed that upon introduction into a different genetic background the mutants carry their wings normally, whereas they continue to sing abnormally.

With the present emphasis on neurobiology cacophony would clearly fall into the category of 'trivial' mutations (Wilcock 1969) if it turned out to be a muscular defect which is responsible for the abnormal song behaviour. Otherwise it could be a promising tool which in conjunction with other, similar mutants might help to open up neurobiology. Unfortunately it is at present impossible to say where exactly cacophony stands.

CHAPTER 5

General discussion

In the introduction to this thesis the ethological and evolutionary significance of Drosophila courtship song has been emphasized. It was considered opportune to start an investigation of the song at the functional and analytical level simultaneously. The functional, evolutionary part of the work consisted mainly of playback experiments, and furthermore of some studies of the mating behaviour of two sibling species and their hybrids and of that of a song mutant.

The analytical part concerned itself with the mechanism of song production, the defects caused by the song mutation cacophony and the inheritance of ipi.

The playback experiments have extended our understanding of the effects of courtship song both on males and females. Both pulse - and sine song increase female receptivity. Pulse song does so only in a stimulation experiment, sine song also under conditions of prestimulation. Therefore sine song is remembered over appreciable periods of time, whereas pulse song is not. This led to the suggestion that pulse song might act like a trigger and sine song in a priming fashion. The action of pulse song is rather ipi-specific. Both songs decrease female locomotor activity to a similar extent.

Section 2.5.3. contains a full discussion of the conclusions which can be drawn from these and earlier experiments about the precise function of ipi. It undoubtedly plays a role in sexual selection, but it was found impossible to decide, with the information presently available, whether it subserves inter - or intraspecific communicative purposes. The experiments reported

in chapters 3 and 4, which also have some bearing on this question, could not in any way change this rather unsatisfactory outlook. The only way out of the dilemma seems to be via long term experiments which manipulate the selection pressures to which the experimental population is exposed. If ipi functions as a species isolator, then in a population of flies which does not need to protect itself against hybridization, this trait should deteriorate. It might be profitable in the future to make detailed ipi comparisons between long established outbred laboratory populations of D. melanogaster and freshly collected flies of the same species coming from an area of sympatry with D. simulans.

As far as the function of sine song is concerned, the playback experiments (section 2.6.4) have not been carried to their conclusion. It would clearly be possible to verify or falsify the alleged summating function of sine song unambiguously with similar experiments. One would have to subject females to different time periods of pre-stimulation with sine song and see whether the effect on receptivity varies accordingly. One would expect to observe some limit to the time over which summation could occur.

Pulse song has a striking effect on male sexual and locomotor activity which could not be demonstrated for sine song. The male response to pulse song is relatively ipi independent. Whether under natural conditions this response is triggered by a male's own song or that of others, or both, could not be decided, but it seems most likely that the second interpretation is the correct one. It must be left to future workers to investigate this question more thoroughly.

The results from the mating speed tests with wingless and winged mutant and control flies have indicated that the mutant song, which is higher in intensity and which has a longer ipi, is actually more effective than wild type song. This was taken to mean that the amplitude of the song as it is found in wild type males is probably not at its optimum level as far as the stimulation of the females is concerned. It is possible that a selective counter pressure has been at work preventing a louder song from evolving. This counter pressure would arise from the adverse effects a loud song has for the performer because it calls in more mating competitors. Therefore the song intensity which has evolved could be a compromise between these detrimental effects and the necessity to sing loud enough to stimulate the females sufficiently.

On the reductionist, analytical side several results have added some information to our understanding of courtship song. From the study of the song of gynandromorphs it has become clear that head structures are necessary and sufficient to enable the mosaic individual to vibrate its wings. To produce a proper song, however, a thoracic structure which maps close to the thoracic cuticle on the blastular surface, must also be male. The nature of this element of song production could not be revealed. It seems desirable and possible to test its nature with neuro-physiological techniques.

The nature or the fate map position of the defect which is responsible for the deviant song of cacophony could not be revealed due to the complications arising out of the sex limited expression of the song. It appears that the next step in the analysis of cacophony must be made with electrophysiological

methods or improved fate mapping techniques.

In Chapter 3 it has been shown that the song of hybrids between D. melanogaster and D. simulans is identical to that of D. simulans. This finding neither contradicts nor verifies the sex linkage hypothesis of ipi. The mating speed tests with the hybrids and their parent species have brought no support for the hypothesis which ascribes ipi receiving and emitting comparator structures a common genetic basis. This conjecture was also not supported by the findings with cacophony, where homozygous females still preferred wild type over mutant males.

Concluding one can say, that this thesis has opened up a few new viewpoints but it has not succeeded very well in clearing old ones. Mutagenesis of courtship song remains a highly promising field of research. Courtship song might well be the behaviour of Drosophila, which, among the ethologically non trivial behaviours, lends itself most conveniently to a genetic dissection approach. Therefore it will be very profitable to extend the mutagenesis experiment with some modifications which permit the screening of larger numbers of males.

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I hereby certify that this thesis has been composed by myself. The experimental work has also been performed by myself with the following exceptions. The data reported in section 2.4.1.1. were collected and analysed in collaboration with Mr. M. Dow. Those of Chapter 3 with Professor A. Manning.

F.v.Schilcher

PUBLICATIONS

The following parts of this thesis have been published or are in press in more or less modified form:

(1) Section 2.5

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Courtship Song and Mating Speed in Hybrids Between *Drosophila melanogaster* and *Drosophila simulans*

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Courtship song and mating speed of hybrids between Drosophila melanogaster *and* D. simulans *were investigated. The courtship song of hybrid males is identical to that of* D. simulans, *suggesting that X chromosome determination, known from the cross between* D. pseudoobscura *and* D. persimilis, *is also possible here. Wingbeat frequency of hybrids is intermediate between that of the two parents, demonstrating that courtship song and wingbeat frequency are inherited independently of each other. In mating tests, hybrid males court and are accepted by* D. simulans *females more than hybrid females (presumably because their song is more "acceptable" to the former).* D. melanogaster *females reject hybrid males. Hybrid females accept* D. melanogaster *males readily, hybrids less readily, and* D. simulans *least.*

KEY WORDS: *Drosophila melanogaster*; *Drosophila simulans*; hybrids; sexual behavior.

INTRODUCTION

The sexual behavior of males of the sibling species *Drosophila melanogaster* and *D. simulans* was described by Spieth (1952) and later was compared in more detail by Manning (1959). The same basic behavioral elements are common to both, but the courtship of *D. simulans* contains a higher proportion of wing scissoring and less vibration than that of *D. melanogaster*. Shorey (1962) first showed that the wing vibration of the males provides acoustic stimuli. Subsequently, Ewing and Bennet-Clark (1968) provided a comparative survey of the courtship sounds of different

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Drosophila species, and showed that the interpulse interval (ipi) was highly species specific.

One of us (F. v. S) is studying the effects of induced mutations in *D. melanogaster* in order to obtain mutations of the song, which might help in elucidating the cause-and-effect chain between genes and this behavior (see the arguments presented by Benzer, 1967, 1973). For mutagenesis it is most economical to examine the X chromosome, and we were anxious to discover whether this chromosome plays a major role in the determination of song characteristics in *D. melanogaster.* We were encouraged in this venture by previous work which has shown that the X chromosome does carry genes of particular importance in the control of courtship song, and perhaps other features of *Drosophila* sexual behavior. First, Ewing (1969), working with *D. pseudoobscura* and *D. persimilis,* could unambiguously locate the control of the major qualitative features of the song on the X chromosome. Quantitative variation of ipi was controlled by dispersed genes, but, as Ewing indicates, his recording method was rather inaccurate for this measurement because he could not control temperature within the desired limits. Second, Futch (1973) found that the mating success of hybrids between *D. pallidosa* and *D. ananassae* depends on the origin of the males' X chromosome. Hybrid males are more successful with females from their mother's species, whose X chromosome they receive.

Since hybrids between *D. melanogaster* and *D. simulans* are uniformly sterile and since male hybrids always carry the *D. simulans* X chromosome, no firm proof of X chromosome involvement can be forthcoming. However, our study is justified because such a hypothesis could be *disproved,* namely by intermediate inheritance in the hybrids.

The hybrids between *D. melanogaster* and *D. simulans* were the first to be described in the genus (Sturtevant, 1920). Sturtevant (1929) found that the cross *D. melanogaster* ♀ × *D. simulans* ♂ was made more easily than the converse, and this has been confirmed by many workers since then (Morgan, 1929; Biddle, 1931; Uphoff, 1948; Manning, 1959; Parsons, 1972; Eoff, 1973). Barker (1962) has reported an apparent exception to this rule, but it could be ascribed to differences in the methods employed for the measurement of the degree of sexual isolation (Barker, 1967). These laboratory findings were confirmed by Sperlich's (1962) field collection, where, in an area where both species occur, he found that 5% of the females produced only sterile female offspring, making it likely that they were *D. melanogaster* females which had been inseminated by *D. simulans* males. No females producing only sterile males were found.

Certainly there is genetic variation for the degree of sexual isolation between the two species. For instance, Parsons (1972) showed that different strains each derived from a single female collected in the wild differ in their

degree of sexual isolation from their sibling species. This difference is primarily determined by the females, but both sexes are the source of the genetic variation. The behavioral basis for this unequal sexual isolation has not been extensively studied. Manning (1959) found that the cross *D. melanogaster* ♀ × *D. simulans* ♂ is easier despite the fact that *D. melanogaster* males are sexually much more active with foreign females than are *D. simulans* males. This study also indicated that male hybrids have a *D. simulans*–like courtship and show a preference for *D. simulans* females. It was also found that hybrid females were very rarely courted by *D. simulans* males.

The present study describes the mating success and sexual behavior of hybrids in more detail. We measured mating success for various combinations of hybrids with each other and with their parental species, and recorded ipi and flight wingbeat frequencies. We shall discuss the different parameters and speculate on their evolution.

METHODS

The *D. simulans* stock used was recently wild-caught in Rome. Two *D. melanogaster* stocks were employed. The first was an attached-X stock producing wild-type males and females carrying an attached-X compound chromosome, homozygous for *yellow, white,* and *forked.* The second stock was derived from the first one by collecting about ten spontaneously produced wild-type females. Thus this second stock was wild type in both sexes, derived its X-chromosome from the males of the attached-X stock, and was otherwise identical to it.

Crossing attached-X females with *D. simulans* males results in hybrid males which carry the X chromosome of their father, whereas crossing females of the wild-type stock with *D. simulans* males results in hybrid females. By using this strategy, we avoided having to rely on the more difficult cross between *D. simulans* ♀♀ and *D. melanogaster* ♂♂ to produce hybrid males. At the same time, the *D. simulans* X chromosome of the hybrid males was combined with *D. melanogaster* cytoplasm. This combination enabled us to control for the possibility that cytoplasmic effects could simulate sex linkage.

The flies were kept in half-pint milk bottles on standard medium at 24 ± 1°C on a 12-hr light, 12-hr dark cycle. To maximize the chances of mating, the interspecific crosses were set up with five 1-day-old females and ten 2-day-old males in 7.5- by 2.5-cm vials containing medium. After 1 day, the contents of four or five such vials were transferred to a stock bottle for egg-laying and development. All virgin flies used in the present experiments were collected within 6 hr of eclosion, sexed under light etherization, and

then transferred to vials. (Since the cultures of hybrids produced only one sex, they were allowed to hatch for 24 hr.) All experiments were carried out at 24 ± 1°C within 3 hr of the flies' dawn, using virgin flies 3–4 days old, except where otherwise indicated. Mating speed tests were performed with 25 pairs of flies in 250-ml flasks. The flasks were held horizontally in a clamp and indirectly illuminated by a 60-W lamp at their base, which was slightly pointed upward so that the flies tended to aggregate at this end and could be sucked out with an aspirator. The time for each copulation was recorded and all tests were concluded after 30 min.

The data thus collected were analyzed according to a technique reported by Bliss (1967) for truncated distributions, possessing several advantages compared with other methods which have been used for the analysis of mating speed data, such as probit analysis (e.g., Manning, 1961) and methods based on the number of pairs copulating in a given time period (e.g., Parsons and Kaul, 1966). Unlike probit analysis, our method does not depend on the assumption that all flies would eventually copulate, if only one could wait long enough. In fact, there are strong indications that the log-time–probit plot is not linear for the mating speed of many *Drosophila* populations. The method also allows us to differentiate between populations which achieve identical numbers of copulations in a given time period but nevertheless, due to different lag times, have different mating speeds. This is because this method utilizes two parameters: mating speed (MS, the mean time to copulation of all pairs available for copulation) and the number of pairs which the method predicts would ever copulate (PC).

Our data seem to indicate that just counting the number of copulations in a given time might be a good measurement, if the period were long enough. The mean number of pairs that did not copulate during our 30-min observation period but that, the method predicts, would have copulated later was only 0.84. We therefore confirm Manning's (1967) suggestion that 30 min is close to the optimum observation period.

Comparisons between the mating speeds of the different genotypes were made using Student's t test on the logarithms of seconds for MS and χ^2 tests for PC.

For recording song, males were introduced with 1-day-old virgin females (to prevent early copulation) into 10- by 15- by 5-mm wire mesh cages with a perspex top and placed on a microphone 1 mm above the ribbon. The song and wingbeat frequencies were recorded after appropriate amplification through a ribbon microphone (Bennet-Clark, 1973) on a Tandberg 3000X tape recorder. The recordings were then displayed on one beam of an oscilloscope with a calibration signal on the other beam. The traces were filmed and measurements could be made from the film.

The flight records were obtained by gluing the flies to entomological

pins under light etherization and suspending them over the microphone. Usually they start flying within 1–3 min. All these measurements were made with temperature controlled at 25 ± 0.5°C because of the high temperature dependence of both courtship song and flight wingbeat frequency (Shorey, 1962; Reed *et al.*, 1942).

RESULTS

Mating Success

Mating speed tests were made using all combinations between the three genotypes, except for the interspecific crosses themselves, which are so slow in mating as to preclude measurements. The results are presented in Table I and the comparisons in Table II. As can be seen from Table I, the two parent species score very similarly on both measures. In fact, there is very little variation in MS between the various combinations. Only one of the comparisons which concern us here is significant: *D. melanogaster* males mate more slowly with hybrid females than with their own females (Table II).

Table I. Mating Speed from Various Crosses Between Genotypes[a]

	D. melanogaster ♂	*D. simulans* ♂	*Hybrid* ♂
D. melanogaster ♀			
No. R	5	—	4
log MS	2.5 ± 0.01	—	—
MS	331.2	—	—
PC	20.8	—	—
D. simulans ♀			
No. R	—	5	4
log MS	—	2.5 ± 0.06	2.7 ± 0.07
MS	—	318.1	488.2
PC	—	21.0	16.7
Hybrid ♀			
No. R	8	6	4
log MS	2.7 ± 0.04	2.6 ± 0.15	2.6 ± 0.05
MS	503.3	365.3	422.1
PC	16.6	3.5	7.5

[a] No. R, number of replicates; log MS, mean mating speed in log seconds ± SE; MS, mean mating speed in seconds; PC, mean number of pairs predicted to copulate out of 25.

Table II. Comparisons of Mating Speed Data Between the Different Genotypes[a]

Constant mate	Mate 1	Mate 2	MS	PC
Hybrid ♂	Hybrid ♀	*D. simulans* ♀	ns	$p < 0.005$
Hybrid ♀	*D. melanogaster* ♂	*D. simulans* ♂	ns	$p < 0.005$
	Hybrid ♂	*D. simulans* ♂	ns	ns
	Hybrid ♂	*D. melanogaster* ♂	ns	$p < 0.01$
D. melanogaster ♂	*D. melanogaster* ♀	Hybrid ♀	$p < 0.01$	ns
D. melanogaster ♀	Hybrid ♂	*D. melanogaster* ♂	Not comparable (see text)	
D. simulans ♂	*D. simulans* ♀	Hybrid ♀	ns	$p < 0.005$
D. simulans ♀	*D. simulans* ♂	Hybrid ♂	ns	ns

[a]Abbreviations as for Table I.

The PC measure shows much higher variability. From Table II, it can be seen that hybrid males are more acceptable to and/or more stimulated by *D. simulans* females than hybrid females. Unfortunately, data for *D. melanogaster* females could not be included in Tables I and II because in four runs only six copulations were observed and the calculation of MS and PC would have been meaningless. Thus hybrid males do well with *D. simulans* females, less well with hybrid females, and very badly indeed with *D. melanogaster* females. This last result is probably not based on female discrimination alone, because the males were extremely sluggish courters in this combination. Hybrid females copulate in a low frequency with *D. simulans* males, are intermediate with hybrids (although the difference is not significant), and accept *D. melanogaster* males most frequently, the difference between hybrid and *D. melanogaster* males accepted being significant at the 1% level. In fact, with regard to the PC measure, hybrid females appear as acceptable to *D. melanogaster* males as their own females, but MS is slower with hybrid females.

D. simulans males mate very slowly with hybrid females. In this case, our observations suggest that the males were again at least partly responsible for the result, because they were extremely inactive in this combination. There is no significant difference between *D. simulans* and hybrid males with *D. simulans* females.

Courtship Song and Wingbeat Frequency

The ipi's of *D. simulans* and hybrid males are almost identical (Table III, Fig. 1). The ipi of *D. melanogaster* males is significantly different from both ($p < 0.001$).

Table III. "Interpulse Intervals" in Courtship Song and Wingbeat Frequency in Flight of the Males of the Two Species and Their Hybrid

	Ipi ± SE	Wingbeat frequency ± SE (cycles/sec)
D. melanogaster	34.6 ± 0.4	218.3 ± 2.4
D. simulans	48.7 ± 1.2	252.7 ± 2.9
Hybrid	47.7 ± 1.2	240.6 ± 2.6

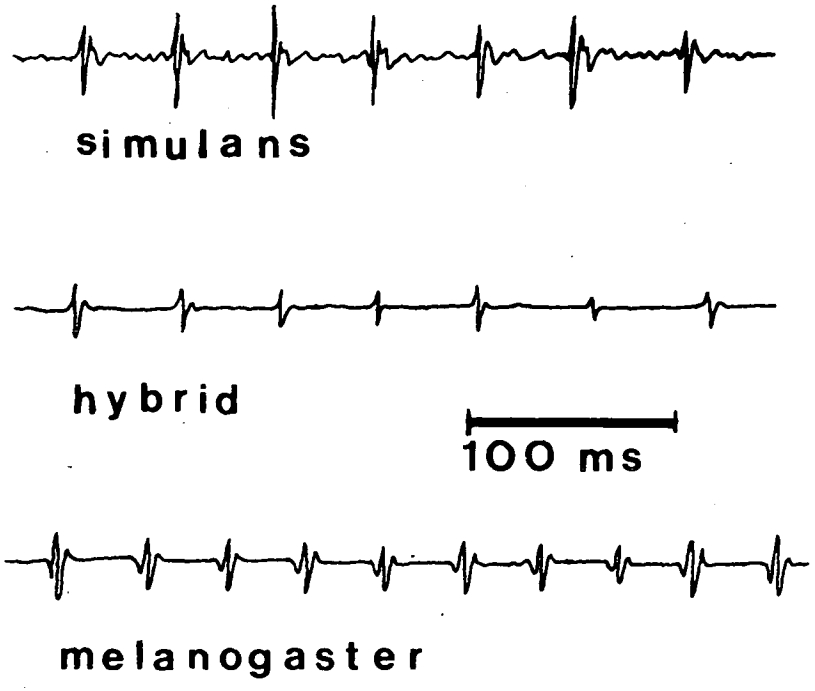

Fig. 1. Oscilloscope records of the songs of males of *D. simulans*, *D. melanogaster*, and their hybrid. Redrawn from a photograph.

Wingbeat frequencies of hybrid and *D. simulans* males are significantly different from each other ($p < 0.01$) and the hybrid frequency is indistinguishable from the midparent value for this trait.

DISCUSSION

Hybrid males are very readily accepted by *D. simulans* females, and we deduce that part of the reason for this is the similarity in ipi between hybrid and *D. simulans* males. Of course, it is possible that hybrid males are inheriting dominant or sex-linked genes from their *D. simulans* fathers which code for the sensory and/or neuronal wiring responsible for the reception and recognition of stimuli functioning as releasers of sexual behavior. In other words, hybrid males could be more stimulated by *D. simulans* females. The latter argument might be supported by the studies which have demonstrated the existence of female sex pheromones in *Drosophila* (Ehrman, 1969; Shorey and Bartell, 1970; Sloane and Spiess, 1971), although they have not conclusively been shown to act as stimulators of sexual behavior.

In fact, the quest for an answer to the question of which sex determines mating speed seems futile as long as one tries to tackle it using matings set up between different strains, mutants, or allopatric species. As our data show, males and females can be very fast with one partner and very slow with another. The courtship system of a population whose gene pool is more or less distinct is so intricate and so dependent on interactions between the sexes that any answer can emerge when males and females are used which have not had the chance of coadapting their communication system. This is in fact what has been found (e.g., Kessler, 1968; Elens *et al.*, 1973). Trivers (1972) has discussed this question in relation to the greater "parental investment" of females, which should make them more discriminating than males. To test this prediction, one would need to look at the respective contributions of the two sexes to mating speed within populations or between sympatric species.

Finally, the fact that *D. simulans* and hybrid males have nearly identical ipi's could be explained by two different modes of inheritance. Ipi could be a sex-linked character, or it could equally well be that *D. simulans* ipi-determining genes are dominant over the homologous *D. melanogaster* genes and distributed throughout the autosomes. We may note that dominance seems to be the rule rather than the exception for the morphology of the male genitals (Tsacas *et al.*, 1971). Nevertheless, these results certainly do not contradict sex linkage, as is found in *D. persimilis* and *D. pseudoobscura*.

The interesting difference in inheritance between ipi and wingbeat fre-

quency proves that the similarity between hybrid and *D. simulans* ipi is not simply due to similarity in their thoracic structure. Clearly, ipi has had an evolutionary past distinct from that of wingbeat frequency. This is not surprising in view of the fact that ipi probably was subjected to the very strong and specific selection pressures characteristic for traits functioning as ethological isolating mechanisms.

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