GENETIC ANALYSIS OF COURTSHIP BEHAVIOUR AND BIOLOGICAL RHYTHMS IN *DROSOPHILA*

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

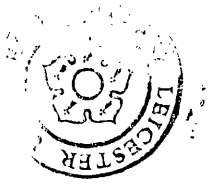
Mary Louise Greenacre BSc (Sheffield)

Department of Genetics

University of Leicester

May 1990

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346



ACKNOWLEDGEMENTS

There are many people whom I would like to thank for their help during my PhD. John Beckett of the Leicester University Computer Centre has been particularly helpful with statistics and computing. The SONGTESTER program of Justin Thackeray made statistical analysis of song rhythms almost pleasurable. I am indebted to Marcus Marvin, Peta, Bambos Kyriacou, Rudi Costa and Alexandre Peixoto for helping me to collect the results of Chapter 5. Keith Baker, Marguerite Lymn, Iris Hallam and Hilda Statham have heroically produced the vast amount of *Drosophila* food required for mating experiments, for which I am eternally grateful.

I should also like to thank my parents for providing moral and financial support and the SERC for my grant. Finally, the help and encouragement provided by Bambos Kyriacou has been much appreciated.

CONTENTS

Chapter 1

Introduction

Sexual reproduction is almost universal within higher animals. Thus, for many animal species, finding a suitable mate is of overwhelming importance if their genes are to be carried to the next generation. The female is usually the sex which invests most in reproduction and parental care (Halliday, 1978) and Bateman (1948) has argued that this will lead to "undiscriminating eagerness" to mate in males and "discriminating passivity" in females. Indeed, the males of many species go to quite surprising lengths to persuade females to mate, often utilising complicated rituals and displays.

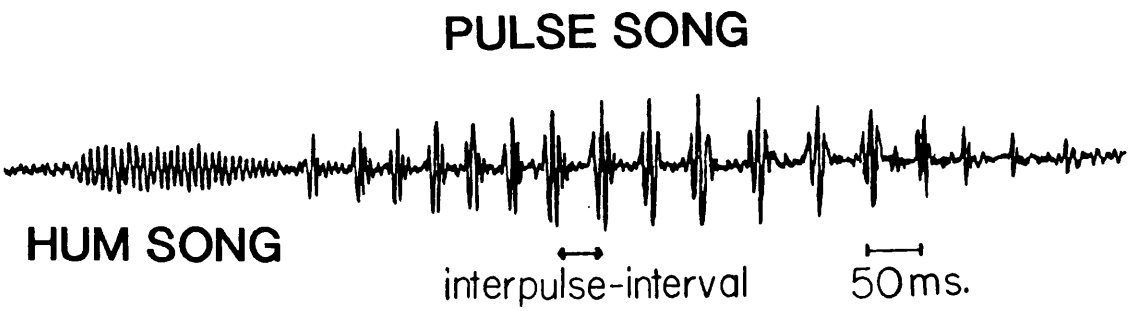
The courtship ritual of the fruitfly, *Drosophila melanogaster*, has been intensively studied (eg Spieth, 1952) and is well understood. Typically, the male orients towards the female and vibrates his wing, producing a species specific lovesong. He then licks the female's genitalia and may attempt to copulate. It is the lovesong which has aroused the most interest in geneticists.

In *D. melanogaster*, the song has 2 components, hum (sine) song and pulse song (Fig 1.1A). Hum song consists of a sinusoidal hum with a frequency of about 160Hz (von Schilcher, 1976a). Pulse song is composed of a series of pulses, with an interpulse interval (IPI) of between 28ms to 40ms (Ewing & Bennet-Clark, 1968). In 1980, Kyriacou & Hall discovered that the IPIs oscillate sinusoidally around values of about 28-40ms, giving a rhythm with a period of 55s to 60s. This was demonstrated by dividing the song into 10s bins and computing a mean IPI for each bin. When plotted against time, the means follow a typical sinusoidal pattern (Fig 1.1B).

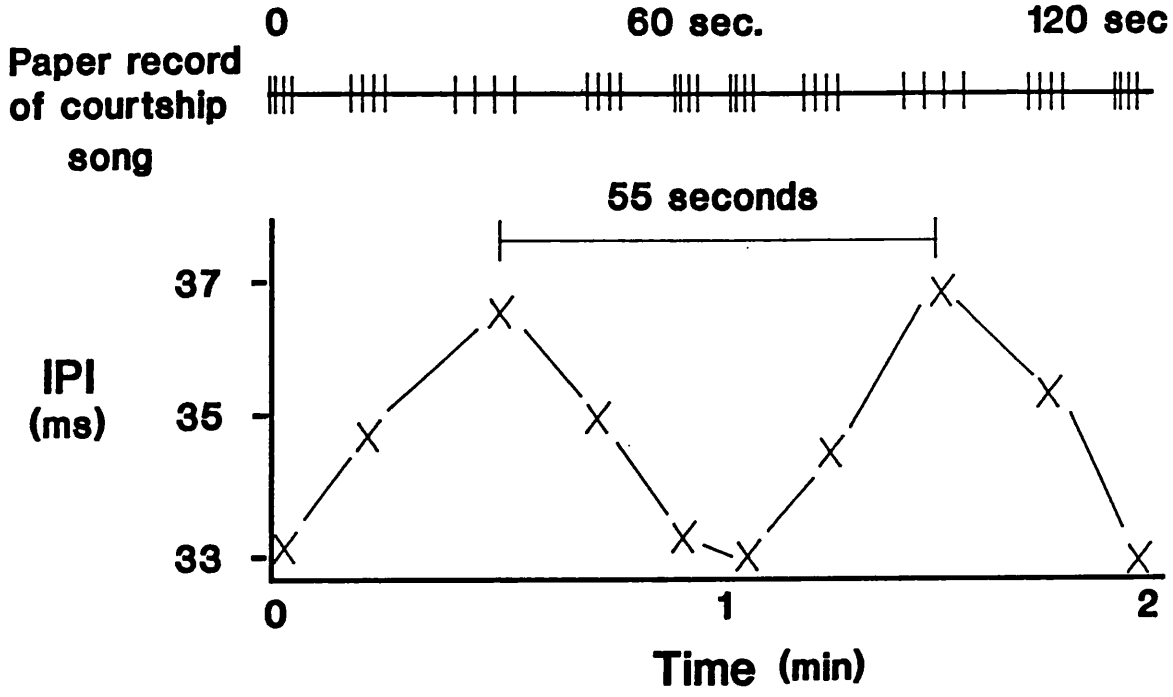
Of obvious interest is the genetical control of the differences in song characteristics between *Drosophila* species. Such interspecific differences are thought to be important for recognition of conspecific flies and thus maintain sexual isolation between species. Cowling & Burnet (1981) studied the songs of six sibling species in the *melanogaster* subgroup, *D. melanogaster*, *D. simulans*, *D. mauritiana*, *D. yakuba*, *D. erecta* and *D. teissieri*. They generated as many interspecific hybrids between these species as possible, and analysed the songs of the hybrids. The characteristics of these songs suggested that the genes controlling the IPI were located on the autosomes and that hum song was controlled by one or more sex linked genes. Interestingly, they found clear differences between the songs of five of the species, the only ambiguous one being that of *mauritiana*. Clearly, sexual isolation mechanisms should be strongest when it is likely that two species may interbreed, for instance if they are sympatric. *D.*

Fig 1.1 Components of the courtship song of *D. melanogaster*

A. Burst of song, including hum and pulse component



B. Diagrammatic representation of how a courtship song rhythm is obtained. The mean IPIs of every 10s of courtship song are computed and plotted, giving the "song rhythm".



mauritiana is found only in Mauritius, so interbreeding with other species under natural conditions is extremely unlikely. Thus a strong degree of sexual isolation is perhaps unecessary for this species. Conversely, *D. melanogaster* and *D. simulans* are sympatric, have different songs and a high degree of sexual isolation. Cowling & Burnet report that *D. simulans* has pulse song with a mean IPI value of 55ms. Other workers have reported a mean IPI value of 48ms for *D. simulans* (Ewing and Bennet-Clark, 1968); all agree, however, that the IPI of *D. simulans* is more variable than that of *D. melanogaster*. The song rhythm of *D. simulans* has a period of of about 35-40s (Kyriacou & Hall, 1980; 1986), whereas that of *D. yakuba* is around 70-80s (Thackeray, 1989).

Presumably, male flies produce song in order to stimulate females to mate. Von Schilcher (1976) showed that if females are pre-stimulated with hum song before being mixed with males, their copulation latency is shorter compared to females which have not been pre-stimulated. However, a similar effect is not observed when females are pre-stimulated with a constant 34ms IPI pulse song. When females are stimulated with the latter song in the presence of wingless males, however, they mate faster with this song than when they hear only white noise. Von Schilcher has suggested that females summate hum song, thereby steadily increasing their receptivity to a threshold level, but that pulse song acts instantaneously as a trigger to mating. Further experiments by von Schilcher (1976) showed that females stimulated with a 34ms IPI had shorter copulation latencies than females stimulated with a 48ms IPI. These experiments must, however, be viewed with caution as a large number of trials were required before marginal statistical significance was achieved (von Schilcher, 1989; Hall & Kyriacou, 1990).

The function of song rhythm has been investigated by Kyriacou & Hall (1982). They played artificial songs incorporating all combinations of a 35ms IPI, a 48ms IPI, a 55s rhythm and a 35s rhythm to *D. melanogaster* and *D. simulans* females. Perhaps not surprisingly, they found that *D. melanogaster* females "prefer" a song incorporating both *melanogaster*-like characteristics - a 55s rhythm and a 34ms IPI. Similarly, *D. simulans* females "prefer" a *D. simulans*-like song, with a 35s rhythm superimposed on a 48ms IPI.

Further work (Kyriacou & Hall, 1984) showed that copulation latency is reduced in females which have been pre-stimulated with rhythmic song oscillating around a 35ms IPI with a period of 55s. Thus, a rhythm is necessary for pre-stimulation with pulse song, but a constant 34ms IPI does not pre-stimulate females (von Schilcher, 1976;

Kyriacou & Hall, 1984). Therefore it appears that females can store rhythmic pulse song. In addition, Kyriacou & Hall (1984) showed that the memory and learning mutants, *dunce*, *rutabaga and amnesiac*, are not effectively pre-stimulated by a 55s/35ms song. Thus, the pre-stimulation effect appears to involve mechanisms of information storage and retrieval.

Given the possible short length of courtships in the wild (Ewing & Ewing, 1987), it is unlikely that females would have time to count IPI cycles. It is possible that females recognise the rate of change of the IPI about a certain value, and that the rhythm is the way in which males achieve a continuous IPI gradient. Alternatively, individual females may have their own preferred IPI value. Thus males singing with a constant IPI may stimulate fewer females than a male who produces many different IPIs. By singing rhythmically, a male can systematically vary his IPI production, thereby stimulating a larger number of females.

Courtship songs are by no means the only manifestation of biological rhythmicity in *Drosophila*. In common with most organisms living on the surface of the planet, *Drosophila* has a variety of behavioural and physiological patterns which are "tuned in" to the normal cycles of the Earth. This results in rhythms which are circadian (daily), circalunar (monthly), circannual (yearly) or ultradian (less than 12 hours) in period length.

Such biological rhythms are controlled by endogenous clocks and are not imposed on the organism by the environment, nor are they learned. One of the first observations of an endogenous rhythm was by De Mairan in 1729 who noticed that the daily leaf movements of *Mimosa* persisted in constant darkness (in Saunders, 1982). However, light and temperature cycles do act as external cues (zeitgebers) to entrain endogenous oscillators to a period of 24 hours. In the absence of such zeitgebers, the rhythms "free-run" and adopt their own natural period. For instance, when human experimental subjects are kept in social and temporal isolation facilities, their temperature rhythm and sleep/wake cycle drift later by one hour each day ie they free-run with a period of 25 hours (Winfree, 1987).

Many species have circadian rhythms which are not exactly 24 hours in length. It is easy to see that, under these circumstances, the clock must be reset each day by external cues in order that it keeps pace with the Earth's movements. At first glance, it may seem that evolution has failed to produce a sufficiently precise clock. However,

Pittendrigh & Daan (1976) have shown that it becomes more difficult to entrain to a 24 hour light/dark cycle as the animal's free-running period becomes closer to 24 hours. Thus, it may be of selective advantage to evolve circadian clocks whose periods deviate from 24 hours, as the circadian clock must be re-set each day in order to keep to a precise 24 hour cycle. This is achieved by the presence of a circadian rhythm in the sensitivity of an organism to a time cue. For example, light will induce a phase delay, a phase advance or no phase shift at all, depending on the subjective circadian time when the animal is exposed to the light. What is a phase shift? Consider an animal with a free-running activity cycle of 25 hours. In order to entrain to a 24 hour light/dark cycle, the animal's activity would have to begin earlier each day than would have been predicted from the free-running rhythm. This is a phase advance. A phase delay would occur if the activity was to begin later each day.

A phase-response-curve (PRC) can be used to predict entrainment by light-dark cycles or other zeitgebers. In a PRC the phase shift obtained from the administration of a light pulse is plotted against the circadian time at which the pulse was administered. PRCs to light pulses appear to follow a very similar pattern in most species (Moore-Ede, Sulzman & Fuller, 1982). The phase shift is usually instantaneous and and depends little on the length of the light pulses. Several transient cycles may occur before a steady state phase shift is obtained.

How does the above relate to natural entrainment by light-dark cycles? The daily light-dark and dark-light transitions (dusk and dawn) have been recognised as the major entraining features (Moore-Ede, Sulzman & Fuller, 1982). Each transition instantaneously phase shifts the rhythm and the net phase shift from each determines the total phase shift. For instance, an animal with a free-running period of 22 hours may undergo a phase delay of 3 hours at dusk and a phase advance of one hour at dawn, a net delay of 2 hours. This would resynchronize a 22 hour period to one of 24 hours. It has been shown that the largest phase delays occur in the early subjective night and the largest phase advances in the late subjective night.

Presumably, circadian rhythms have developed to ensure that behaviour and metabolism are appropriately timed with respect to the environment. Pittendrigh (1965) showed that populations of D. pseudoobscura raised in light-dark cycles emerge from the pupae in a rhythmic fashion with peaks close to dawn. Success in the act of

eclosion is greatest at this time (Saunders, 1982). The relative humidity of the air is at its highest then, and this may prevent the delicate wings of the newly emerged adults from drying out.

Biological rhythmicity has tremendous implications for clinical practice. For example, patients suffering from immobilisation following an accident excrete an increased amount of calcium during a few hours in the middle of the day (Moore-Ede & Burr, 1973). An antidemineralisation drug, oxymetholone, was found to be most effective if administered during this midday peak. The rhythm of oxymetholone effectivenes appears to be related to a rhythm of bone salt mobilisation, which peaks in the early morning. Drugs used in cancer chemotherapy are highly toxic, many showing a rhythm of toxicity. It is possible that a higher dose can be tolerated by the body at times of least sensitivity, thus perhaps increasing the chance of recovery (Moore-Ede, Sulzman & Fuller, 1982).

Certain disorders of sleep patterns and psychiatric illnesses are known to be related to circadian sleep-wake cycles. Many depressed patients show symptoms of early awakening and diurnal mood swings, and it is possible that they are phase-advanced with respect to the 24 hour environment (Wehr & Wirz-Justice, 1982). Sleep deprivation studies by these workers have suggested that a critical period occurs in the early morning, during which the patient must be awake if depressive symptoms are to be alleviated. If circadian rhythms in depressive patients are phase-advanced, then this critical period may occur earlier when they are still asleep. Kripke *et al* (1983) have hypothesised that light could be the effective stimulus during this period. Awakening depressed patients in the early morning and exposing them to bright white light alleviates depressive symptoms. A control treatment with dim red light shows only a small improvement in the patients' condition (Kripke *et al*, 1983).

A group of drugs, the benzodiazepines, are widely used in the treatment of insomnia and anxiety. These drugs are thought to work by potentiating the action of a neurotransmittor, γ-aminobutyric acid (GABA) (Turek & Losee-Olson, 1986). The suprachiasmatic nucleus (SCN) of the hypothalamus is thought to be the central pacemaker for many mammalian circadian rhythms (Turek, 1985) and may act by exerting control over the secretion of melatonin from the pineal gland. Melatonin can entrain the circadian oscillation of locomotor activity rhythm in rats to a 24 hour light/dark cycle (Redman *et al*, 1983). Both cell bodies and axons containing GABA are found in the SCN (Card &

Moore, 1984; Van den Pol & Tsujimoto, 1985). It seems, then, that the benzadiazepines may directly affect the SCN. Turek and Losee-Olson (1986) showed that a benzodiazepine drug can phase-shift the locomotor activity rhythm in golden hamsters. The phase-response-curve obtained demonstrates that the phase-shifting effects of the drug are dependent upon the circadian time of its administration. Such knowledge could be useful in designing an appropriate clinical regime for administration of the benzodiazepines to human patients.

Thus the study of biological rhythmicity has the potential for the alleviation of many medical or social problems eg jet lag and shift work. A good understanding of the anatomy and physiology of oscillating systems has been achieved (eg Turek, 1985; Saunders, 1982). However, little is known about the organisation of such systems or how they are assembled at the molecular level. Fortunately, the well documented genetics of *Drosophila* and other organisms are currently allowing genetic and molecular analyses of biological rhythmicity to proceed. It is hoped that this approach will reveal much information about the ticking of the biological clock.

In 1971, Konopka & Benzer isolated 3 mutations in *D. melanogaster* which drastically altered the normal 24 hour rhythms of eclosion and locomotor activity. Complementation tests revealed these mutants to be alleles of the same gene, the *period* (*per*) gene, located close to the tip of the X-chromosome. One mutant, per^{s}, was found to have a short, 19 hour rhythm. The per^{L1} mutant had a long, 28 hour rhythm and the per^{01} mutant was found to be arrhythmic. Konopka and Benzer also investigated the effect on the locomotor activity rhythm of having the 3 mutant and the wild type alleles in combination with one another in heterozygous females. The per^{L1} and per^{01} alleles were found to be recessive to the wild type, but the per^{s} allele is partially dominant, as per^{+}/per^{s} females have intermediate periods. The per^{01} allele is also recessive to the per^{s} and per^{L1} alleles; when the per^{s} and per^{L1} alleles were tested together, an intermediate period was produced, close to the normal value.

In addition to its effects on the eclosion and activity rhythms, the per gene also affects the ultradian rhythm present in courtship songs of *D. melanogaster* (Kyriacou & Hall, 1980). In parallel to their effects on the circadian rhythm, per^{s} shortens the song rhythm to a period of around 40s, the per^{L1} allele lengthens it to about 80s and the rhythm is seemingly abolished in per^{01} flies. Kyriacou and Hall also constructed males which were heterozygous for various combinations

of the *per* alleles by making diplo-X flies and converting them to males using the *transformer* mutation. Interestingly, the effects of heterozygosity on the song rhythm did not exactly parallel those on the circadian rhythm reported by Konopka and Benzer. The per^{S} allele was found to be dominant to per^{+}, the per^{L1} allele recessive to per^{+} and the per^{S}/per^{L1} combination again gives an intermediate phenotype. However, the song period is reduced in length when per^{01} is in combination with each of the other 3 *per* alleles.

The normal 24 hour period of the locomotor activity and eclosion rhythms can be altered by changes in dosage of the *per* locus (Smith & Konopka, 1982). A decrease in *per* dosage lengthens the period, whilst an increase shortens it. Thus per^{S} may be a hypermorph and per^{L1} may be a hypomorph. This could be due to a change in the activity of the mutant gene product or to a change in the rate of production of the wild type product. In fact, the former appears to be true; if per^{S} was simply an overproducer, then per^{S}/per^{+} females would have shorter periods than per^{S}/per^{-} (deficiency) females. However, this has been found not to be the case (Smith & Konopka, 1982). This was borne out by a theoretical study (Coté and Brody, 1986) which calculated that the per^{S} protein is a hypermorph with 34 times the activity of the per^{+} protein.

The *per* phenotype has been under constant study since the early 1980's. An important result has been the demonstration that per^{01} flies are not totally arrhythmic after all. Using more sophisticated analytical techniques, Dowse *et al* showed that per^{01} and per^{-} (deficiency) flies have activity rhythms with short and ultradian periodicities. Multiple periodicities are also common in these flies. Kyriacou & Hall (1989) have since observed that ultradian periodicities may be present in song rhythms. Further work has recently confirmed this view (Kyriacou & Hall, 1990b).

Since Konopka and Benzer's original EMS mutagenesis (1971), several new *per* mutations have been isolated. per^{02} and per^{03} (Smith & Konopka, 1982; Hamblen *et al*, 1986) are molecularly identical to per^{01}. per^{04} mutants show both ultradian and long period rhythms in locomotor activity and also have tendency towards hyperactivity (Hamblen-Coyle *et al*, 1989). The per^{L2} mutant (Smith & Konopka, 1982) is phenotypically very similar to per^{L1}. However, the per^{Lvar} (variegated) mutation, when homozygous in females or hemizygous in males, produces arrhythmic locomotor activity. When the mutation is uncovered by certain deletions, most flies are still arrhythmic, but some demonstrate long

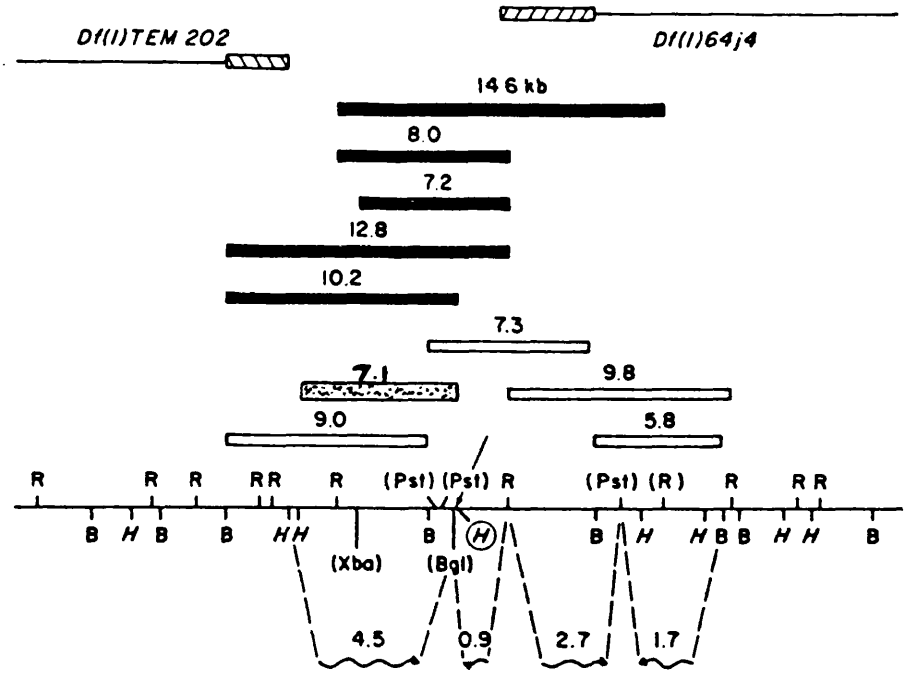
periodicities (Hall & Kyriacou, 1990).

The *per* gene has been cloned by two separate groups, from the Rockefeller University (Bargiello & Young, 1984) and from Brandeis University (Reddy *et al*, 1984). The *per* gene is located in the 3B1-2 region of the X chromosome (Smith & Konopka, 1981; 1982). The Rockefeller group cloned DNA from this region, via a chromosome walk from the nearby *Notch* locus; the Brandeis group utilised DNA which had been previously microexcised directly from the polytene chromosomes (Pirrotta *et al*, 1983). There are several chromosomal deletions and translocations in the vicinity of the *per* gene which are known to affect circadian rhythmicity (Smith & Konopka, 1981). By comparing RNA transcripts from these deletion and translocation strains to transcripts expressed from the cloned DNA libraries, both groups were able to identify several species of RNA which may have been transcribed from the *per* gene. These included a 0.9kb and a 4.5kb RNA message. The 0.9kb transcript was originally reported by Reddy *et al* (1984) to cycle during a light/dark cycle, although this was later found to be an artefact (Lorenz et al, 1989).

P-element mediated transformation of overlapping DNA fragments into arrhythmic per^{01} flies allowed both sets of workers to determine which fragments could rescue arrhythmicity. The Brandeis group (Zehring *et al*, 1984) identified a 14.6kb and an 8.0kb DNA fragment, both of which encoded the entire 0.9kb RNA transcript, as well as part of the 4.5kb transcript. In Bargiello *et al*'s study (1984), a 7.1kb DNA fragment, encoding all the 4.5kb RNA, was found to rescue arrhythmicity (see Figure 1.2). Subsequently, Hamblen *et al* (1986) confirmed that the 4.5kb RNA transcript was necessary and sufficient for the restoration of rhythmicity. However, the transformed flies described above showed incomplete penetrance for rhythmicity, with longer than normal circadian and courtship song periods. This problem was solved when a 13.2kb DNA fragment, carrying more information at the 5′ end of the gene, was used in transformations. The resulting flies had almost normal circadian and song periods (Citri *et al*, 1987; Yu *et al*, 1987b). Presumably, this upstream DNA contains regulatory sequences necessary for the correct expression of *per*.

Thus, the 4.5kb RNA transcript appears to be the *per* mRNA. However, this transcript is equally abundant in the *per* mutants, including per^{01}, compared to the wild type (Bargiello & Young, 1984). Interestingly, Baylies *et al* (1987) have shown that different lines of per^{01} flies transformed with the same 7.1kb DNA fragment have greatly

Fig 1.2 Molecular biology of the per locus - DNA cloned and RNAs expressed from the 3B1-2 interval of the X chromosome



The solid bars represent DNA fragments effecting rescue of arrythmicity. The open bars represent DNA fragments which do not recue arrhythmicity.

The dotted bar represents the DNA fragment from Bargiello et al's (1984) study which rescues rhythmicity.

The hatched bars represent deletion breakpoints, with the missing DNA to the right (TEM 202) or the left (64j4) of the bars.

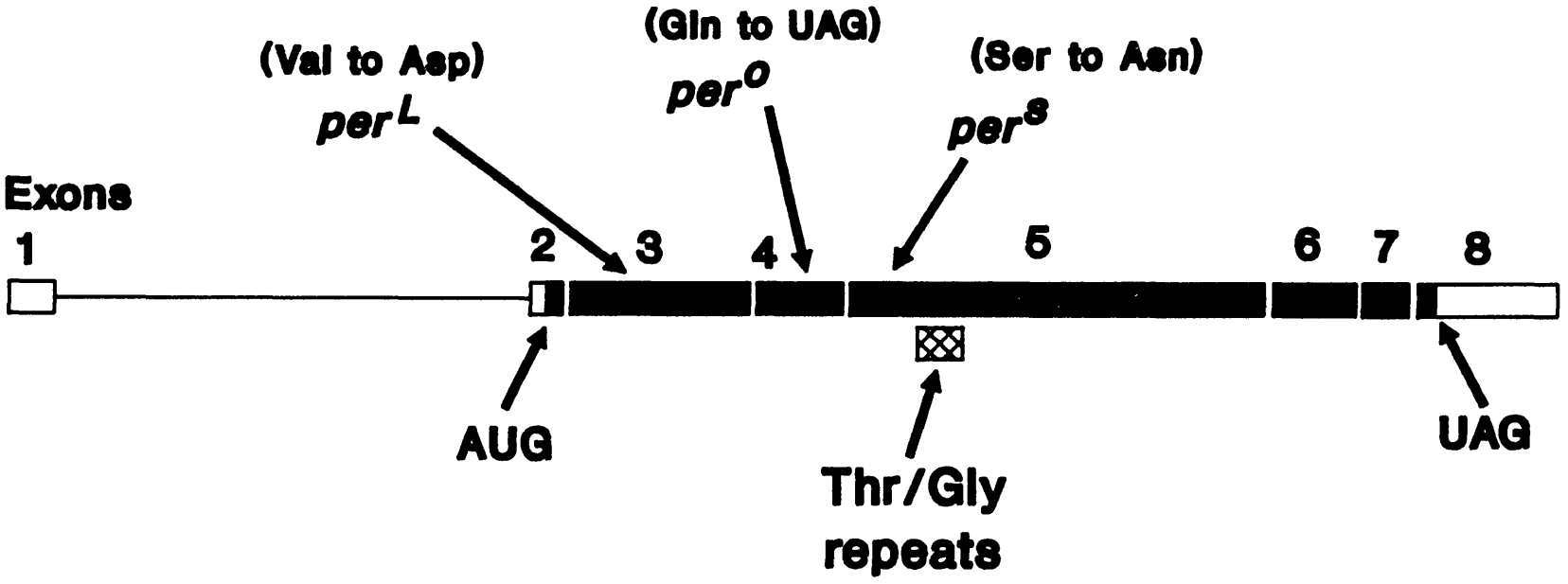
varying levels of 4.5kb RNA transcript. Furthermore, the abundance of the transcript is inversely correlated with period length, so that flies with the highest levels have the shortest circadian periods. This fits in with the work on *per* dosage as previously described (Smith & Konopka, 1982), suggesting that per^{s} and per^{L1} produce hyper- and hypoactive proteins respectively. The 7.1kb DNA fragment used by Baylies is missing some of the non-coding information at the 3′ end of the gene, implying that such sequences are necessary to regulate the abundance of the *per* transcript.

Citri *et al* (1987) have constructed complementary DNAs (cDNAs) for the *per* locus. Such cDNAs lack any intron information. Three types of cDNA were found, corresponding to three species of messenger RNA. The most abundant (70%) was designated Type A, with Types B & C having much lower levels of expression. The structure of Type A cDNA is shown in Figure 1.3. There are 8 exons; the first exon is non-coding, and the coding sequence begins in exon 2. The translated exons code for about 1200 amino acids, giving rise to a protein with a relative molecular mass of 127000. Type B cDNA has exon 5 and exon 8 split into two exons each; the division of exon 8 occurs downstream of the stop codon shared by Type A and Type B, so does not alter the putative protein sequence. The splicing event in exon 5, however, results in the deletion of 96 amino acids from the putative Type B protein compared to Type A. Type C does not splice out the three most 3′ introns, resulting in one very long exon. This produces a change in the reading frame, so that the sequence of the carboxy end of the Type C protein is entirely different from Type A. The cDNAs are capable of restoring rhythmicity to per^{01} transformants, but the rescue is weak, resulting in long periods and poor penetrance. This is possibly due to the absence of regulatory sequences in the introns, especially the large intron 1.

Exon 5 contains a sequence coding for a series of Threonine-Glycine (Thr-Gly) repeats, with 20 Thr-Gly pairs in the Oregon-R strain (Citri *et al*, 1987). The per^{s} mutation is also located in exon 5, causing a single amino acid substitution of serine to asparagine. A single amino acid substitution is also responsible for the per^{L1} phenotype, with a valine being replaced by aspartic acid. In per^{01}, a stop codon is produced, resulting in a truncated protein of around 400 amino acids (Baylies *et al*, 1987; Yu *et al*, 1987a). The intron/exon structure of *per* is illustrated in Figure 1.3.

The conceptual amino acid sequence suggests that the *per*

Fig 1.3 Intron/exon structure of the *D. melanogaster per* gene



filled boxes - translated exons

open boxes - untranslated exons

1 Kb

protein may be a proteoglycan (Jackson et al, 1986; Reddy et al, 1986). The Thr-Gly repeat is similar to a Serine-Glycine repeat found in the rat chondroitin sulphate proteoglycan. The serine residues of the rat protein can be glycosylated ie have have glycosaminoglycan (GAG) side chains attached, and serine is structurally similar to threonine. Biochemical studies (Reddy et al, 1986; Bargiello et al, 1987) have shown that the per protein is extremely large, becoming much smaller when treated with an enzyme which removes GAG side chains. However, there are some difficulties associated with the proteoglycan hypothesis, and these are summarised in Hall & Kyriacou (1990). Briefly, glycosylation can be disrupted in mammals when Ser-Gly repeats are altered to Thr-Gly repeats. In addition, the Thr-Gly region can be deleted without affecting the 24 hour period of the locomotor activity rhythm, and thus is clearly not vital for circadian rhythmicity (Yu et al, 1987b).

Proteoglycans are heterogeneous in both function and location. Thus, the homology of *per* to proteoglycans may not be particularly useful in characterising the function of clocks in general and the *per* gene in particular. A fundamental question relates to the organisation of circadian systems - is there a single master clock driving all observed rhythmicities or are organisms made up of a population of clocks? A master clock seems to operate in single-celled organisms, but higher organisms probably have large numbers of oscillators, controlling a number of behavioural and physiological rhythms (Saunders, 1982). How might a population of oscillators serve to produce stable rhythms relating to environmental conditions?

It has been suggested that biological rhythms may be based on several oscillators, each with a short, ultradian period, coupled together so that a longer period is produced (Pavlidis, 1969). Some evidence for this is provided by the phenomenon of the "splitting" of rhythms into 2 or more components which can free-run with different periods. When arctic ground squirrels (*Spermophilus undulatus*) are kept in conditions of constant light, the locomotor activity rhythm free-runs for several days and then splits into 2 components (Pittendrigh, 1960). A circadian rhythm in the frequency of compound action potentials (CAPs) exists in the eye of the mollusc, *Aplysia*. Jacklet & Geronimo (1971) showed that ablation of cells from the optic nerve of the eye reduces the period of the rhythm from circadian to ultradian, suggesting a population of interacting neuronal oscillators.

As already mentioned, per^{01} flies have short, ultradian

periods in their locomotor activity rhythms (Dowse et al, 1987). This suggests that the *per* gene may act by coupling ultradian oscillators to produce a circadian rhythm. Only when the *per* gene is deleted or non-functional can the effects of the ultradian oscillators be seen. It is possible that per^{S} and per^{L1} can alter periodicity by "tightening" or "loosening" coupling respectively. Interestingly, raising flies in constant darkness produces individuals with strong ultradian components in their locomotor activity rhythms (Dowse & Ringo, 1989). It is suggested that light may act to couple a population of ultradian oscillators into a composite circadian clock and that this clock therefore cannot be formed in individuals which have been deprived of light.

Weitzel & Rensing (1981) labelled wild type and per^{01} *Drosophila* salivary gland cells with a fluorescent dye which is useful in measuring changes in electrical potential across biological membranes. They discovered that a higher proportion of individual per^{+} cells showed a circadian rhythm of fluorescence and that the amplitude of the rhythm is higher in per^{+} cells than in per^{01} cells. Salivary gland cells are thought to be in constant communication with one another, so that if one cell has the information to express a rhythm, that information should be shared with other cells. The fact that fewer per^{01} cells express a rhythm may suggest that communication between per^{01} cells is less efficient (Bargiello et al, 1987).

Communication between salivary gland cells in *per* mutants and wild type flies was further investigated by Bargiello et al (1987). They found that Lucifer Yellow dye will diffuse readily between salivary gland cells of wild type, 3rd instar larvae, but does not easily move from cell to cell in per^{01} larvae. When injected into the cells of per^{S} larvae, the dye is transferred from cell to cell more rapidly than in per^{+}. These effects were paralleled when the strength of electrotonic coupling between salivary gland cells was also measured. Based on these results, Bargiello et al suggested that the coupling between cells is mediated by gap junction channels in the cell membranes. Gap junctions constitute the electrical synapses between cells, providing a means of communication. Interestingly, proteoglycans are involved in the formation of rat hepatocyte gap junctions (Spray et al, 1987), so it seems possible that the *per* proteoglycan product may be involved in mediating intercellular communication via gap junction. Unfortunately, it is not known whether the *per* product affects gap junction mediated communication in cells of *Drosophila* tissues other than the salivary

glands.

An alternative model for the action of *per* has been suggested by Konopka & Orr (1979), again involving cell membranes. They envisaged the establishment of an ion gradient via an ion pump during the subjective day, with the gradient being depleted during the subjective night through photosensitive ion channels. It is difficult to see how this model could be incorporated into the coupling hypothesis for the action of *per*.

Although individual cells may constitute clocks in their own right, it is likely that groups of cells or organs may have a particular clock function, particularly in higher organisms. As already mentioned, the suprachiasmatic nucleus is thought to be the central pacemaker in mammals. The anatomical location of such clocks in *Drosophila* was first investigated using the technique of genetic mosaicism. Genetic mosaics can be created by X-chromosome loss, resulting in a fly with male and female tissue which can be separately marked using visible mutations. Such flies (gynandromorphs) were originally used to fate-map surface structures (Garcia-Bellido & Merriam, 1969), but the technique has been extended to include internal tissue (Kankel & Hall, 1976). This has enabled the anatomical site of action of behavioural mutations to be located.

Such gynandromorphs showing mosaicism for the per^{s} mutation were produced by Konopka *et al* (1983). They found that the oscillator controlling the locomotor activity rhythm was located in the brain. In addition, they discovered some mosaics with one half of the brain of per^{s} genotype and the other half of per^{+}; these flies displayed both short and normal rhythms. This suggests that there are oscillators on both sides of the brain, which can function independently of one another. This putative brain location is supported by experiments in which a per^{s} brain was transplanted into the abdomen of an arrhythmic, per^{01} fly, conferring a short period rhythm on the recipient fly (Handler & Konopka, 1979). This experiment also suggested a diffusible, neurohumoral factor may be involved. However, molecular analysis of the *per* gene does not reveal any DNA sequence which could encode a peptide signal sequence which is required for secretion of a protein (Jackson *et al*, 1986; Citri *et al*, 1987). Thus it seems unlikely that the *per* protein is secreted.

The control of courtship song has been fate-mapped using mosaics, and suggests a thoracic focus (von Schilcher & Hall, 1979). Conceivably, the song rhythm could be produced by the resonant

properties of the thoracic box, be due to myogenic factors, or be determined by the nervous system. An experiment by Kyriacou & Hall (1985) suggests that the nervous system is involved. These workers showed that mutations affecting neuronal excitability seem to have the ability to stop the clock which controls the rhythm in the courtship song. The nap^{ts} (*no-action-potential temperature sensitive*) and $para^{ts}$ (*paralytic temperature sensitive*) mutations prevent the generation of action potentials, possibly by causing abnormalities in sodium channels (Wu & Ganetsky, 1980; Suzuki *et al*, 1971). Kyriacou and Hall (1985) allowed mutant males to begin singing to females and then rapidly immobilised them by transferring them to the restrictive temperature ($35^{o}C$) of the mutations. When the males recovered and resumed their courtship, it was observed that a phase-delay had occurred in the courtship song rhythm. This delay was proportional to the length of the heat pulse. Control flies were treated with carbon dioxide, which does not affect membrane excitability. These flies resumed their courtship song cycle approximately in-phase with the rhythm established earlier. Tetrodotoxin (TTX) blocks the sodium channels of wild type flies, producing a temperature-sensitive paralysis similar to that seen in nap^{ts} (Jackson et al, 1984). Thus it appears that the blocking of membrane excitability is capable of blocking the biological clock controlling song rhythms in *Drosophila*. However, TTX does not phase-shift the circadian rhythm seen in the firing of compound action potentials in the eye of *Aplysia* (Eskin, 1977). It is possible that the blocking of sodium channels may not affect the circadian clock of *Drosophila* in the same way as the song clock.

Genetic mosaics produced by Konopka *et al* (in Hall, 1984) have suggested a thoracic ganglion location for the song clock. Mosaics were produced which had a per^{s} brain and a per^{+} thoracic ganglion; such flies showed a short circadian activity rhythm and a normal song rhythm. Conversely, mosaic flies with a per^{+} brain and a per^{s} thoracic ganglion showed a normal activity rhythm and a short period song rhythm. This suggests that there are separate pacemakers for the courtship song rhythm and the locomotor activity rhythm. The pacemaker for the activity rhythm appears to be located in the head and the song clock in the thoracic ganglion.

There is much experimental evidence to demonstrate that the pacemaker controlling the locomotor activity rhythm in Orthopterans is located in the optic lobes (eg Saunders, 1982). Cutting the optic tracts (between the optic lobes and the rest of the brain) of *L. maderae*

causes the locomotor activity rhythm to be abolished (Nishiitsusuji-Uwo & Pittendrigh, 1968). However, cutting the optic nerves between the eye and the brain causes the rhythm to free-run in a light/dark cycle. It was concluded that the clock lies in the optic lobes and that connection to the compound eyes is required for entrainment to a light/dark cycle.

Interestingly, some optic lobe mutants of *D. melanogaster* show mild disturbances of rhythmicity. The double mutants, *minibrain/sine oculis* and *small optic lobes/sine oculis*, have their optic lobes reduced to less than 5% of normal. These flies show complex rhythmicity of locomotor activity, having either an unstable single rhythm or multiple periodicities occurring simultaneously (Helfrich, 1986). This further suggests that the locomotor activity rhythm is controlled by a population of oscillators.

Several studies have localised *per* RNA and protein to various *Drosophila* tissues. The *per* transcript and its protein product are abundantly expressed in the salivary glands of embryos and 3rd instar larvae (Bargiello *et al*, 1987). James *et al* (1986) noted that *per* RNA is enriched in adult *Drosophila* heads, although also detectable in other parts of the body. Furthermore, embryonic expression of the transcript is limited to the CNS and localised within the brain and ventral ganglia. It was suggested that expression of *per* RNA in the embryo is required for construction of tissues which are involved in expression of rhythmicity in later life, although it was recognised that *per* may play a role in the actual process of development (James *et al*, 1986).

Using antibodies directed against a *per* epitope and RNA hybridisation, Saez and Young (1988) localised *per* proteins and transcripts to the brain, the thoracic ganglia, the gonadal tissues and the ring gland complex of *Drosophila* pupae. In the adult, RNA and proteins were found in the eyes and optic lobes, as well as in the thoracic ganglia and gonadal tissue. Also using immunoreactive techniques, Siwicki *et al* (1988) reported a similar but less widespread pattern of *per* protein localisation. These workers found particularly strong staining in the lateral brain neurons, between the optic lobes and the protocerebrum, in both adults and pupae. They hence speculated that these neurons will be a particularly important part of the clock function.

Some of the major sites of *per* protein and RNA localisation can be correlated with known *per* functions. Genetic mosaics have suggested a thoracic location for the song clock (Hall, 1984) and *per*

protein and RNA is located in the thoracic ganglion. The localisation of per RNA and protein to the brain, including the optic lobes, fits in with the genetic mosaic location of the activity clock to the brain and with Helfrich's (1986) observation of disrupted rhythms in optic lobe mutants. Eclosion is controlled by the secretion of an eclosion hormone from the ring gland complex in pupae and this secretion is known to be controlled by a circadian pacemaker (Truman, 1985). The fact that per is strongly expressed in the ring gland suggests that this organ may function as a clock (Saez & Young, 1988). If this is true then it would appear that separate clocks control the circadian rhythms of eclosion and locomotor activity, as is the case in *D. pseudoobscura* (Engelmann & Mack, 1978).

Using an antibody directed against a per epitope, Zerr et al (ms submitted) observed a circadian rhythm in the intensity of immunoreactive staining in sections of the adult brain and visual system. In a 12:12 light/dark cycle, per^{+}, per^{s} and per^{L1} mutants showed a 24 hour rhythm, although the amplitude of the rhythm was rather shallow in per^{L1} flies. In constant darkness the free-running staining patterns of per^{+} were again about 24 hours, but those of per^{s} were nearer 20 hours - thus the cycling of the per product parallels the behavioural rhythms that it controls. This may suggest that the per protein can autoregulate, probably by involving oscillations in the rate of protein synthesis, as per mRNA also has a circadian rhythm of abundance (Hardin et al, 1990). The mRNA rhythm is phase-advanced with respect to the protein rhythm by approximately 6 hours (Hardin et al, op cit). Interestingly, the staining patterns observed by Zerr et al (ms submitted) back up the conclusion that the lateral neurons of the brain may be particularly important in the control of circadian rhythmicity (Siwicki et al, 1988). The staining patterns of per transformants which have rather weak and variable rhythmicity were also examined. It was discovered that the intensity of staining in the lateral neurons of individual flies was correlated with the strength of their activity rhythm.

The fact that per mRNA shows a circadian rhythm of abundance (Hardin et al, 1990) may cause problems for the hypothesis that per acts as a "coupler" between cells, with the "tightness" of the coupling affecting intercellular communication. If the per protein shows a circadian rhythm of abundance, then the level of communication may be expected to be more or less according to the level of the per protein. This is highly unlikely and could not explain per's effect on

non-circadian rhythms, such as the lovesong. However, there is likely to be a good reason why the *per* protein demonstrates a circadian rhythm. Work by Petersen *et al* (1988) shows that *per* may also be responsible for the pattern of activity within the 24 hour locomotor activity rhythm. It is possible that the cycling of the *per* protein may be important in controlling the pattern of activity.

The fact that the *per* gene is expressed throughout development raises an interesting question - is the *per* protein involved in the "building" of clock structures or is it merely necessary "on the spot" for the manifestation of rhythmicity? By fusing a heat-shock promotor to the *per* coding sequence, Ewer *et al* (1988) were able to investigate at which developmental stages the *per* gene was required for the expression of a locomotor activity rhythm in the adult. They discovered that *per* transcription is necessary only in the adult for rhythmicity to be obtained. When the gene is turned "on" in the adult, but "off" during earlier developmental stages, a locomotor activity rhythm is still seen in the adult. This suggests that the *per* gene does not contribute to the building of clock structures controlling the activity rhythm. The results are consistent with the idea that *per* acts as a coupler via gap junctional communication, as it would be necessary only on the spot in order to mediate rhythmicity.

Thus modern molecular and genetic techniques have considerably advanced the study of the *per* gene and its effects on biological rhythmicity. Similar techniques are currently being employed in other organisms, most notably *Neurospora crassa*, *Aplysia*, *Bulla* and the golden hamster. A circadian clock in *Neurospora* controls the timing of conidiation in a mycelium culture growing vegetatively (Sargent *et al*, 1966) and the period of the rhythm is altered by mutations at the *frequency* (*frq*) locus. Four alleles produce periods which are shorter than wild type and three produce periods which are longer than normal (McClung *et al*, 1989). The *frq* gene has been cloned and sequenced and, interestingly, was found to contain a Thr-Gly repeat region. The *frq* gene product, like that of *per*, has numerous potential sites for glycosylation (McClung *et al*, 1989).

Neurons in the eyes of marine molluscs such as *Aplysia* and *Bulla* are capable of generating circadian rhythms in the frequency of compound action potentials recorded from the optic nerve (Jacklet & Geronimo, 1971; Block *et al*, 1982). Siwicki *et al* (1989) showed that an anti-*per* antibody would stain the pacemaker cells in the eyes of *Aplysia* and *Bulla* (review: Jacklet, 1989). Furthermore, the anti-*per* antibody

identifies a protein in *Aplysia* which demonstrates a 24 hour rhythm of abundance, similar to that of the *per* protein.

Thus it appears that proteins involved in the control of biological rhythmicity from *Neurospora*, *Aplysia* and *Bulla* all bear some resemblance to the *per* protein. It is entirely possible that the genes encoding these proteins share a common ancestry and that some vital sequences have been conserved throughout evolution. Alternatively, convergent evolution could perhaps have produced a protein structural element necessary for the function of biological clocks. The antibody used by Siwicki *et al* (1988, 1989) was raised against the region of the *per* protein near the site of the per^{s} mutation. This region is highly conserved amongst *Drosophila* species (Colot *et al*, 1988; Thackeray, 1989), suggesting that it may be functionally important. Notably, the region does not include the Thr-Gly encoding repeat. Interestingly, an antibody against a less well-conserved region of the *per* protein fails to label the mollusc pacemaker cells. It is possible that only a small structural motif needs to be conserved within clock genes in order to provide a pacemaker function.

The study of the genetics of biological rhythmicity has now been extended to mammals. A mutation which shortens the period of the locomotor activity rhythm in golden hamsters has been found (Ralph & Menaker, 1988). The mutation, *tau*, occurs at a single, autosomal locus. Wild type hamsters have a free-running period of about 24 hours - animals heterozygous for the *tau* gene have periods of about 22 hours and homozygotes of about 20 hours. Subsequent work from Ralph & Menaker (reported in Mrosovsky, 1989) has shown that the period of the activity rhythm of mutant hamsters can be lengthened by replacing their suprachiasmatic nucleus (SCN) with wild type fetal SCN. This confirms the pacemaker role of the SCN. Attempts to unravel the molecular biology of this locus have begun (Mrosovsky, 1989) and it is hoped that the hamster will provide insight into the functioning of the biological clock in mammals. It will be interesting to see if the protein product of the *tau* gene bears any resemblance to that of the *per* gene. It is worth noting that DNA with some homology to *per* has been detected in another mammal, the mouse, (Shin *et al*, 1985) and this DNA appears to code for a Thr-Gly repeat. However, this homology may not be particularly informative, as the DNA sequence encoding the Thr-Gly repeat forms a hypervariable minisatellite in the human genome (Georges *et al*, 1988) and thus might be expected to be found at high frequency in other mammalian genomes.

It is perhaps surprising that the Thr-Gly encoding region appears to be of such importance in the *per* gene, as the repeat can be deleted without affecting the locomotor activity rhythm (Yu et al, 1987). However, the repeat does appear to be important in the control of the courtship song rhythm. When Yu et al (1987) transformed arrhythmic, per^{01} flies with *per* DNA from which the Thr-Gly encoding region had been deleted, the transformed flies demonstrated normal locomotor activity rhythms, but short, 40s song rhythms. Interestingly, a polymorphism exists at the Thr-Gly region, with different strains of *D. melanogaster* having either 17, 20 or 23 Thr-Gly pairs.

The *per* genes from *D. virilis* and *D. pseudoobscura* have been cloned and their DNA sequences compared to *D. melanogaster* (Colot et al, 1988). About 30% of the protein is non-conserved between the species, with the non-conserved sequences interspersed between the conserved areas. The longest conserved region contains the sites of the per^{s} and per^{L1} mutations, perhaps suggesting that the region has particular functional significance. The region in which the Thr-Gly region is encoded is particularly divergent between the three species, and the Thr-Gly repeat itself is non-conserved. The *D. virilis* protein has only two Thr-Gly tandem repeats, whilst the *D. pseudoobscura* protein has 7 Thr-Gly or Thr-Val repeats, interrupted by another sequence.

Thus, the *per* gene seems to contain sequences that are highly conserved between different species of *Drosophila* and sequences that are highly diverged. This is perhaps not surprising when one considers that that the *per* gene affects both a well-conserved behaviour (the circadian locomotor activity rhythm) and the highly variable courtship song rhythm (Kyriacou & Hall, 1980; Thackeray, 1989). The Thr-Gly region is particularly variable between species (Colot et al, 1988) and has an important function in the control of the courtship song rhythm (Yu et al, 1987). The song acts as a species recognition signal and the song rhythm appears to be important in allowing *Drosophila* females to recognise conspecific males (Kyriacou & Hall, 1982). Thus, the *per* gene, especially its Thr-Gly encoding region, may be important in sexual selection and speciation.

The locomotor activity patterns of *Drosophila* may also be important in maintaining isolation between species. Clearly, a species which is active only in the mornings will be behaviourally isolated from a species which is active only in the evenings. This type of isolating mechanism may be even more important in maintaining barriers to interspecific matings than sexual selection for species-specific

courtship characteristics. *D. melanogaster* and *D. pseudoobscura* both show circadian rhythmicity in their locomotor activity, but have different activity patterns (Petersen *et al*, 1988). *D. pseudoobscura* have shorter locomotor activity periods (around 23 hours) and are lacking the subjective morning peak of activity found in *D. melanogaster*. When transformed with a *pseudoobscura* wild type per gene, per^{+} (*melanogaster*) flies show activity patterns characteristic of *D. pseudoobscura*. Thus it seems likely that the *per* gene is responsible for the pattern of activity within a 24 hour period and hence may affect another characteristic important in maintaining isolation between species.

A somewhat contentious issue in the arena of isolation of species through mate choice is that of genetic coupling. Alexander (1962) proposed the theory of "genetic coupling", whereby the same gene or genes control the expression of both the male sexual character and female response to that character. Thus, a mutation in such a gene could produce a corresponding change in both male and female characters. Alternatively, the coordination between male and females characters may be achieved by the co-evolution of different genes controlling each character (von Helversen & von Helversen, 1975). A change in the male character would induce selection for a corresponding change in the female character, or vice versa.

It is vital that animals can distinguish between members of their own and other species. Sexual selection and mate recognition systems act to allow animals to choose a conspecific mate and thus have important consequences for speciation. Genetic coupling is an appealing hypothesis because of the rapidity in which a change in the male character could produce a corresponding change in the female or vice versa. This would enable an acceleration in the process of speciation. In contrast, the co-evolution hypothesis would produce a much slower adaptation of one sex to a change in the other. Of course, the distinction between genetic coupling and co-evolution may only be useful when one or a few major loci are involved. The practical consequences of the two alternatives may not be very different in a polygenic system.

The two alternatives have been tested by several workers using the hybridisation of closely related species and examination of male/female communication during courtship. Unfortunately, on the whole, this approach has not been particularly successful in distinguishing between co-evolution and genetic coupling. Interspecific hybridisation was used to investigate the genetic control of differences

in courtship song production and reception between *Drosophila melanogaster* and *D. simulans* (Kyriacou & Hall, 1986). Male hybrids from both reciprocal crosses have IPI (interpulse interval) values intermediate between the parental values (around 44ms). This suggests that the IPI is controlled by autosomal genes. The hybrid carrying the *D. simulans* X-chromosome has a *D. simulans*-like rhythm of around 40s, whilst the reciprocal hybrid has a *D. melanogaster*-like rhythm of around 55s, demonstrating that the species-specific IPI cycle is controlled by a gene or genes on the X chromosome. Hybrid females respond best to artificial songs which have an intermediate IPI value and an intermediate rhythm. However, they also mate well when presented with the two parental songs. This experiment hints at the possibility of genetic coupling, conceivably involving the X-linked *per* locus, but cannot provide any conclusive evidence.

Male production of courtship song and female reception have been examined in the Australian field crickets, *Teleogryllus commodus* and *T. oceanicus* (Hoy et *al*, 1977). Both species produce a species specific courtship song, which is genetically determined. Reciprocal crosses between *T. commodus* and *T. oceanicus* produce two types of male F1 hybrid which have distinctly different songs, suggesting that the gene(s) controlling song pattern are sex linked. When the song preferences of the F1 hybrid females were examined, the females were found to prefer the song of males from their own reciprocal cross. Hoy et al suggested that these results could be explained by genetic coupling, with selective inactivation of the paternal X-chromosome, as, of course, hybrid females are genetically identical. However, it has since been acknowledged that the result obtained could also be explained by co-evolution (Doherty & Hoy, 1985).

Ewing (1969) has suggested that sex linkage may be important in the evolution of mate recognition systems, as the effects of a recessive mutation will be seen in the heterogametic sex. Both examples described above involve sex likage, but this is not seen in the acoustic communication system of tree frogs (Doherty & Gerhardt, 1984). There is no difference between the call structure of male reciprocal hybrids between *Hyla chrysoscelis* and *H. femoralis*. Female hybrids prefer a hybrid call over that of *H. chrysoscelis*, but do not distinguish between hybrid calls and calls of *H. femoralis*. Again, this experiment does not distinguish between genetic coupling and co-evolution.

The courtship songs and responses of interspecific hybrids between the grasshoppers *Chorthippus biguttulus* and *Ch. mollis* have been

investigated (von Helversen & von Helversen, 1975: see review in Elsner & Popov, 1978). Female grasshoppers also produce a song in response to the male, signalling acceptance. Songs of the hybrids (male and female) differ considerably from one another, between reciprocal crosses and within individual crosses. Variation between reciprocal crosses could be explained by sex-liked differences in the males, but female F1 hybrids are genetically identical. Maternal inheritance is a possibility, but cannot explain the differences seen in progeny from within an individual cross. It is suggested that the song patterns are produced by two independent neuronal networks, corresponding to information from the two parental genomes. The song patterns are then superimposed in the hybrid and may interact unpredictably, producing the unexpected variation (von Helversen & von Helversen, op.cit.). F1 hybrid females respond weakly to the songs of hybrid males and prefer the songs of one or both parental species. Interestingly, some females prefer the song of one parental type, but respond with the song of the other parent. This experiment is taken as evidence for the co-evolution theory (von Helversen & von Helversen, op. cit.). However, if two separate output/receiver neuronal networks exist, then these networks may interact in an unpredictable fashion. Thus it would not be surprising that male song and female response were uncorrelated, as each arises by chance from the interaction of two independent systems (Doherty & Hoy, 1985; Butlin & Ritchie, 1990). Thus, this experiment does not provide unequivocal evidence for co-evolution.

At first glance, the work of Zouros (1981) demonstrates a lack of genetic coupling in the male and female mating behaviours of *Drosophila mojavensis* and *D. arizonensis*. The mating preferences of males and females carrying various interspecific chromosomal combinations were tested. Electrophoretic variants were used as chromosome markers. Male mating behaviour appears to be determined by the Y-chromosome and an autosome, whilst female behaviour is mainly controlled by loci on two other autosomes. The problem with this work (with respect to the study of genetic coupling) is that it

... does not focus on an individual aspect of mating behaviour (eg song or pheromones) which could form the basis of a mating preference. Asymmetries may exist in the relative importance of stimuli which determine the mating preferences of the two species (eg pheromones may be the most important aspect of courtship to *D. mojavensis*, but song may be more important to *D. arizonensis*). If this was the case, then

genetic coupling would not be seen even if it did exist. There is some evidence for asymmetries in the importance of courtship stimuli between species of the *D. virilis* group (Hoikkala, 1988). Removal of the antennae or aristae of females affect their mating propensities in different ways in different species. In *D. lummei*, for instance, the operation has little effect, but courtship is almost completely blocked in *D. montana* and *D. ezoana*.

Female European corn borer moths (*Ostrinia nubilalis*) produce a sex pheromone which is detected by olfactory cells on the antennae of males, producing a male flight response (Roelofs et al, 1987). Specific pheromone blends distinguish an E morph and a Z morph, which are behaviourally isolated in the field, but which produce viable, fertile hybrids. Female pheromone production was found to be controlled by a single autosomal gene with two alleles. Male pheromone detection is also controlled by a single autosomal gene with two alleles, whilst flight response was found to be sex linked (Roelofs et al, 1987). Unfortunately, it was not possible to tell if it was the same autosomal gene controlling pheromone production in females and reception in males. Thus, the evidence for or against genetic coupling is again ambiguous.

Alexander (1962) suggested that output and reception mechanisms in courtship communication may share a common neuronal network, specified by the same genes. This idea has often been taken to imply that a common neuronal network is a necessary prerequisite for genetic coupling (eg Doherty & Hoy, 1985; Bauer & von Helversen, 1987). Male grasshoppers (*Ch. parallelus* and *Ch. montanus*) produce a courtship song whose pattern varies with temperature. When at low temperatures, female grasshoppers respond best to songs recorded at low temperature. At high temperatures, they respond best to high temperature songs. The temperature dependence of the female song is the same as that of the male's (Bauer & von Helversen, 1987).

By selective warming of single ganglia in various parts of the grasshopper nervous system, Bauer & von Helversen (1987) tried to affect female song production and recognition. They found that song recognition depends upon the temperature of the head and song production upon the temperature of the thoracic ganglia. The work clearly demonstrates the existence of separate neuronal networks for song production and reception in female grasshoppers. Bauer and von Helversen appear to assume that song production in the male grasshopper will also be controlled from the thorax.

It is suggested (Bauer & von Helversen, 1987) that this work

provides strong evidence against the genetic coupling hypothesis. However, a more careful consideration of gene action reveals that a common neuronal pathway for song reception and production is by no means a condition for genetic coupling. Many genes have pleiotropic effects, both on behavioural and physical characteristics. There is no reason to suppose that a gene cannot encode components of more than one neuronal system. Indeed, the *per* gene provides an example, as previously mentioned. Genetic mosaic studies have shown that the *per* gene affects nerves in the thorax controlling the song rhythm and nerves in the head controlling the locomotor activity rhythm (Hall, 1984).

Thus, no clear evidence for the genetic coupling theory versus the co-evolution theory has been provided. It seems entirely possible that both scenarios may have been important during the course of evolution. This may also be the case in what is currently one of the most contentious issues among population and evolutionary geneticists - that of intrasexual selection by female choice. Most of the examples discussed so far have concentrated on interspecies selection, but it seems likely that mate choice also occurs within species. Sexual selection was originally proposed by Darwin to explain the existence of secondary sexual characteristics in males, which at first glance would appear to be deleterious eg bright plumage in some male birds can make them more vulnerable to predation. Despite Darwin's early interest in the subject, the idea of sexual selection by female choice was largely ignored by biologists until fairly recently. At first, evidence for sexual selection rested mainly with comparative observations (eg Poulton, 1890), but more modern studies have involved carefully planned experimental manipulations.

Perhaps the best known of such studies was performed by Andersson (1982) on the long tailed widow bird. He altered the tail lengths of various male birds by cutting out sections of the tail of some male birds and using these to artificially lengthen the tails of other males. He discovered that males with the longest tails were the most successful in mating and those with the shortest tails were the least. The notion that differences in mating success may be due to the quality of the territory held by the male, or his ability to defend it, were discounted. This experiment, then, appears to provide clear evidence that females prefer males with longer tails.

What possible mechanisms are there for the evolution of such female preferences? In 1930 Fisher proposed his so-called 'runaway process of sexual selection' whereby an inherited male character with a

slight natural selective advantage might become the object of an inherited female mating preference. The sons of these females will inherit the preferred character and hence are more likely to mate and reproduce than males without the character. In addition the daughters inherit the female mating preference. This goes on and on, with the mating preference and the preferred character advancing together. Unless checked by some other evolutionary factor, the rate of advancement will increase geometrically.

Several genetical models for Fisher's theory have been proposed. O'Donald (1980) suggested that the preferred character might be controlled by alleles at one locus and the preference by alleles at another locus. In this model, increase in the preference would be geometrical at first but would then slow down. In addition, it suggests that an initial natural selective advantage for the character is not essential. In this system equilibrium would be reached when the disadvantages of the character to viability began to outweigh the advantages of gaining a mate. In other words, there would be a negative genetic correlation between male viability and male mating success.

Fisher's theory depends upon the idea that the secondary sexual characteristics of males, and females' preferences for them, can be inherited. This has been demonstrated in a series of experiments with ladybirds. The two spot ladybird, *Adalia bipunctata*, is polymorphic for a melanic and a non-melanic form. Majerus et al (1982) showed that in a mixed population more melanic than non-melanic males find a mate. By artificially selecting females which mated with melanic males and allowing only these females to produce the next generation, they increased the preference for melanic males from 20% to 50% in three generations. These experiments were subsequently extended (O'Donald & Majerus, 1985; Majerus et al, 1986) to ten generations and estimates of heritability were consistant with the hypothesis that female preference is controlled by a single, dominant gene.

Fisher's theory predicts that ever-increasing expression of the preferred male character will occur until it becomes so extreme so as to be more disadvantageous to survival than beneficial to mating success. Using an experimental technique similar to Andersson's (1982), described earlier, Pape Moller (1988) artificially shortened or elongated the tails of the monogamous swallow, *Hirundo rustica*. It was found that males with longer tails obtained mates more quickly than males with short tails and also enjoyed increased reproductive success. However, subsequent work (Pape Moller, 1989) has shown that males with

elongated tails catch smaller, less profitable prey. In addition, males with elongated tails decrease their natural tail length after moulting and suffer a subsequent decrease in reproductive success in the next breeding season. Of course, the decrease in tail length after moulting could have been due to the effects of experimental manipulation, rather than to the effects of a long tail *per se*. However, the fact that males with long tails suffer an impairment in foraging ability does seem to suggest that male tail ornaments in the swallow incur costs in the viability of the animal.

A balance between sexual and viability selection appears to have been achieved in the three spine stickleback (*Gasterostens aculeatus*). Female sticklebacks prefer to lay their eggs in the nests of red throated males in preference to the nests of non-red males (Semler, 1971). This behaviour is not due to male-male competition - when females are given a choice between two genetically non-red males, one of which has an artificially reddened throat, the "red throated" males are again more successful. However, red throated males suffer for their increased reproductive success. When both red and non-red males are present, a predator, the rainbow trout, will strike preferentially at red males (Moodie, 1972).

This counterbalance between natural and sexual selection predicts that geographical variation in natural selection against a preferred character should lead to a parallel variation in preference (Lande, 1982). This prediction has been tested in the Trinidadian guppie, *Poecilia reticulata* (Breden & Stoner, 1987). Adult male guppies differ in the extent and brightness of their colour patterns and males from high predation populations tend to be less bright than males from low predation populations. This difference in brightness seems to be caused by selective predation of bright males (Endler, 1980). Naive female guppies from high and low predation populations were tested for their sexual preferences for models of bright or drab male guppies. Aerators were placed near the bright models so that brightness and movement were correlated. Male movement and brightness both contribute to male mating success, as well as to increased risk of predation (Breden & Stoner, 1987; Breden, 1988). It was found that females from low predation populations prefer bright male models and females from high predation populations prefer drab models. If high predation females were to mate with bright males then they would experience a loss of fitness in terms of higher predation of their bright male offspring. Thus, their preference has evolved as a correlated response to natural

selection acting against bright males.

This work has been criticised by Endler (1988) on the grounds that the models used were much larger than real guppies, so that females from high predation populations could have avoided the models because they were frightened of them. Low predation females are likely to be less wary of possible predators. However, Breden (1988) has pointed out that females responded to the models in a similar fashion to those tested with live males, and so were unlikely to perceive the models as predators.

Some biologists have felt uncomfortable with the idea that female preferences could come about through Fisher's process of "runaway sexual selection" and have sought a more adaptive explanation. This has led to the "good genes theory" whereby secondary sexual characteristics influence female mate choice because they indicate viability in other respects. In other words, females acquire "good genes" to pass onto their offspring by mating with males possessing these characteristics. Of course, females may choose to mate with males who have something to offer other than their genes, such as territory or parental care. The selective advantages of choosing such a mate are obvious.

Male *Colias* butterflies demonstrate a courtship flight, and persistance in flight increases a male's chance of mating (Watt et al, 1986). Flight depends on a narrow range of body temperatures and hence on thermoregulation, in which a glycolytic enzyme, phosphoglucose isomerase (PGI), is important. There are several different alleles at the PGI locus in *Colias* which confer different effects on enzyme kinetics. Some genotypes can maintain fuel supply to flight more effectively than others (Watt et al, 1986). It seems likely that males with kinetically better PGI genotypes should better sustain courtship flight and hence increase their mating success. Watt et al examined female choice for different PGI genotypes and found females mated more frequently with males with kinetically better genotypes. Of course, this observation could result from male/male competition, rather than from female choice. However, older *Colias* females are more discriminating than young females and, interestingly, the preference for kinetically better males is even more marked in older females than in young ones (Watt et al, 1986). These results suggest that *Colias* females select males with "good genotypes" as mates.

According to the good genes theory, females should increase their fitness by exercising mate choice. Partridge (1980) compared the viability of larvae of females which had been allowed to choose their

own mates to the viability of the larvae of those which had not. She found that the former were more competitive in the scramble for food than the latter. This seems to suggest that there is additive genetic variance for this fitness component, and also that females choose mates with 'good' genes for this character. Unfortunately in this experiment it is impossible to tell whether females have actually chosen a mate or whether in fact males have outcompeted each other for females.

An increase in the fitness of females which have exercised mate choice may be seen in pheasants (*Phasianus colchicus*). These animals are sexually dimorphic, with males possessing spurs on their legs. Spur length is positively correlated with male mating success and viability (von Schantz et al, 1989). Von Schantz et al showed that in natural conditions, females select males with long spurs. A similar result was obtained when spur length was experimentally manipulated, suggesting that other variables perhaps normally correlated with spur length are not the target of female preference. In addition, there was no correlation between the number of females selecting a particular male and the quality of his territory. It is possible that the effect of spur length could be due to male-male competition, but again no correlation between spur length and social dominance was observed. Females which mated with a long spurred male had a higher reproductive success, suceeding in raising a larger number of chicks. Thus it appears that females can increase their own fitness by selecting mates with long spurs. Proponents of the good gene theory would suggest that spur length is an indicator of viability in another, as yet unknown, respect.

The work on pheasants described above would appear to support the good genes theory. Males possessing long spurs have a high viability as well as increased attractiveness. Fisher's theory suggests that increased attractiveness should be correlated with a decrease in viability - a trade-off between natural and sexual selection. In addition, by exercising sexual selection, female pheasants increase their fitness, presumably because their offspring inherit traits which increase their viability from their father. However, this experiment does not conclusively prove that there is a genetic component in the increased viability in the chicks of long-spurred males. It is possible, for instance, that females give a larger amount of care to chicks from a mating with a preferred male. Burley (1988) has shown that this may occur in zebra finches.

Developing the good genes theory, Hamilton and Zuk (1982)

have suggested that some secondary sexual characteristics may serve to advertise freedom from parasitic infection. Females will choose to mate with such males because their offspring would inherit genes promoting parasite resistance. Hamilton and Zuk assume that parasite resistant males will be brighter or have longer tails etc and that parasite resistance is heritable. The theory predicts that sexual selection for characters indicating parasite load will be particularly important in species which are prone to parasite infection. Hence, the prevalence of such characters should correlate positively with the extent of parasite load across species. This has been investigated in North American and European passerine birds (Hamilton & Zuk, 1982; Read, 1987). The work is subject to much debate (Zuk, 1989; Read & Harvey, 1989; Cox, 1989; Hamilton & Zuk, 1989) but as yet, no conclusion has been reached about the importance of parasite infection in the evolution of sexual selection.

One of the main problems with the "good genes" theory is that populations at equilibrium under constant selective conditions are not expected to display any heritability for fitness (Fisher, 1958). If there is no genetic variance for fitness in the population then clearly choosing between one mate and another cannot result in increased fitness of the progeny. A lack of genetic variance would also be a problem for Fisher's runaway selection theory. If there was little genetic variance for the male character, then there would be little leeway for the character to develop in the manner predicted by Fisher. However, although the overall heritability of fitness should be zero, it is now thought that individual components of fitness might have some genetic variance (eg Jones, 1987). This heritability could be maintained by changes in the environment (and hence in selection pressure) or by mutation and migration. There are large difficulties in measuring fitness heritabilities, mainly in distinguishing between environmental and genetic variance. A series of separate experiments have examined several components of fitness such as egg to adult viability, egg laying ability, longevity and male mating success (Mukai, 1983; Mackay, 1986). They found that some of these components retain a fairly high level of genetic variance, which would allow the good genes theory to operate.

Cyclical changes in the environment (eg seasons) may result in ever-changing selection pressures so that a particular genotype may have different levels of fitness at different times. This would allow for genetic variance in fitness components. Relating to the Hamilton-Zuk theory, host/parasite relations are cyclical in nature; a

gene conferring resistance to a host will increase in frequency until it is countered by a gene producing increased virulence in the parasite. There is some evidence in ring-necked pheasants (*Phasianus colchicus*) that parasite resistance can be inherited. When one group of chicks was brought up in a hygienic environment, fed with an anti-parasite drug, and another group raised in unhygienic conditions, more of the latter died, as expected. However, the offspring of the unhygienic group had increased resistance to parasitic infection, suggesting that there had been selection for resistance in the previous generation (N Hillgarth, University of Oxford. In Pomiankowski, 1989).

The argument between proponents of Fisher's runaway selection theory and the good genes theory seems no closer to a solution. As described here, there is experimental evidence for various facets of both theories, making it impossible to deny the importance of one or the other. Thus the intertwined fields of sexual selection and genetic coupling are still the subjects for much discussion. The genetics of mate choice are central to the problem and yet the organisms used in most studies are not well-suited to genetical research. More precise and carefully planned genetic experiments may shed some light on the topics under discussion.

The well understood genetics of *Drosophila* make it an ideal candidate for such studies. The courtship song has been extensively studied and its individual components analysed (eg Ewing & Bennet-Clark, 1968; Kyriacou & Hall, 1980). It is known that courtship song affects female mating response and hence is important in sexual selection (eg von Schilcher, 1976; Kyriacou & Hall, 1982). Thus it is possible to focus directly on a very precise behavioural element which is important in sexual communication and hence in sexual selection and evolution.

Courtship song in *Drosophila* is also of interest because it demonstrates a biological rhythm. The *per* gene affects the song rhythm and other biological rhythms in *Drosophila* and hence is of interest to evolutionary biologists, chronobiologists and neuroethologists. The projects described in Chapters 6-9 of this thesis have attempted to further elucidate the role of the courtship song of *Drosophila* in sexual selection. A selection experiment has been performed in an attempt to select genes important in the control of female preferences for aspects of the courtship song. This experiment may also reveal a possible function for the production of IPI cycles by the male. In addition, genetic and temperature coupling with respect to the courtship song have been examined. Chapters 3 and 4 investigate the effect of clock

mutations, both at per and other loci, on the courtship song. The results reveal interesting neurogenetic and evolutionary aspects of how these genes determine song and perhaps circadian oscillators. Chapter 5 further investigates the effects of these mutations on development time, another phenotype recently shown to be under clock control (Kyriacou et *al*, 1990a).

Chapter 2

Materials and Methods

2.1 STOCK MAINTENANCE

All stocks were maintained in one-third pint milk bottles on a food medium of oatmeal-agar-treacle, seeded with a few drops of aqueous yeast solution. Unless stated otherwise, cultures were always kept in a light-dark cycle of 12 hours dark and 12 hours light, with lights-on at 9am, at a temperature of either 18°C or 25°C. Parents of flies which were to be used for experiments were removed from the bottle after 5 days to prevent the overcrowding of progeny.

2.2 VIRGIN COLLECTION FOR MATING EXPERIMENTS

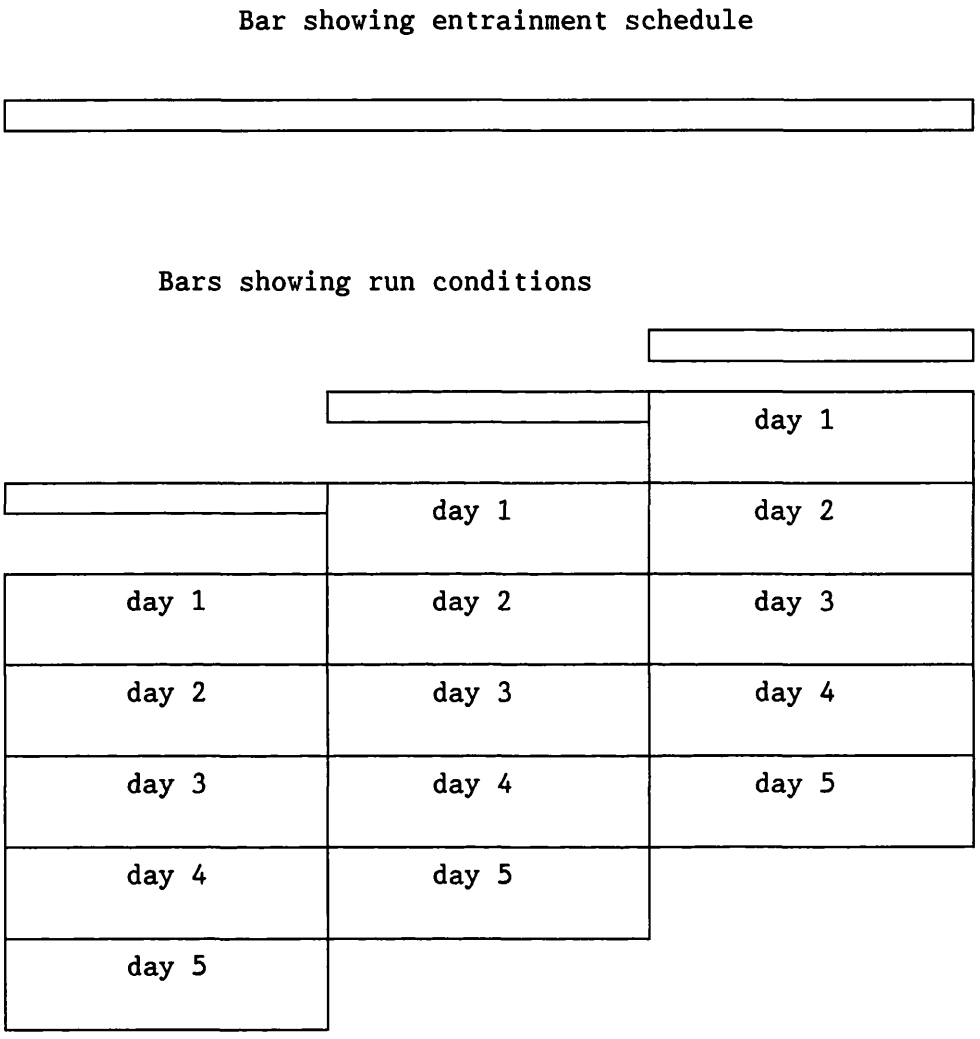
It was essential for all mating experiments that the females used were virgins, as once a female has mated, she will not mate again for several days. A female will generally remain virgin for up to 8 hours after eclosion at 25°C. Two virgin collections were made per day - one in the early morning and one in the early evening. To ensure virginity in the morning collection, bottles were transferred to 18°C overnight.

Flies were collected under CO_2 anaesthesia and stored in vials (2.5cm diameter, 10cm in height) at a density of 9 to 13 flies per vial. Vials in which females had been stored were carefully checked before experimentation to ensure that no larvae were present, and hence that the females were indeed virgin. Only rarely were females found to be non-virgin, and such females were discarded. All flies collected for subsequent experimentation were stored at 25°C unless stated otherwise.

2.3 LOCOMOTOR ACTIVITY RECORDING

Flies were collected within one day of eclosion and entrained in a light-dark cycle of LD12:12, at 25°C. At 3 or 4 days of age, the flies were aspirated into glass tubes (8cm long, 3mm internal diameter) containing about 1.5cm of sugar food (100g sucrose, 20g dried yeast, 14g Sterilin agar per litre, 0.2% Nipagin fungicide). Each tube contained only one fly. The end of the tube containing the food was sealed with Nescofilm to prevent the food from drying out and the other end was plugged with cotton wool. Only male flies were used, as egg laying by females can result in rapid deterioration of the food and hence early

Figure 2.1 ACTIVITY PLOT - A program to triple plot locomotor activity data to produce an actogram (after Thackeray, 1989)



The data in each 30 minute bin is represented by one of four shades, according to the number of events in that bin - white (few events), light grey, dark grey and black (many events). Each day's data is drawn as a row of 48 boxes, with each box representing one half hour bin. The data is triple plotted (see diagram above), with one day's data plotted underneath the preceding day's. In the bars showing entrainment and run conditions, a white rectangle means lights on; a black rectangle means lights off.

death of the fly. Monitoring of activity was always undertaken in free-running conditions ie constant darkness (DD) with no external cues to indicate the time of day. After being placed in the monitoring apparatus, the flies were allowed to acclimatise for 24 hours in the dark before recording of activity was begun. Activity was continuously recorded for 6 or 7 days.

The activity monitoring apparatus consisted of an infra-red emittor/detector system. The tubes containing the flies were placed between the emittor and detector; an event was recorded every time the fly broke the beam. Events were stored in a computer (Commodore PET) in successive time bins of 30 minutes duration.

The activity data thus obtained was analysed using the Flymon program (P G Caryl, 1985, unpublished). This uses an autocorrelation procedure (Chatfield, 1984), whereby data from bins a certain distance apart (called the "lag") are correlated. The lag used here ranged from 1, where bin 1 and bin 2, bin 2 and bin 3, bin 3 and bin 4 etc were correlated, to 100, where bin 1 and bin 101, bin 2 and bin 102 etc were correlated. The Flymon program also calculates whether the correlation at each lag is significant; the lag with the highest positive correlation represents the strongest periodicity in the data (Chatfield, 1984).

A further computer program, ACTIVITY_PLOT, was written by J R Thackeray (PhD thesis, 1989) to allow visual inspection of activity data. A diagram and explanation of the output from this program is shown in Fig 2.1.

2.4 COURTSHIP SONG RECORDING AND ANALYSIS

2.4.1 Song recording

Male flies whose courtship song was to be analysed were collected within one day of eclosion and stored in vials at 25°C, in a 12L:12D cycle. Wild type flies were stored at a density of between 5-10 flies per vial. Mutant flies, which were generally reluctant to sing, were stored singly; this can have the effect of increasing the amount of song productio (Hall & Kyyriacou, 1990). The song was recorded when the male was 4 or 5 days old.

The recording equipment consisted of a small, circular perspex cell, with a mesh underside into which one male & one or two females were introduced without anaesthesia. The cell used for recording wild type

songs had dimensions of 19mm diameter x 8mm deep. A smaller cell than usual (10mm diameter x 4mm deep) was used for mutant males in order to encourage them to court the female. The circular shape of this small cell allowed the flies to run around the walls of the chamber, so the small size of the chamber did not interfere with their courtship pattern (see Kyriacou & Hall, 1989; Hall & Kyriacou, 1990). The females were one or two day old virgins with their wings removed in order to reduce background noise. The cell was placed in a foam lined box with its mesh underside over a condenser microphone (an Insectovox from Brandeis University - Fig 2.2). Courtship songs were then amplified and recorded on a Revox tape recorder for about 6 minutes onto reel-to-reel tape. A "picture" of the song was obtained by tracing the recording onto light sensitive paper (Kodak Linagraph direct print, Type 1895) using an oscillograph. The song was first filtered (below 150 Hz and above 900 Hz) to remove extraneous noise.

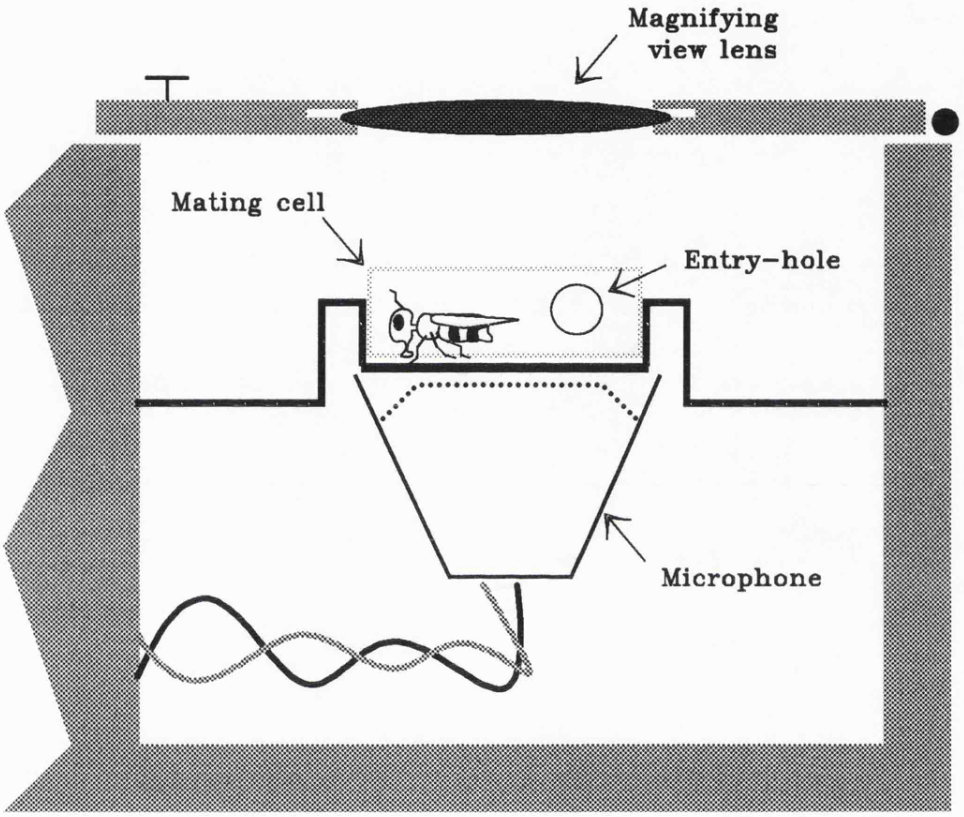
The song was analysed by dividing it into 10s "bins" and measuring the distances between pulses (interpulse interval or IPI) using a ruler. For a courtship with a mean IPI of 35ms lower and upper cut-off values of 15 and 65ms were set respectively. These were adjusted according to the value of the mean IPI of individual courtships. For example, a courtship with a mean IPI of 40ms had cut-off values of 15-80ms. Bins with less than 15 IPIs were not included in the analysis. Courtships with more than one third of the mean IPI points missing were discarded.

2.4.2 Statistical analysis

Three methods of assessing the statistical significance of songs were employed - a non-linear regression analysis using the BMDP3R (Dixon, 1985) program and two independently derived spectral analyses, one which used a CLEAN algorithm (Roberts *et al*, 1987) and one which was a modification of a standard Fourier analysis (van den Berg, University of Groningen, unpublished).

The CLEAN spectral analysis estimates missing data points by a procedure approximating to a least squares interpolation. The highest peak or frequency from the spectral analysis is taken as the cycle length or period. Sometimes, the 2nd peak of the spectrogram is used, when the 1st peak gives a value of more than half the length of the song. This occurs most frequently when the data points of a song rise systematically during the courtship. Songs with this characteristic are known as

Fig. 2.2 Schematic diagram showing cross section through Insectavox (after Thackeray, 1989)



The mating cell used for courtship song recording is shown within the Insectavox unit (Gorczyca and Hall, 1987), positioned over a condenser microphone.

"climbers". On analysis of climbers with CLEAN, the highest peak obtained often corresponds to a very long cycle. This cycle represents the upward trend in the data, which may be considered to be part of a very slowly oscillating sine wave. The 2nd peak value is also used when a period of less than 20s is obtained. This is the nyquist value ie twice the value of the bin length of 10s. An artifact of spectral analyses is that they will occasionally find the highest frequency in the spectrogram to be around the nyquist frequency.

The van den Berg program is a basic Fourier analysis, but differs from CLEAN in that missing data points are omitted from the analysis and are not estimated. However the phase of the data points is maintained (Hall & Kyriacou, 1990). As with CLEAN, the value of the 2nd peak of the spectrogram is taken as the true cycle length when the 1st peak gives a value of less than 20s or a value of more than half the length of the song.

The BMDP3R program (Dixon, 1985) requires four input parameters in order to run. These are mean IPI, cycle amplitude, starting point or phase of the cycle and cycle length. An additional parameter is required for analysis of "climbers". The first three parameters are obtained from visual inspection of the data; cycle length is obtained by first running the data through the CLEAN and van den Berg spectral analyses and taking the highest peak as the cycle length. If there is a disagreement between the periods obtained from CLEAN and van den Berg, both are tried in the BMDP3R analysis, with the period producing the best fit (measured by the highest F ratio) taken as the correct cycle length. The BMDP3R nonlinear regression analysis ignores any missing data points.

The total sum of squares (SS_T) is computed from the difference between the mean and the observed data points. By a process of iteration, BMDP3R estimates the sine wave of best fit and calculates the residual sum of squares (SS_R) from the difference between the predicted sine wave and the observed data points. The SS_R is then subtracted from the total sum of squares to give the sum of squares accounted for (SS_{AC}) by the sine wave. The SS_{AC} can then be used to obtain an F ratio which represents a signal-to-noise ratio (Kyriacou & Hall, 1989). If the F ratio is significant according to F tables commonly used in an analysis of variance, then the song under investigation can be said to have a significant rhythm.

F tables designed for use in an analysis of variance were used by Kyriacou & Hall (1980) to operationally define "weaker" rhythms (ie

ones with a small F-ratio) from "stronger" ones. This approach allowed them to separate per^{01} songs which have much weaker cycles from the other *per* variants (Kyriacou & Hall, 1989). A more stringent analysis has been performed by Kyriacou & Hall (1989) where they examined the F-ratios obtained after fitting sine waves (using a van den Berg analysis) to computer generated random songs where the IPIs fluctuated randomly about a mean IPI (see Section 2.4.3). They observed that 5% of random songs had F-ratios of 4.8 or higher (see Kyriacou & Hall, 1989, Table V, p855). Under a null hypothesis that no rhythms are present in the data, the minimum F-ratio for significance at the 0.05 level should be 4.8. However, this assumes that all periodicities from 20s to infinity are scanned and that no linear trends (ie "climbers", giving very long periodicities) are present in the randomised songs. If more limited periods are scanned (see Kyriacou & Hall, 1989) the F-ratio required for significance drops considerably, in fact to a value very close to that found in the F-tables.

Consequently, the F-tables give a good indication of the strength of a rhythm, as long as it is remembered that arrhythmic songs are being defined operationally. There is at present no method to decide whether a rhythm found in a time series analysis containing gaps is statistically significant or not. Thus the use of the F-ratio provides a reasonable alternative method.

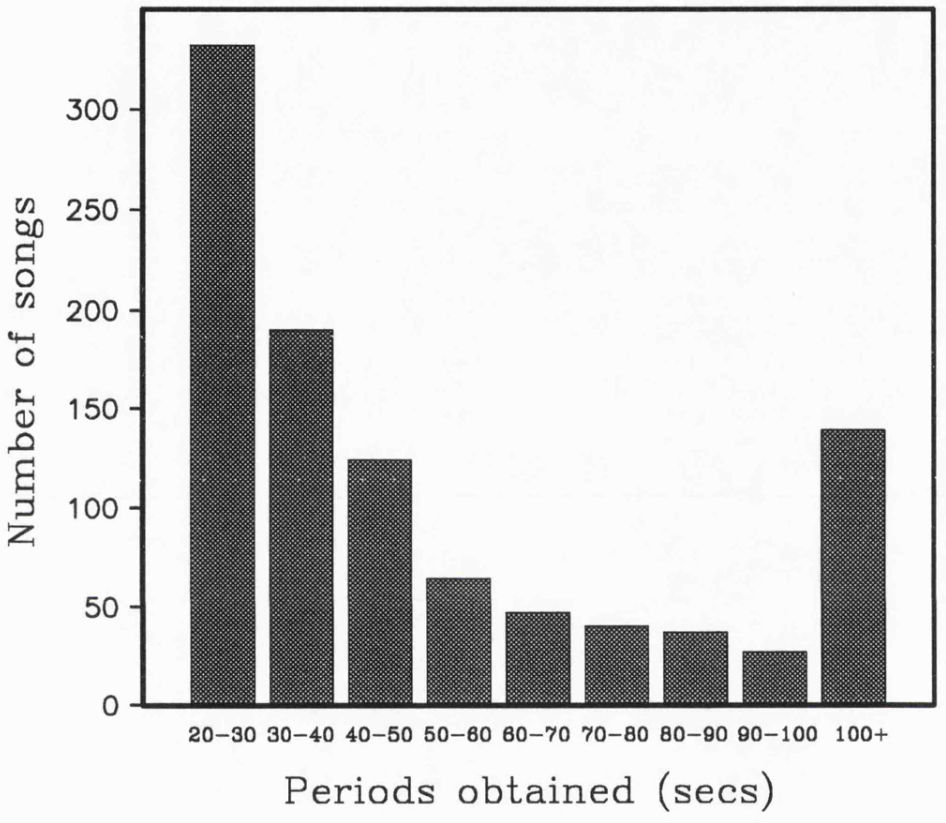
When analysing "climbers" with BMDP3R, there is a danger that a significant F ratio will be found simply because the SS_R obtained from a climbing sine wave (or, indeed, any climbing line) is almost certain to be small in comparison to the SS_T. This would lead to a large SS_{AC} (SS_T - SS_R) and hence a large F ratio. In fact a large F ratio could arise through fitting any line through such data.

For this reason, it is necessary to check that the best linear or curvilinear fit is not so good a fit as a "climbing" sine wave. This is done by calculating the SS_R after fitting the best line. The difference between the residuals obtained from the line versus the "climbing" sine wave (SS_Rstraight - SS_Rsine) can be used to calculate a further F ratio. If the F ratio is significant, it implies that the sine wave is a better fit to the observed data than is the best line.

2.4.3 Random song simulations (see Kyriacou & Hall, 1989)

8 sets of 1000 songs were artificially generated using a sub routine from the NAG library (Thackeray, PhD thesis, 1989). Each song

Fig.2.3 Distribution of periods obtained from a van den Berg analysis of 1000 random songs



The parameters used in generating the random songs used in this analysis were mean IPI = 35ms, sd = 2 and N = 25 (5 missing bins).

Data from Kyriacou & Hall (1989)

contained N=30 data points, each point representing an IPI mean. Each point fell randomly about the mean, with various means and standard deviations being employed. Further songs were simulated in which 5 out of the 30 data points were missing (N=25) in order to mimic natural songs which often have IPI means missing. The 5 missing data points fell at random.

Each of these random songs was then analysed using both CLEAN and the van den Berg analysis and the distribution of the highest peaks in the spectrogram obtained (Kyriacou & Hall, 1989; Thackeray, 1989). The distribution of the periods obtained from each of the 8 sets of random songs were very similar to one another. Thus, altering the number of IPI points, the mean or the standard deviation does not appear to much alter the periods obtained. In addition, the CLEAN and the van den Berg analyses produced very similar results (Thackeray, 1989). The distribution of periods arising from a van den Berg analysis of a set of random songs with a mean IPI of 35ms, a standard deviation of 2 and N=25 is shown in Fig 2.3. It can be seen that the majority of the cycle lengths from the random songs fall in the 20-40s range ie towards the nyquist frequency of 20s.

2.5 MEASUREMENT OF DEVELOPMENT TIME

2.5.1 Collection of eggs and measurement of rate of egg hatching

40 pairs of flies of mixed ages (1-3 days) were placed in a one-third pint milk bottle, with an egg laying plate suspended fom the top. The plate contained a special egg laying medium (yeast, 1% acetic acid, agar, 0.2% nipagin fungicide), coloured pink with neutral red dye, and spread with a yeast paste also coloured pink. The pink colour enabled easier identification of eggs. Flies were allowed to acclimatise in these bottles for 3 days before collection of eggs. The egg plates were changed 3 times per day during this period. Flies were maintained at 25^{o}C on a reverse light/dark cycle (12L:12D) ie light during the night and dark during the day as previous experience has shown that egg laying is increased under these conditions (C P Kyriacou, pers comm). After 3 days, the male flies were removed from the bottles.

The following day, eggs were collected for experimentation. Three collections were carried out; one from 9-11am, one from 11am-1.30pm and one from 1.30-4.30pm. New egg plates were placed in the bottles at

the beginning of the collection period and removed at the end. Changing of all the egg plates of all genotypes was accomplished within 5 minutes.

Eggs were then carefully picked off the plates using a mounted needle and placed in rows on a clean agar plate which was not covered with a yeast paste. The plates were maintained in the reverse light/dark cycle, except during scoring, and at 25°C throughout. One hundred eggs of each genotype were collected from the 4.30pm collection; a varying number of eggs were collected at 11am and 1.30pm. Scoring of the number of eggs hatching was begun 18 hours after the first collection (ie at 5am the following morning) and continued until the afternoon of the following day. Hatching was scored as often as possible (about every half to one hour) during periods in which many larvae were emerging; scoring was less frequent when few larvae were emerging.

Female flies are able to retain eggs for several hours before laying. If this were to occur in this experiment, then some eggs would be considerably older than others in the same collection. This has hopefully been avoided by regularly replacing the old egg plates during the acclimatization period; evidence has shown (Roberts, 1986) that females do not normally retain eggs when fresh, clean medium is available for laying.

2.5.2 Measurement of pupation and adult emergence times

Eggs from the 4.30pm collection were also used to examine pupation and adult emergence times. Ten eggs were scooped up using a drinking straw and transferred to a vial containing a standard amount of food medium (see Section 2.2, p31). 200 eggs of each genotype (250 of one genotype) were collected. The vials were then placed in a light box consisting of a large, wooden box, illuminated with a strip light. The level of illumination, the photon fluence rate (pfr), was measured as 100 mol$\mu m^{-2}s^{-1}$. Normal laboratory lighting has a pfr of 1.5μmol $m^{-2}s^{-1}$. Thus the vials were maintained in constant bright light at a temperature of 25°C.

The number of larvae pupating was scored every 3 or 4 hours for 24 hours after the onset of pupation. After this period, the number pupating was scored less frequently for another 2 days. The number of adults eclosing in the vials was similarly measured.

2.6 SELECTION FOR FEMALE SONG PREFERENCES

A song simulator was used to generate artificial fly song. This machine could be set to produce either a rhythmic song, or a song with a constant IPI (see Kyriacou & Hall, 1982). A comparison of artificial song and natural song is shown in Fig 2.4.

2.6.1 Experiment 1

The selection procedure was as follows:

1. Male and female virgin flies from the base population were collected as described in Section 2.2 (p31) and stored at a density of 10 per vial for 4 or 5 days. The wings of the males were removed using watchmakers forceps under CO_2 anaesthesia; this prevented them from singing. The day before experimentation, all flies were transferred without anaesthesia to clean food vials.

2. 40 females and 40 males with their wings removed were placed in a mating chamber (5 x 5 x 4cm) with a fine mesh underside and top. This apparatus was placed above the output speaker of the song simulator. The sexes could be kept separate until it was necessary to mix them.

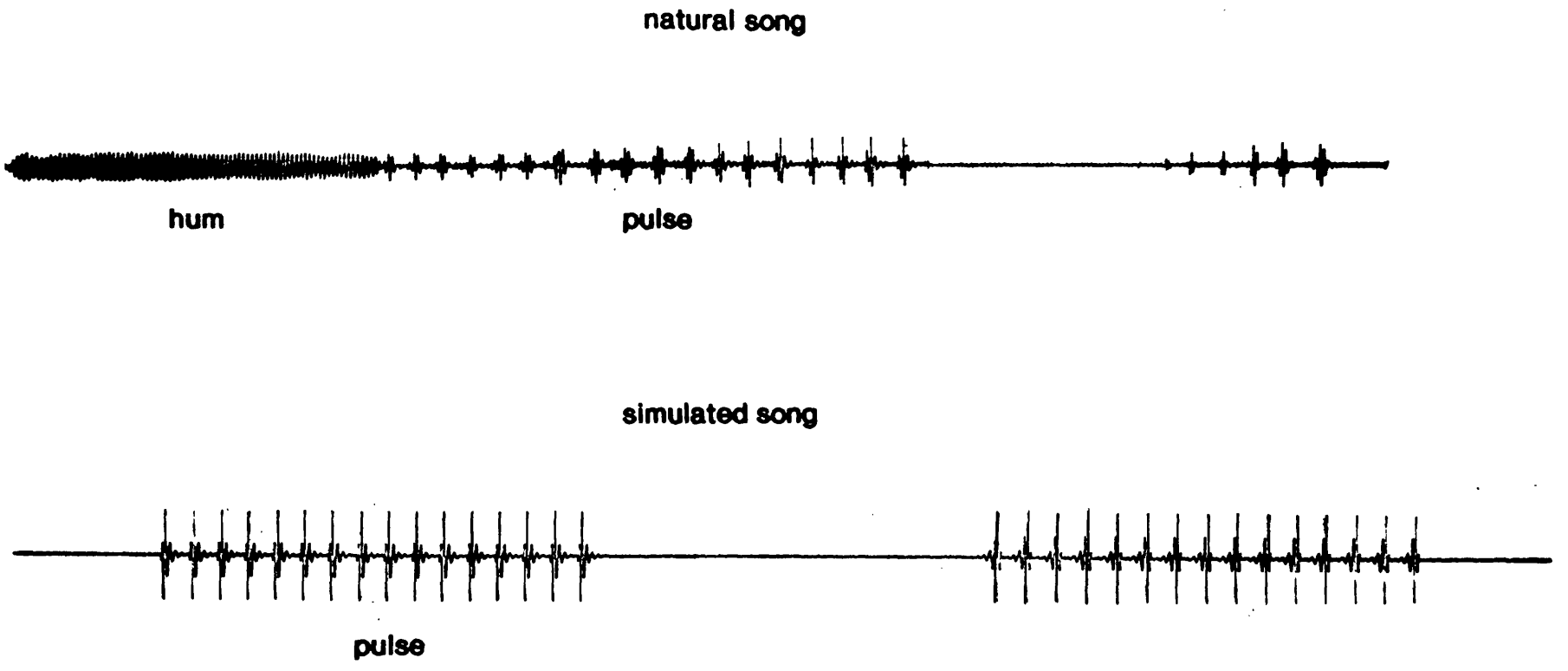
3. The song simulator was set to produce a song with a constant 40ms IPI, with a pattern of 0.5s of song followed by 2s of silence. This pattern was similar to the natural song pattern of the base population.

4. The first 7 pairs to copulate were removed rapidly with an aspirater and the male shaken off the female before insemination could occur. This was done in order to prevent any genes from the males being selected. The females were then placed in a vial with a single male taken at random from the base population. These flies produced the next generation, G1.

5. The procedure was repeated, again using a song with a 40ms IPI. Thus two selection lines selected at a 40ms IPI were obtained. These were called the HiA and HiB lines.

6. The procedure was repeated twice, this time using 30ms IPI as the stimulus. The 2 selection lines thus obtained were called the LoA and LoB lines.

Fig 2.4 Comparison of natural song and simulated song



No hum song is present in the simulated song

7. Two control lines were also set up, called ConA and ConB. These were obtained by again mixing 40 males and 40 females and selecting the first 7 to mate. This time, however, the wings were not removed from the males and the females were allowed to hear natural song. The males were again shaken off the females before insemination, and the females remated with males taken at random from the base population.

8. In subsequent generations, the selection procedure was repeated exactly, except that females were taken from the current generation of selected flies. Hi line females were selected with 40ms IPI song, Lo line females with 30ms IPI song, and control females with natural song. Males for the selection procedure were always obtained from the base population in order to minimise the possibility of assortative mating of females with their own selection line. The 7 selected females were placed in a vial with a single male taken at random, and allowed to produce the next generation, G2, G3, G4 etc. This time, however, the male was not taken from the base population, but from the same generation of the same selection line as the female. Any genes controlling female choice will also be present in males, even though they may, of course, have a completely different function or no function at all.

2.6.2 Experiment 2

For reasons which will be discussed later, it was found necessary to start the selection experiment again, changing the procedure slightly in order to prevent selection of 'fast-mating' genes. This was achieved by playing 30ms song to Hi line females for 5 minutes and discarding any that mated. There was then 1.5 minutes of silence whilst the IPI on the simulator was adjusted to 40ms IPI. Any females mating during this time were discarded. Subsequently, 40ms IPI song was played to the remaining females, and the first 7 to copulate were selected. The procedure was repeated with the Lo line females, this time playing them 40ms IPI song for 5 minutes, discarding those which mated and then playing the remainder 30ms IPI. Hopefully, this would remove (a) those females who preferred the 'wrong' song and (b) those females who would mate quickly whichever song they heard. The control lines again heard natural song; any females mating in the first 6.5 minutes were discarded and the first 7 to mate after this time were selected to produce the next control generation.

2.6.3 Testing female IPI preference

In order to test whether or not the selection process had changed female IPI preference, 20 females from each line were mixed with 20 males from the base population, in the same mating chamber as previously described. A song with a constant 30ms IPI was played to them for 20 minutes and the time of copulation of each fly noted. This procedure was repeated using a different set of 20 females from each line; this time a 40ms IPI song was played.

If the selection process had worked, then it would be expected that Hi line females (selected at 40ms IPI) would mate faster when played 40ms IPI song than when played 30ms IPI song. Conversely, Lo line females (selected at 30ms IPI) should mate faster with an IPI of 30ms compared to a 40ms IPI. Control flies should show no sustained preference for either type of song.

In addition to the control lines, ConA and ConB, mentioned earlier, an additional control of unselected, base population females was also used in the test procedure.

2.7 GENETIC COUPLING - FEMALE SONG RHYTHM PREFERENCES

2.7.1 Generation of simulated song

The song simulator was used to generate three types of artificial song - one with a 55s rhythm, one with a 40s rhythm and one with a 80s rhythm. All three songs had a mean IPI of 35ms and a cycle amplitude of 5ms ie the individual IPIs varied between 30-40ms, following the cyclical pattern of a sine wave. The simulator was set to produce a song pattern of 0.5s song, followed by 2s of silence.

The 3 songs were then recorded onto reel-to-reel tape, oscillographed onto photographic paper (see Section 2.4.1, p32) and analysed to check the accuracy of the song produced. Unfortunately, it was not possible to set the simulator to begin the song at a particular point in the song cycle. It is possible that the starting point of the cycle may influence female preference, so it was decided that each song should start at the "trough" - the lowest point in the IPI cycle. The song pattern consisted of bursts of pulses lasting 0.5s, followed by 2s of silence. The mean IPI of the first few bursts were calculated; the burst with the lowest mean IPI was the first trough of the cycle. This

burst was located by listening to the tape and following the song bursts on the oscilloscope and on the trace on the photographic paper. Once the trough had been located, all preceding song was wiped off the tape, so that the song cycle now began at the trough. See Figure 2.5.

2.7.2 Experimental Procedure

1. Female virgins were collected from the stocks under investigation as described in Section 2.2 (p31). All flies were 4 or 5 days old on the day of the experiment. Males from the Oregon-K stock were used throughout and had their wings surgically removed to prevent them from singing. All flies were transferred to clean food vials the day before experimentation. The experiment was performed at 25°C.

2. 25 females and 25 males were placed in a mating chamber as described in Section 2.6.1 (p38). This apparatus was placed above the output speaker attached to an amplifier and tape recorder (Fig 2.6).

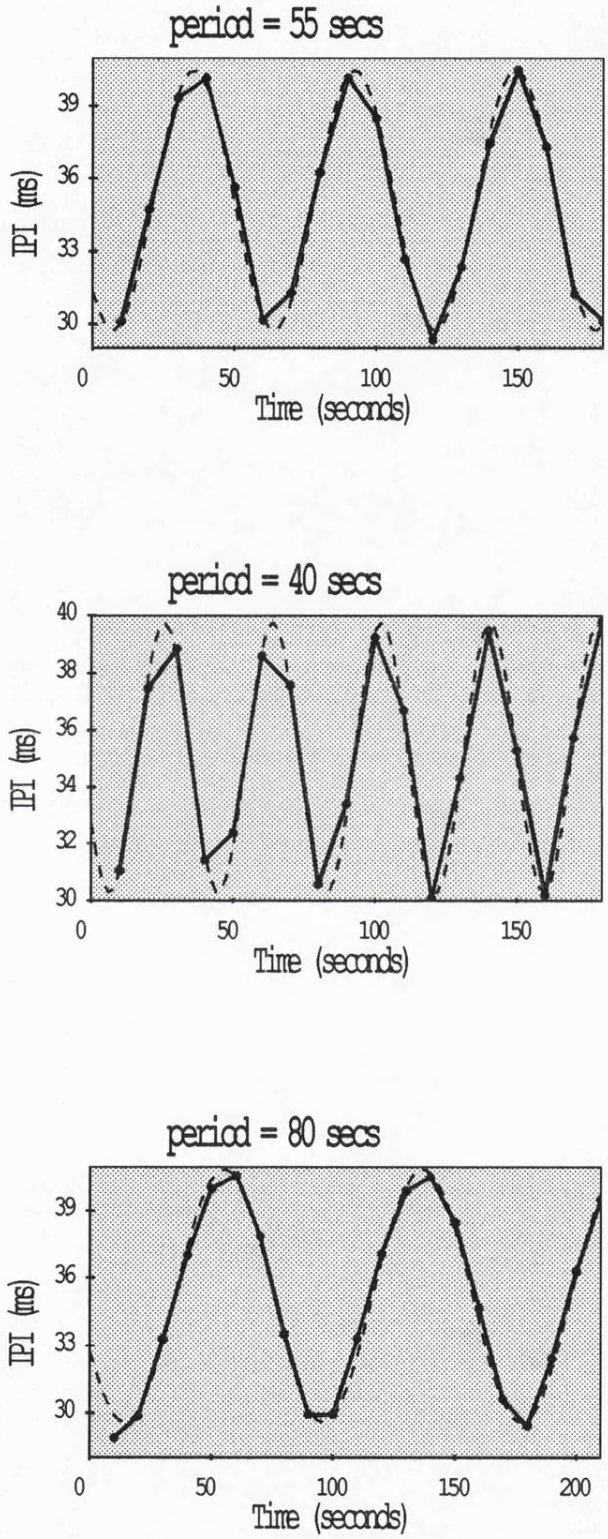
3. The mesh door of the mating chamber was opened to allow the males and females to mix, and the tape recording of the simulated song was started. The song was played at a level of 90 decibels, measured by a sound level meter (Realistic), in the mating chamber.

4. The time of each mating to occur within 15 minutes was noted.

5. This procedure was repeated a total of 4 times, each time using a different group of flies, each group with the same genotype. Each group would receive a different stimulation - either a song with a 40s rhythm, a song with a 55s rhythm, a song with an 80s rhythm or silence. The order in which the different songs and silence were tested was randomised. This procedure made up one run of the experiment.

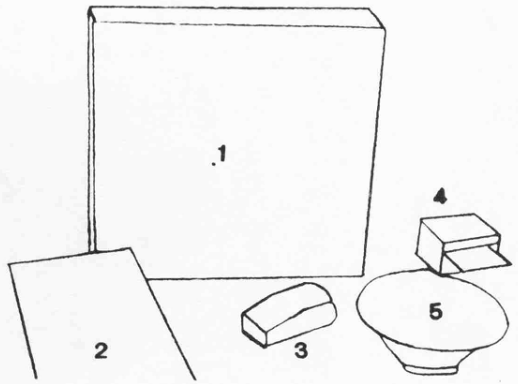
6. Any run with a total of less than 10 matings was discarded and not used in the analysis of the results. Between 10 and 20 runs of the experiment were performed for each genotype investigated.

Fig 2.5 Rhythms produced by the artificial song simulator



The songs were analysed by dividing them into 10 second bins and computing a mean IPI for each bin. This produces a slight distortion of the rhythm, so that it appears not to follow a perfect sine wave.

Fig 2.6 Arrangement of equipment for mating experiments



KEY

1 tape recorder
2 amplifier
3 sound meter
4 mating chamber
5 speaker

2.8 GENETIC COUPLING - POSSIBLE MECHANISMS INFLUENCING FEMALE SONG PREFERENCES

The general experimental procedure was identical to that followed in Section 2.7.2. However, flies were not tested with a simulated song carrying an 80s rhythm. In addition, flies were observed for 10 minutes instead of 15 minutes. Runs with less than 7 matings were discarded. Between 14-18 runs of the experiment were performed.

Song rhythm preferences of flies raised entirely in constant bright light were also investigated using the same method as above. Flies to be tested were raised in the light box described in Section 2.5.2 throughout their development from egg to adult. After eclosion, flies were collected according to Section 2.2 (p31), and subsequently stored in the light box until experimentation.

2.9 TEMPERATURE COUPLING - THE EFFECT OF TEMPERATURE ON MALE SONG PRODUCTION AND FEMALE SONG RECEPTION

2.9.1 Effect of low temperature on male song rhythm production

Flies whose songs were to be recorded were raised and maintained at a temperature of 18°C. The songs of these flies were recorded as described in Section 2.4.1 (p32), but at a temperature of between 15-18°C. Unfortunately, no constant temperature room was available for song recording at the time of this experiment. The temperature was monitored throughout the recording and did not change within recordings of individual flies. The songs were then analysed as described in Sections 2.4.1 and 2.4.2 (p32).

2.9.2 Effect of low temperature on female song rhythm preferences

Song rhythm preferences of females at 18°C were tested, using the same method as described in Section 2.8. Briefly, per^+ females were tested with simulated songs, with a cycle of 55s or 40s (mean IPI=35ms), and with silence. Flies to be tested were raised and maintained at 18°C and the experimental procedure performed at 18°C.

2.9.3 Effect of temperature on female IPI preferences

2.9.3.1 Song recording at 18°C and 25°C

Changes in temperature are known to affect the mean IPI of songs produced by male fruitflies (Shorey, 1962). In order to test the effect of temperature on female IPI preferences, it was necessary to ascertain the mean IPI of males of the same strain (see Section 9.2.1) at 18°C and 25°C. Female preferences could then be tested using songs which mimicked the natural songs of males of the same strain. Songs of this strain were recorded as described in Section 2.4.1 (p32), at 25°C and at 18°C. Flies were raised and maintained at the same temperature at which they were to be recorded. As only the mean IPI, and not the rhythm, was of interest, only 1 minute of song was recorded and analysed. Five songs were recorded at 25°C and four at 18°C. The mean IPI was calculated by finding the mean IPI of each song and calculating the mean of the means. The songs recorded at 25°C had a mean IPI of 32.92ms (SE=0.66) and songs recorded at 18°C had a mean IPI of 48.15ms (SE=1.22).

2.9.3.2 Generation of simulated song

Simulated song was generated and recorded using the method described in Section 2.7.1 (p40). Two songs were produced, both with a rhythm of 55s. One song had IPIs ranging between 28-38ms, with a mean of 33ms and the other had IPIs ranging between 43-53ms, with a mean of 48ms. These songs mimicked the natural songs of the males described above at a temperature of 25°C and 18°C respectively. As previously described, both songs were edited so that they began at the trough of a cycle.

2.9.3.3 Testing female IPI preferences

The experimental procedure was as described in Section 2.8 (p41), except that flies were tested with the simulated songs described above (55s cycle, superimposed on a 33ms or 48ms IPI). This made up one run of the experiment. The experiment was performed at 25°C and 18°C; 25 runs were carried out at 25°C and 20 runs at 18°C. Flies were raised and maintained at the temperature at which they were to be tested.

2.9.4 Effect of temperature on IPI production of *D. simulans*

The songs of *D. simulans* were recorded in the same way as those of *D. melanogaster*, as described in Section 2.4.1 (p32). Flies were raised and maintained at the temperature at which they were to be recorded, either 18°C or 25°C. Two minutes of song were recorded and analysed. Five songs were recorded at 25°C and four at 18°C.

Chapter 3

Locomotor activity and courtship song rhythms of three clock mutants

3.1 INTRODUCTION

The study of the *per* gene has contributed several ideas as to how biological clocks may function at the molecular level (eg Bargiello *et al*, 1987; Kyriacou, 1990). However, such a widespread phenomenon as biological rhythmicity cannot be controlled by one gene alone and the action of other clock genes must be investigated to build up a more complete picture of the underlying mechanisms. Three autosomal loci in *Drosophila* have been isolated which principally affect the phasing of locomotor activity and eclosion cycles (Jackson, 1983). *phase-angle-2* and *phase-angle-3* (*psi-2* & *psi-3*) result in slightly longer than normal activity periods. In addition, the adults tend to eclose from the pupae several hours before dawn, instead of after dawn, as is usually the case in wild type flies (Jackson, 1983). The song periods of these mutant males are slightly lengthened, mirroring their effects on circadian behaviour (Jackson, 1983). The *gate* (*gat*) mutation causes a desynchronisation of adult eclosion, so that it appears arrhythmic. The mutation also affects song cycles such that both long and short periodicities are observed (Jackson, 1983).

This Chapter has investigated the locomotor activity and courtship song cycles in three mutants, $Clock^{K06}$, *andante* and *disconnected*.

3.1.1 *Clock*^{k06} (*Clk6*)

This genetic variant was originally isolated by Konopka (1987a) and found to be a semi-dominant mutation. The locomotor activity and eclosion rhythms of *Clk6* mutants are shortened to about 22.5 hours (Dushay *et al*, 1990). The locomotor activity rhythm of *Clk6* can be entrained by light/dark cycles to a period of 24 hours, but the evening peak of activity is seen at a different time to the wild type (Dushay *et al*, 1990). The *Clk6* locus is situated very close to the *per* locus, but can be separated from per^{01} by recombination (Dushay *et al*, 1990). The estimated distance between these two mutations is short enough to suggest that *Clk6* could be an allele of the *per* locus.

3.1.2 *andante*

The *andante* mutation was originally isolated by Smith (1982). It is an X-chromosomal locus, situated at position 10E3 (Konopka, 1987b),

and is in fact an allele of *dusky*, a wing colour mutant (F R Jackson, pers comm to C P Kyriacou). *andante* is a semi-dominant mutation, which lengthens the locomotor activity and eclosion rhythms by about two hours (Konopka, 1987b).

3.1.3 *disconnected (disco)*

The basic phenotype of the *disco* mutant is fully described in Steller *et al* (1987). It is an X-chromosomal mutation falling within the cytogenetic interval 14B3-4. Briefly, the compound eyes of the *disco* adult are disconnected from the optic lobes of the brain. A larval visual nerve, Bolwig's nerve, is unable to establish its correct synaptic connections during embryonic development of *disco* larvae. The nerve is thus misrouted, leading to a lack of innervation of the developing optic lobes. In turn, this results in a massive degeneration of these structures, so that *disco* adults have only rudimentary optic lobes.

The effect of *disco* on circadian rhythmicity has been investigated by Dushay *et al* (1989). The mutation abolishes both the eclosion rhythm and the locomotor activity rhythm, although *disco* flies can be entrained to a 24 hour activity rhythm by light/dark cycles. As in per^{01} mutants, *disco* mutants demonstrate very short, ultradian periodicities (Dowse *et al*, 1990). The optic lobes of *Drosophila* have been previously implicated in the control of circadian rhythmicity. Analysis of the activity rhythms of several optic lobe mutants revealed multiple but significant periodicities (Helfrich, 1986). It is therefore interesting that *disco* mutants are arrhythmic in this behaviour, as degeneration of the optic lobes does not in itself abolish circadian rhythmicity. It seems possible that *disco* may affect rhythmicity in some other way; if this is so, then song rhythms may also be disrupted in this mutant.

3.1.4 Existence of song rhythm - the controversy

The existence of cycles in courtship songs has recently been questioned (Ewing, 1988; Crossley, 1988). Lengthy and detailed replies to this criticism have been published (Kyriacou & Hall, 1989; Kyriacou & Hall, 1990; Hall & Kyriacou, 1990) and the interested reader is referred to these reports. A brief consideration of the main points of dissention will be given here.

Ewing and Crossley (1988) have separately failed to detect

cycles in courtship songs. They criticise the use of non-linear regression using the BMDP3R program (Kyriacou & Hall, 1980; also see Section 2.4.2, p33) on the grounds that it requires good initial estimates of input parameters. Ewing and Crossley suggest that spectral analysis is a more appropriate method of analysing song data. However, when applying such methods to their own data, they still fail to observe rhythmicity. Kyriacou & Hall (1989) have subsequently adopted the use of spectral analysis (see Section 2.4.2, p33) and have confirmed the results originally obtained from the BMDP3R analysis. Thus Crossley and Ewing's contention that inappropriate use of a statistical program has produced spurious rhythmicity does not seem to be correct. Why, then, do they fail to detect rhythmicity in their own data?

Several suggestions have been put forward to account for this failure (Kyriacou & Hall, 1989; Kyriacou & Hall, 1990; Hall & Kyriacou, 1990). One of the problems with the BMDPIT program (spectral analysis) used by Crossley and Ewing is that it cannot run with missing data values. Male *Drosophila* frequently interrupt their courtship and so 10s bins (see Section 2.4.1, p32) with little or no data are inevitable. This laboratory normally considers any bin containing less than 15 IPIs as being empty; however, Ewing and Crossley use means based on as few as two IPIs. When a bin contains no data, they estimate a value mid-way between the values immediately preceding and following the empty bin. Thus, some of the means used in their analyses are based on tiny amounts of data or even no data at all.

Another problem relates specifically to the work of Ewing (1988). He uses very small mating cells in order to increase the amount of song produced by the male. Kyriacou & Hall (1989) have demonstrated that male song production is inhibited in small cells, with a high proportion of the song falling into short song bursts. In addition, there is apparently an increase in aggressive interactions between males and females, leading to increased noise on the song recordings. This can have the effect of making the song records more difficult to interpret.

It is clear that it must be possible to distinguish between IPIs (interpulse intervals) and interbout intervals between successive song bursts. For this reason an upper cut-off value for the measurement of IPIs is set in this laboratory at 65ms for a song with a mean IPI of 35ms. The upper cut-off varies according to the overall mean IPI (see Section 2.4.1, p32). This is particularly important when songs are recorded at different temperatures, as the mean IPI becomes longer at lower temperatures. Ewing (1988) sticks to a cut-off value of 70ms

regardless of the mean IPI value, and Crossley (1988) to one of 80ms, even though the latter author records songs over a temperature range of at least 5°C (S Crossley, pers comm to C P Kyriacou). Kyriacou & Hall (1989 & 1990) have demonstrated that this procedure has prevented Crossley from finding a rhythm in the song data of a wild type male which had been analysed both by Crossley and themselves. In this particular fly, a rhythm of around 60s was found by Kyriacou & Hall (1989), irrespective of whether the primary IPI means had been computed by Crossley or by Kyriacou & Hall.

A subset of Crossley's (1988) data was deemed suitable for analysis under criteria adopted in this laboratory (Kyriacou & Hall, 1989). Eight songs which had no empty bins or mean IPIs based on less than 15 individual IPIs are presented in Crossley (1989). Six out of eight of these songs gave a period in the range of 50-65s, using Crossley's BMDPIT program. Kyriacou & Hall generated sets of 1000 random songs and subjected them to the CLEAN analysis (see Section 2.4.3, p35). The maximum proportion of random songs giving periods in the 50-65s range was 11%. Using this figure, the probability of 6 out of 8 of Crossley's songs having periods in the 50-65s range by chance was calculated to be 0.000036. Kyriacou & Hall (1989) conclude that wild type periods do exist in Crossley's songs. Thus, the discrepancies between the results of Crossley and Ewing and those of Kyriacou & Hall are probably due to differences in methodology and to the inappropriate application of the BMDPIT program by Ewing and Crossley.

3.2 MATERIALS AND METHODS

3.2.1 Locomotor activity recording

The locomotor activity of 3 genotypes was recorded; *Clk6 cho* and *andante* (both from J Hall, Brandeis University) and a wild type strain, Brighton (from L Partridge, University of Edinburgh).

Locomotor activity recording was carried out as described in Section 2.3, p31.

3.2.2 Courtship song recording and analysis

Flies of 3 different *Clk6* strains were used in this experiment; *Clk6* nos 1-34 were of the genotype *Clk6 cho (chocolate)*, *Clk6* nos 45 & 46

carried the markers *yellow*, *chocolate* and *crossveinless* whilst *Clk6* nos 48-50 had the genotype *Clk6* $w^{\#98}$ (*white*). These markers are fully described in Lindsley & Grell (1968). Many songs (over 50) were recorded, but a large number of flies sang too poorly for their songs to be subsequently analysed by time series methods. Eventually, 15 songs were obtained for analysis.

One strain of *andante* was investigated. 15 songs were eventually analysed; again many were too poor to be included.

Males from two strains of the *disco* mutant were examined; one unmarked strain and one marked with *yellow*, *white* and *forked*. Originally, it was believed that these 2 strains carried different alleles of *disco* (see Dushay *et al*, 1989), but it is now known that the 2 alleles are identical at the molecular level (M Freeman, pers comm to C P Kyriacou). Flies labelled "a" (eg *disco* no3a) were of the unmarked *disco* strain; flies labelled "b" were marked with *yellow*, *white* and *forked* (eg *disco* no5b). 18 songs were analysed; more were recorded, but were too poor to be included.

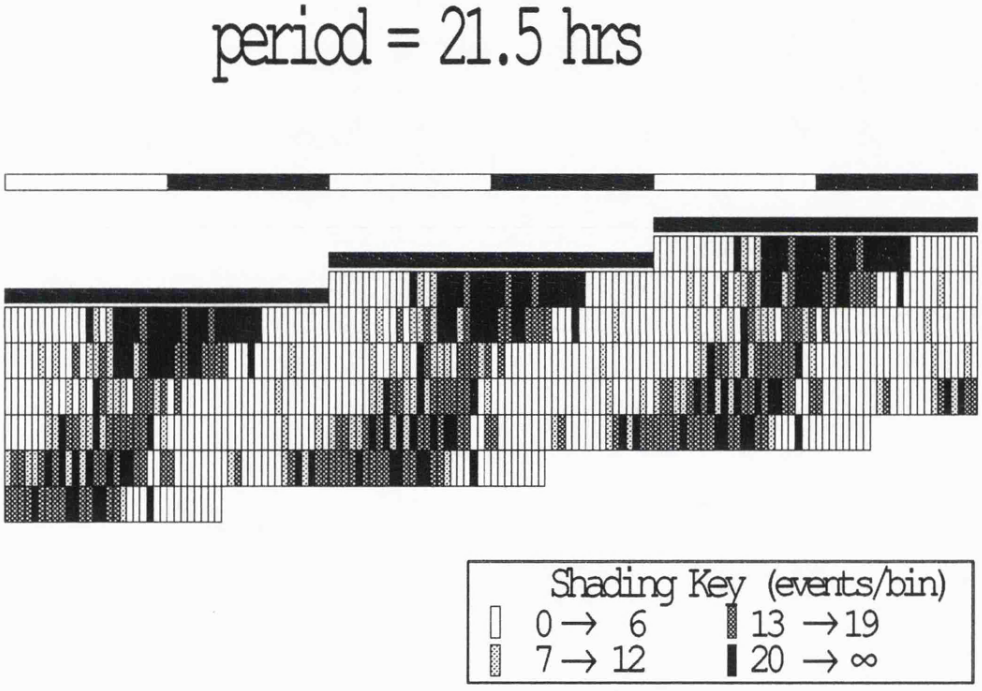
Song recording and analysis were carried out as described in Section 2.4.

3.3 RESULTS

3.3.1 Locomotor activity

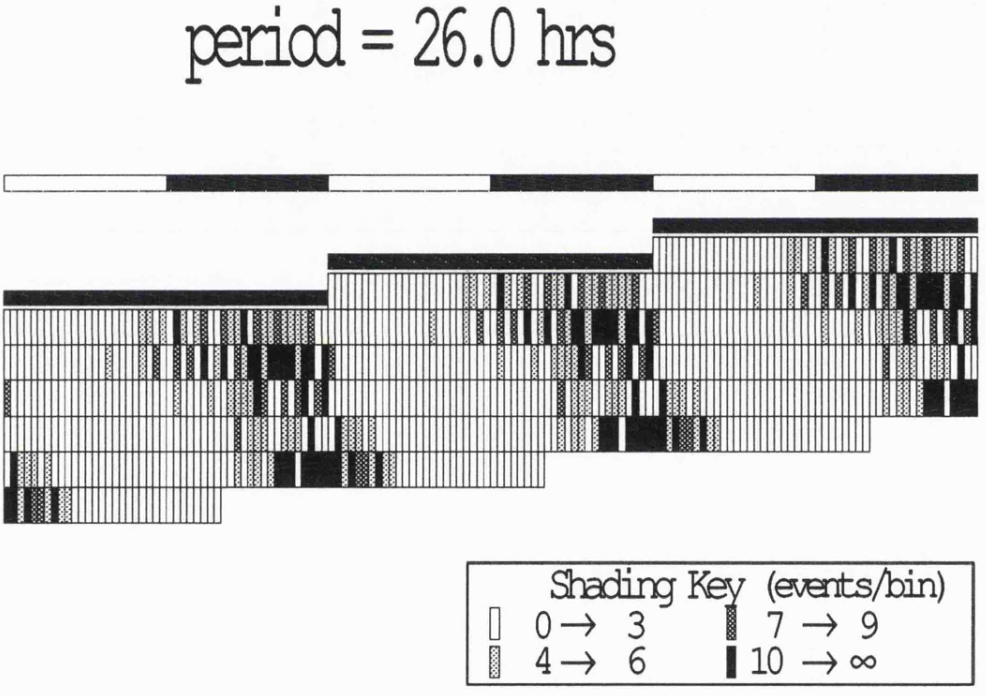
The number of flies recorded and the mean locomotor activity period obtained for each genotype are shown in Table 3.1. The mean period of the *Clk6* flies is 22.3 hrs (SEM=0.228) and that of the *andante* flies is 25.6 hrs (SEM=0.189). The mean period of the wild type Brighton strain is 24.2 hrs (SEM=0.189). A one way analysis of variance shows that there is a significant difference between the genotypes ($F=72.33$, $df=2,41$ $p<<0.01$). A Newman-Keuls test shows that each genotype is significantly different from the others ($p<<0.01$).

Fig 3.1 Locomotor activity of *Clk6* under free-running conditions



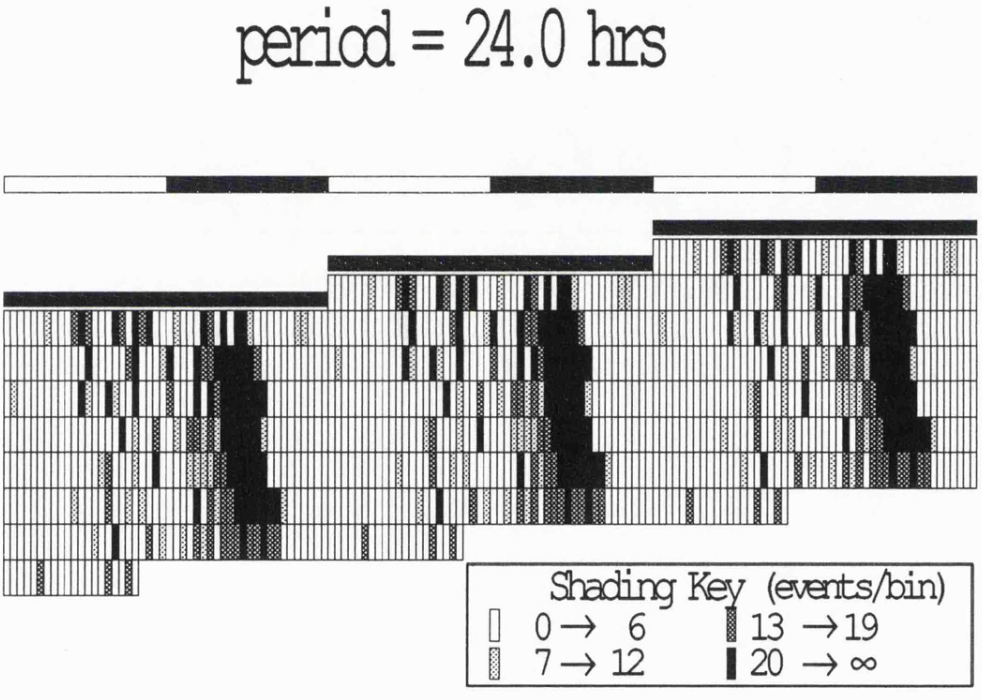
Actogram of locomotor activity recorded in constant darkness after entrainment in LD12:12. See Fig 2.1 for explanation of actogram.

Fig 3.2 Locomotor activity of *andante* under free-running conditions



Actogram of locomotor activity recorded in constant darkness after entrainment in LD12:12. See Fig 2.1 for explanation of actogram.

Fig 3.3 Locomotor activity of Brighton under free-running conditions



Actogram of locomotor activity recorded in constant darkness after entrainment in LD12:12. See Fig 2.1 for explanation of actogram.

Table 3.1 Locomotor activity of 3 strains of *D melanogaster*

Genotype	No. recorded	Rhythmic (n)	Arrhythmic (n)	Mean period (hrs) ± SEM
Clk6 cho	15	15	0	22.3 ± 0.228
andante	20	17	3	25.6 ± 0.189
Brighton	13	12	1	24.2 ± 0.189

A locomotor activity plot (actogram), representative for each strain, is shown in Figs 3.1 - 3.3. An explanation of these plots is given in Section 2.3, p31.

3.3.2 Song rhythms

3.3.2.1 *Clk6*

Table 3.2 shows the periods obtained from the BMDP3R, CLEAN and van den Berg analyses of the *Clk6* songs. It can be seen that 9 out of 15 songs have cycles which are significant using the BMDP3R analysis. The periods range from 44-63s, with the exception of *Clk6* no7, which has an unusually long period of 107.7s. The mean period is 62.1s (SEM=6.01); excluding *Clk6* no7, this value reduces to 56.4s (SEM=2.16). Both means are very close to the value of 50-60s usually quoted as the wild type range (eg Kyriacou & Hall, 1980). A one way ANOVA of the significant periods of *Clk6* and per^+ (from Kyriacou & Hall, 1989) shows no significant difference between the two genotypes ($F=3.22$; $df=1,24$).

Table 3.3 shows the distribution of song periods from a CLEAN and van den Berg analysis of *Clk6*, *andante*, *disco*, per^+ and random simulated songs. The random songs were generated using parameters of N=25 (5 missing data points), sd=2 and mean IPI of 35ms, as discussed in Section 2.4.3, p35. These parameters resemble those of the natural songs

Table 3.2 Song periods obtained from Clk^{k06} males by analysis with BMDP3R, CLEAN and van den Berg

FLY	BMDP3R secs (if sig)	F	df	CLEAN secs	van den Berg secs	
1	57.9	6.06	3,19	56.0	58.2	
3	62.7	5.99	4,22	64.4	63.0	*
6				29.5	29.4	
7	107.7	4.06	3,23	112.0	108.6	
11	44.0	4.44	3,20	31.2	42.9	
12				64.0	21.0	
17	54.7	5.84	3,30	56.7	54.9	
24				54.5	26.6	*
33	60.9	3.28	3,16	28.7	61.4	
34				51.7*	52.6	*
45				66.7	21.7	
46	51.7	8.78	4,20	51.2*	65.0	*
48	58.9	3.41	3,27	59.0	58.9	
49				25.8	173.1	
50	60.2	6.43	4,26	60.0	61.1	
mean	62.1					

* periods obtained from 2nd peak of the spectrogram

Table 3.3 Distribution of song periods from analyses by CLEAN and van den Berg of Clk^{k06} andante, disco, wild type (per^{+}) and a random distribution

	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100	100+	N
CLEAN analysis										
Clk^{k06}	3	1	0	6	4	0	0	1	1	15
andante	1	1	4	2	4	0	1	1	1	15
disco	2	2	3	2	4	2	1	0	2	18
per^{+} *	0	0	2	13	3	0	0	0	0	18
random	332	190	124	64	47	40	37	27	139	1000
van den Berg analysis										
Clk^{k06}	4	0	1	4	4	0	0	0	2	15
andante	0	1	2	2	3	2	1	2	2	15
disco	2	0	2	6	3	2	0	3	0	18
per^{+} @	0	0	4	16	4	0	1	0	0	25
random	381	189	114	53	45	36	33	20	129	1000

* From Kyriacou & Hall, 1989
@ From Kyriacou et al (1990b)

seen in this study. The distribution of the periods obtained from a van den Berg analysis of the random songs is seen in Fig 2.3.

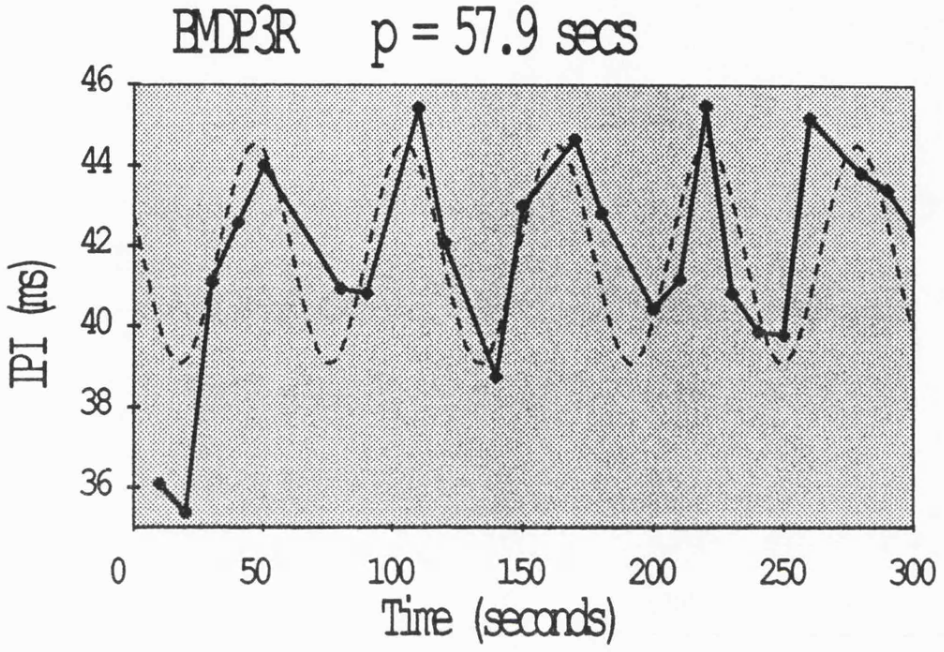
On analysis with CLEAN (see Table 3.3), 10 out of 15 *Clk6* song periods fall in the 50-70s range. A contingency test demonstrates that the distribution of *Clk6* periods is not significantly different to that of 18 wild type songs analysed by Kyriacou & Hall (1989) (χ^2=9.60, df=8). However, the difference between the distributions of the Clk6 periods and the periods of the random songs is highly significant ($p<<0.01$, χ^2=44.59, df=8). With the van den Berg analysis, 8 out of 15 song periods fall into the 50-70s range, and the difference between the distributions of the *Clk6* periods and the periods of the random songs is highly significant ($p>0.001$, χ^2=32.21, df=8). There is no significant difference between the distribution of the per^+ song periods and that of the *Clk6* song periods when analysed with the van den Berg procedure (χ^2=14.40; df=8).

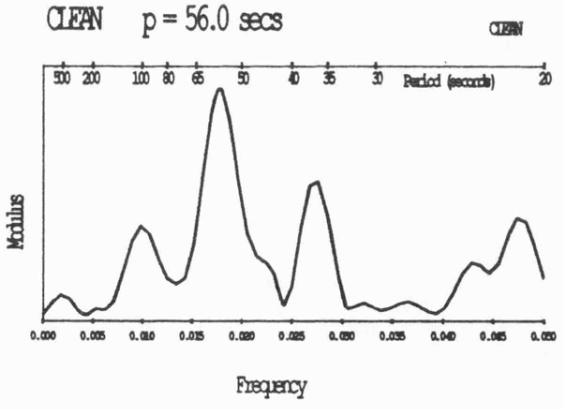
The proportion of song periods falling into the 50-70s range from the CLEAN analysis of the random songs is 0.111. 10 out of 15 *Clk6* songs fall into this range on analysis with CLEAN. The probability of 10 or more *Clk6* songs falling into this range by chance can thus be calculated. In fact, this probability (using the distribution from the CLEAN analysis) is 5.01×10^{-7}. Thus it is clear that the distribution of the song periods obtained from the *Clk6* flies cannot be due to chance.

The periods obtained from the van den Berg and CLEAN analyses differ by more than 10s in Clk6 nos 11, 12, 24, 33, 45, 46 and 49. In these cases, both periods were tried in the BMDP3R analysis and the period which gave the highest F ratio was taken as correct. For instance, in *Clk6* no11 the periods obtained from a CLEAN and van den Berg analysis were 31.2s and 42.9s respectively. When a value of 31s was used in a BMDP3R analysis, a period of 30.4s was obtained, with a F ratio of 1.78 (df=3,22). This is a much lower value than the F ratio of 4.44 (df=3,20) achieved with a period of 44.0s. Thus, the latter value was taken as being correct.

Figure 3.4 shows the BMDP3R, CLEAN and van den Berg analyses of the *Clk6* no1 song. This song has periods of 57.9s, 56.0s and 58.2s from the BMDP3R, CLEAN and van den Berg analyses respectively. Figure 3.5 shows the analysis of Clk6 no46, the IPIs of which fall around a climbing sine wave. The dominant peak in the spectrum from a CLEAN analysis is at 640s, which merely reflects the general upward trend in the data. The second peak is at 51.2s and this is taken as being the true periodic value. The van den Berg analysis gives a period of 65.0s (2nd peak

Fig 3.4 BMDP3R, CLEAN and van den Berg analyses of *Clk6* no1





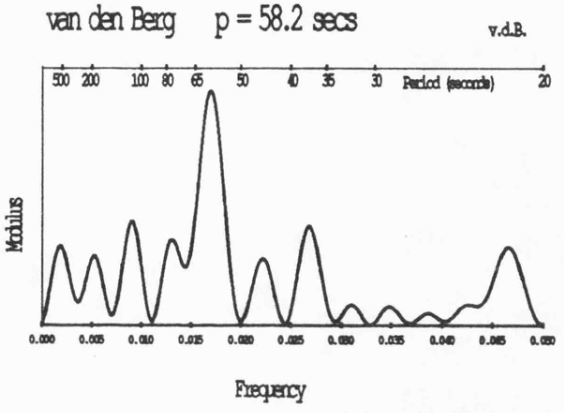
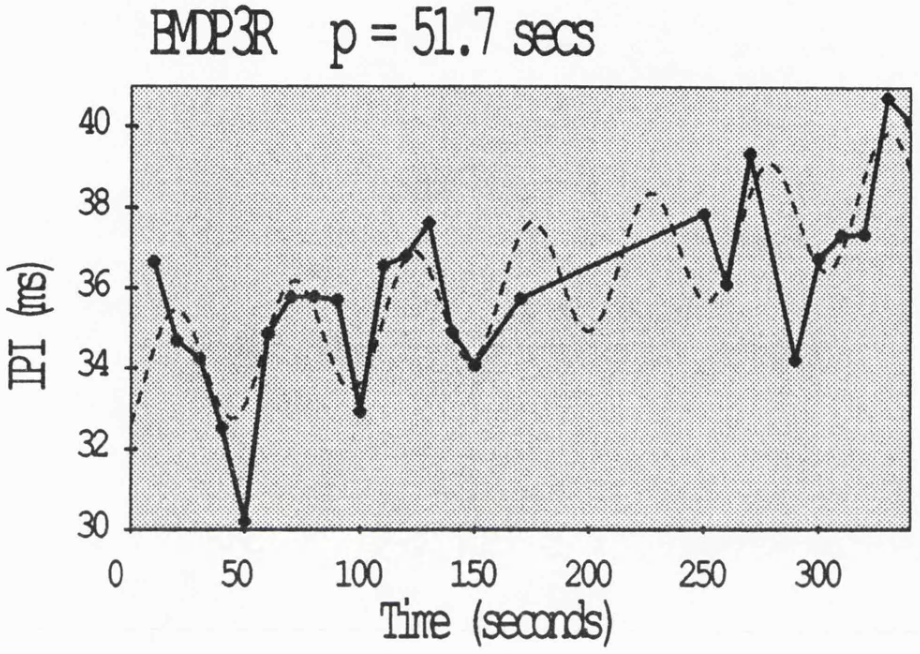
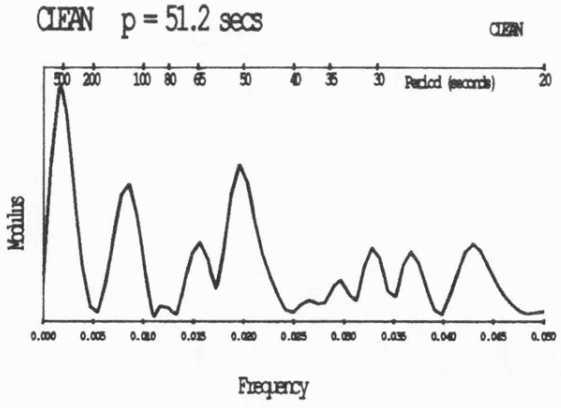
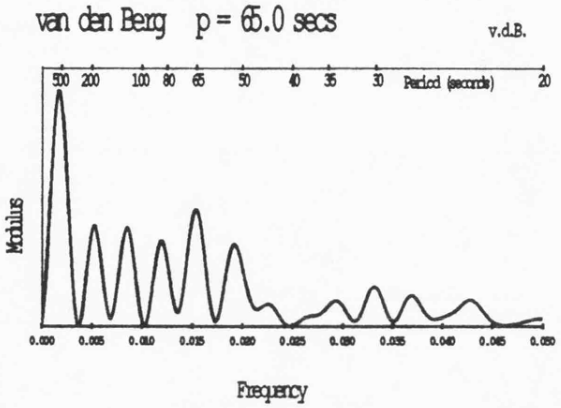


Fig 3.5 BMDP3R, CLEAN and van den Berg analyses of *Clk6* no46







value). When the period from the CLEAN analysis is used as the starting parameter in the BMDP3R analysis, the period obtained is 51.7s (F=8.78; df=4,20). When the period from the van den Berg analysis is used, the BMDP3R period obtained is 64.9s (F=5.86; df=5,19). Both F ratios are significant, but only the F ratio using the CLEAN period is significant against a straight line (see Section 2.4.2, p33). Note that the peak at 51.2s in the CLEAN analysis is fairly strong, but that the peak at 65.0s in the van den Berg analysis is weak (see Fig 3.5). Thus it is not surprising that a higher F-ratio from the BMDP3R analysis is obtained when the CLEAN period is used as a starting parameter.

3.3.2.2 *andante*

Table 3.4 shows the periods obtained from the BMDP3R, CLEAN and van den Berg analyses of the *andante* songs. 9 out of 15 songs have cycles which are significant using the BMDP3R analysis. The periods of these songs varies greatly - from 40.8s to 96.6s. The mean is 65.9s, with a standard error of 6.50. The song of *andante* no3 is illustrated in Figure 3.6. A one way ANOVA of the significant periods (with BMDP3R) of *andante* and per^+ (from Kyriacou & Hall, 1989) shows a significant difference between the two genotypes (F=6.04; df=1,24).

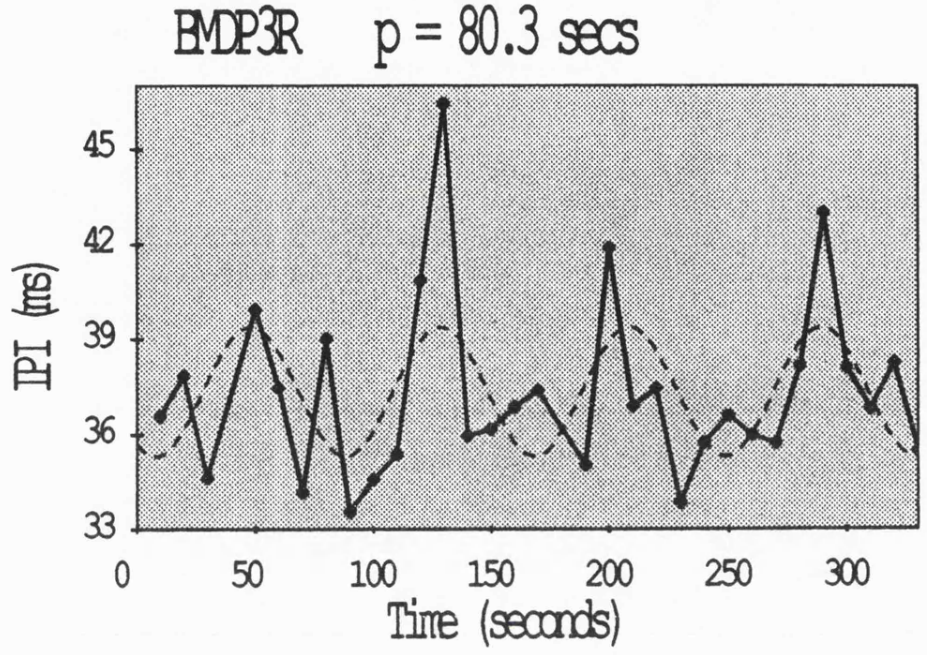
Table 3.3 shows the distribution of the periods obtained from analysis of *andante*, per^+ and the random songs with CLEAN and van den Berg. A contingency test demonstrates no significant difference between the song periods of per^+ and *andante* with the CLEAN analysis (χ^2=13.83; df=8). However, with the van den Berg analysis, a significant difference between per^+ and *andante* is observed (χ^2=17.28; df=8). The *andante* song periods were also compared to those of per^{L1} (from Kyriacou et al, 1990b; data not shown). With a CLEAN analysis, the song periods of *andante* and per^{L1} are just significantly different (χ^2=15.52; df=8). However, with a van den Berg analysis, there is no significant difference between per^{L1} and *andante* (χ^2=7.92; df=8). There is a highly significant difference between the *andante* periods and those of the random songs from both the CLEAN and van den Berg analyses (χ^2=24.38 & 28.97 for the CLEAN and van den Berg analyses respectively; df=8). It is noteworthy that a different distribution of the *andante* song periods is obtained with the van den Berg and CLEAN analyses and the possible reasons for this are discussed in Appendix 1. It would appear from these results that the song periods of the *andante* mutant are slightly longer than wild type.

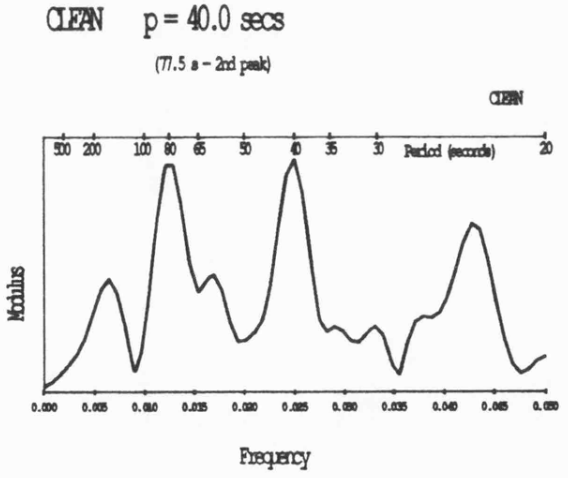
Table 3.4 Song periods obtained from andante males by analysis with BMDP3R, CLEAN and van den Berg

FLY	BMDP3R secs (if sig)	F	df	CLEAN secs	van den Berg secs
2				27.8	98.8
3	80.3	3.47	3,27	40.0	79.7
4	87.6	5.67	3,26	62.9	87.4
5				83.3*	69.0*
7				33.5*	33.1*
9	63.3	4.12	3,19	61.3	64.0
20	40.8	3.54	3,20	41.4	40.8
21	44.1	5.22	3,20	44.4	43.9
24	96.6	8.16	5,21	95.4*	96.4*
25				52.2	214.0
28	71.8	3.40	3,26	69.5	72.0
29				63.2	64.8
30				182.9	171.3
36	55.7	3.61	3,19	40.0	56.3
40	52.9	5.45	3,35	53.3	53.0
mean	65.9				
SEM	6.50				

* periods obtained from the 2nd peak of the spectrogram

Fig 3.6 BMDP3R, CLEAN and van den Berg analyses of *andante* no3





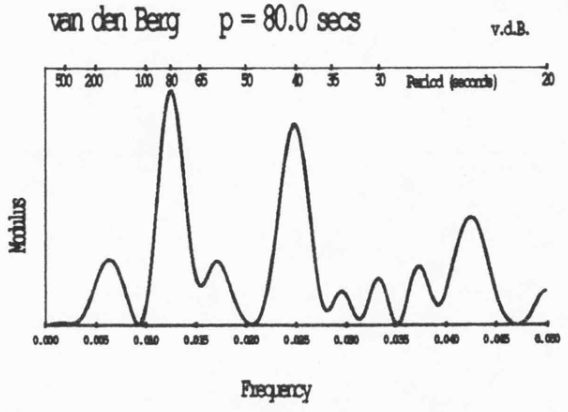
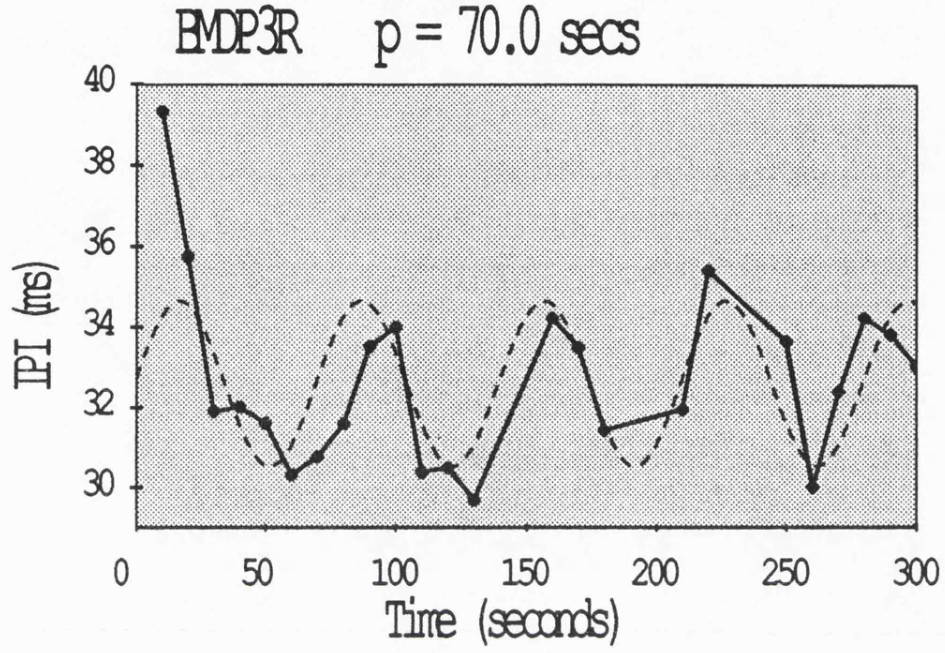


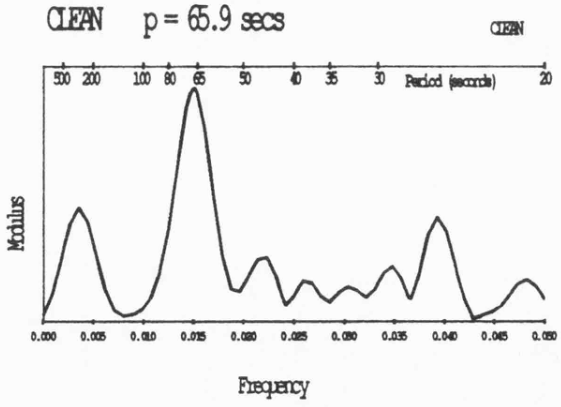
Table 3.5 Song periods obtained from *disco* flies by analysis with BMDP3R, CLEAN and van den Berg

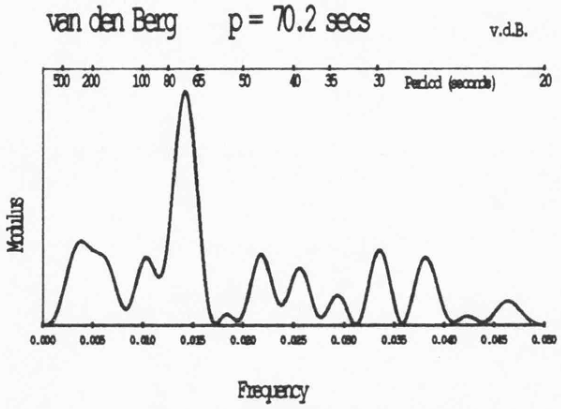
FLY	BMDP3R secs (if sig)	F	df	CLEAN secs	van den Berg secs
3a				33.5	20.0
6a	70.0	5.82	3,20	65.9	69.9
11a	53.3	6.34	3,21	51.7	53.2
16a				49.2	54.6
19a				22.0*	59.7*
5b	68.2	3.18	3,25	67.4	68.1
6b				104.0	90.4
9b	91.7	4.75	3,21	88.6	91.5
10b	94.9	3.90	3,17	100.0	93.8
11b	74.2	3.82	3,25	71.1	74.4
15b				72.5	73.7
16b	64.9	4.56	3,26	65.3	65.0
22b	60.5	3.70	3,18	28.9	59.9
34b				40.0	40.1
37b	59.6	5.14	3,18	61.1	59.6
38b	53.6	3.74	3,17	52.0	52.9
39b	29.7	3.66	3,24	30.0	29.8
40b	46.4	4.67	3,23	46.2	46.4
mean	63.9				
SEM	5.24				

* period obtained from the 2nd peak of the spectrogram

Fig 3.7 BMDP3R, CLEAN and van den Berg analyses of *disco* no6a







3.3.2.3 *disconnected*

Table 3.5 shows the periods obtained from the BMDP3R, CLEAN and van den Berg analyses. It can be seen that 12 out of 18 songs have cycles which are significant using the BMDP3R analysis. The mean period is 63.9s (SEM=5.24). A one way ANOVA of the significant periods of *disco* versus per^+ songs (from Kyriacou & Hall, 1989) shows a significant difference between the two genotypes ($F=4.80$; $df=1,27$). The song of *disco* no6a is shown in Figure 3.7.

Table 3.3 shows the distribution of the periods obtained from the CLEAN and van den Berg analyses of the *disco* songs. A contingency test demonstrates no significant difference between the song periods of *disco* and per^+ with the van den Berg analysis. However, a significant difference between *disco* and per^+ is seen with the CLEAN analysis ($\chi^2=17.41$; $df=8$). There is a significant difference between the *disco* songs and those of the random songs with both the CLEAN and van den Berg analyses ($\chi^2=18.13$ & 61.01 for the CLEAN & van den Berg analyses respectively; $df=8$). Overall, the results would suggest that the song periods of the *disco* mutant are slightly longer than wild type periods.

3.4 DISCUSSION

From the results presented here, it appears that *Clk6* males produce songs with wild type periods. It is clear, however, that song rhythmicity is not as strong as in the wild type. In the CLEAN distribution, 4 periods are found at the extreme edges of the spectrum in *Clk6*; in the wild type, all periods are clustered in the 40-70 second range. Males of the *Clk6* genotype also sing less vigorously than their wild type counterparts. Most males tested did not sing at all and those that did sang poorly. This could explain why song rhythmicity appears weaker in *Clk6* than in wild type. It is not clear why *Clk6* males are reluctant to sing. Certainly results presented in Chapter 5 show that *Clk6* flies are viable and give little impression of being a "sick" strain. Dushay et al (1990) also conclude that *Clk6* flies are vigorous in terms of general viability.

Meiotic recombination experiments (Dushay et al, 1990) have mapped the Clk6 mutation to a position just right of per^{01} on the X-chromosome. The two loci are estimated to be about 0.015 map units apart, close enough for *Clk6* to be an allele of the *per* gene, perhaps

defining a regulatory site. Alternatively, the *Clk6* mutation could be located just proximal to the site of *per* mRNA transcription (Dushay *et al*, 1990). Encoded there are two RNA transcripts of 0.9Kb and 2.7Kb (Bargiello & Young, 1984; Reddy *et al*, 1984). These RNA species have no specific phenotypes assigned to them. Whether *Clk6* is found to be a novel clock gene or part of the *per* locus, it provides the first example of a mutation which confers short periodicity in one rhythmic character (activity) and wild type periodicity in another (song). The *Clk6* mutation may be a "circadian variant" in that eclosion rhythms are shortened to the same extent as circadian rhythms of locomotor activity (Dushay *et al*, 1990). It will be interesting to investigate the effects of *Clk6* on other rhythmic and temporal events (see Chapter 5). All biological rhythm mutations so far studied in *Drosophila* have had corresponding effects on different rhythms eg the per^{S} allele confers a short periodicity on both activity and song rhythms.

The *andante* mutation results in the lengthening of the locomotor activity rhythm by about 2 hours (Konopka, 1987b & this thesis). It appears that the songs of this genotype may also have periods in the long range, but the results are not clear-cut. The *andante* song periods obtained from the van den Berg analysis are significantly different from per^{+}, but not from per^{L1}, suggesting that *andante* song periods do indeed fall into the long range. In contrast, the CLEAN analysis shows that *andante* is significantly different from per^{L1} but not from per^{+}. However, the χ^2 value of the *andante* versus per^{L1} (CLEAN) comparison is barely significant (χ^2=15.79; df=8). Overall, the evidence suggests that the *andante* mutation results in song periods which are longer than wild type, but shorter than per^{L1}, thus paralleling its effect on the locomotor activity rhythm.

The known alleles of the *per* gene have parallel effects on all rhythmic and temporal characteristics thus far examined - eclosion, locomotor activity, courtship song and development time (Konopka & Benzer, 1971; Kyriacou & Hall, 1980; Kyriacou *et al*, 1990a). This fits in with the proposed function of *per* in intercellular communication (Bargiello *et al*, 1987), as intercellular communication may be expected to be pertinent to most, if not all, biological rhythms. The expression of *per* is also widespread throughout *Drosophila* tissues. The *per* gene may mediate rhythmicity by providing a communication function to cells which oscillate. Other clock genes may contribute towards the building up of an oscillator or towards the "ticking" of individual oscillating cells. These latter clock genes may therefore be expected to contribute

towards some oscillators, but not others. It is clear from mosaic studies that different oscillators exist for different rhythms. The song oscillator has been located to the thorax, whilst the activity oscillator is found in the head (Konopka *et al*, 1983; Hall, 1984).

It is not yet possible to speculate on the molecular function of the *andante* and *Clk6* genes. Like *per*, *andante* affects all rhythmic phenotypes so far investigated. The molecular analysis of *andante* is currently underway (F R Jackson, pers comm to C P Kyriacou) and the results are awaited with interest. In contrast, *Clk6* seems to affect only circadian behaviour and hence may be expected to play a less fundamental role in determining overall rhythmicity. If *Clk6* is a novel clock gene then it may perhaps contribute some, as yet unknown, function to some oscillators but not to others. However, *Clk6* may define a regulatory site of the *per* gene. The locomotor activity periods of *Clk6* are short, reflecting the effect of many doses of the per^{+} gene (Smith & Konopka, 1982; Coté & Brody, 1986). The normal function of the wild type *Clk6* gene thus may be to repress production of the *per* protein. This putative regulatory role for *Clk6* would appear to be tissue specific, as *Clk6* seems to affect the locomotor activity clock in the brain, but not the song clock in the thorax.

Oscillators may be built up from the products of all or some clock genes. It is possible to imagine a hierarchy of clock genes in which some genes contribute to all the oscillators and some genes towards only some or even just one oscillator. More biological rhythms and the genes affecting them must be identified before a possible structure of this hierarchy can begin to be formulated.

The compound eyes of *disco* mutants are disconnected from the optic lobes, leading to massive degeneration of these ganglia (Steller *et al*, 1987). The pacemaker which controls locomotor activity rhythms in Orthopteran insects is thought to lie in the optic lobes (eg Saunders, 1982). However, this does not appear to be true of *Drosophila*, as the optic lobes of *sol;so* double mutants are reduced to less than 5% of their wild type value, yet these flies show only mild disturbances in their locomotor activity rhythms (Helfrich, 1986). Thus, degeneration of the optic lobes in *disco* mutants would not seem to be the explanation for the arrhythmic locomotor activity phenotype observed in these flies (Dushay *et al*, 1989).

Some input of light is necessary to "start" the locomotor activity clock (Dowse & Ringo, 1989). However, blind *norp-A* (*no-receptor-potential*) flies and the eyeless, ocelliless *so*

(*small-optic-lobes*) mutants exhibit quasi-normal circadian rhythmicity (Dushay *et al*, 1989). Thus, a lack of light input caused by the disconnection of the eyes from the brain in *disco* mutants would be unlikely to explain the arrhythmic locomotor activity phenotype. Extraocular photoreceptors would seem to be more important in mediating circadian rhythmicity. Indeed, the importance of extraocular receptors for rhythmicity has been demonstrated in silkworms (Truman, 1974). Centrally located, light sensitive structures may be identified by an antibody called 24B10, which detects an antigen encoded by the *chaoptic* gene (van Vactor *et al*, 1988). 24B10 expression has been detected in the brain region close to where anti-*per* antibodies stain certain nerve cells in the lateral, dorsal and anterior region of the fly's head ganglia (in Dushay *et al*, 1989; cf Siwicki *et al*, 1988). Extraocular receptors and the circadian pacemaker may be in close proximity in order to facilitate interactions between them. This hypothesized communication may mediate circadian rhythmicity, and may be disrupted by the *disco* mutation (Dushay *et al*, 1989). Steller *et al* (1987) have shown that the primary function of the *disco* gene is in neuronal cell recognition; if communication between the extraocular photoreceptors and the pacemaker occurs via neuronal connections, then this may explain the circadian defect in *disco* mutants. Alternatively, it is possible that *disco* may have some direct effect on the pacemaker.

The *disco* mutant demonstrates strong rhythmicity in its courtship songs, but with periods which are rather longer than wild type. Thus, *disco* is the first mutant in which arrhythmia in one characteristic (locomotor activity) and rhythmicity in another (song) has been shown. If *disco* does facilitate the communication of extraocular photoreceptors with the pacemaker, then it seems likely that the song pacemaker in the thorax also requires some light input to function correctly. This could be tested by recording the songs of wild type flies raised entirely in darkness. This has been previously attempted, but insufficient song was obtained for analysis under these conditions (C P Kyriacou, pers comm). The long song rhythms observed in *disco* flies may result from a lack of communication, direct or indirect, between the extraocular photoreceptor cells and the song pacemaker. Given that the songs of *disco* mutants are not arrhythmic, it seems likely that light stimulation is not as important for the correct functioning of the song clock as it is for the locomotor activity clock. This is supported by the finding that constant bright light abolishes the locomotor activity rhythm, but has no effect on the courtship song rhythm (Zerr *et al*, submitted; Kyriacou & Hall,

1980).

However, *disco* may directly affect the song pacemaker in the thorax. The *disco* mutation has been reported to produce defects in a small number of peripheral neurons in the thorax and abdomen (Steller et al, 1987). At present, it is impossible to state with any certainty how *disco* may affect either the song or circadian cycles. If the role of *disco* lies in the facilitation of photoreceptor/pacemaker communication, then this work may have demonstrated a previously unrecognised requirement for light in the production of wild type rhythmicity in courtship songs.

Chapter 4

Song rhythms in naturally occuring Threonine-Glycine length variants

4.1 INTRODUCTION

Sequencing of the *per* gene has revealed a region in exon 5 which codes for a Threonine-Glycine (Thr-Gly) repeat (Citri et *al*, 1987). The repeat is similar to a Serine-Glycine (Ser-Gly) repeat found in the rat chondroitin sulphate protein, and this has led to suggestions that the *per* protein is a proteoglycan (Jackson et *al*, 1986; Reddy et *al*, 1986). Biochemical studies (Reddy et *al*, 1986; Bargiello et *al*, 1987) provide some support for this conclusion. Bargiello et *al* (1987) have suggested that biological rhythmicity may be mediated by intercellular communication via gap junction channels and proteoglycans are known to be involved in the formation of rat hepatocyte gap junctions (Spray et *al*, 1987). Thus, a possible mechanism for the action of *per* is suggested, based on its proteoglycan-like properties (see Introduction). It would appear, then, that the Thr-Gly region is important for circadian behaviour.

However, Yu et *al* (1987b) have shown that the Thr-Gly encoding region is not vital for circadian rhythmicity. They transformed per^{01} flies with *per* DNA from which the Thr-Gly encoding region had been deleted - the resulting transformants had normal locomotor activity rhythms. However, the deletion did affect the courtship song rhythm, which was reduced to a value of around 40s, instead of the normal 55s. In addition, a polymorphism within the Thr-Gly region was found - different strains of *D. melanogaster* have either 17, 20 or 23 Thr-Gly pairs (Yu et *al*, 1987b) and within strain Thr-Gly length polymorphisms have also been discovered (R Costa, pers comm). The Thr-Gly regions of *D. simulans* and *D. yakuba* have also been investigated - different strains of *D. simulans* have between 15-24 pairs, whilst the one strain of *D. yakuba* so far examined has 15 pairs (Wheeler et *al*, ms submitted; Thackeray, 1989).

Interspecific variation in the courtship song rhythm is controlled by a gene or genes on the X chromosome, possibly the *per* gene (Kyriacou & Hall, 1986). The mapping of interspecific genetic differences has been made possible with molecular techniques. per^{01} *melanogaster* flies have been transformed with the *per* gene from *D. simulans*. The transformants sing with a *D. simulans*-like rhythm of about 35s (Wheeler et *al*, op cit). Thus the *per* gene is strongly implicated in the control of interspecific differences in song rhythm. However, it has been possible to further identify a small region within the *per* gene responsible for this difference. Two reciprocal chimaeric *per* genes have

been constructed, in which a 700 base pair DNA fragment (containing the Thr-Gly encoding region and some flanking DNA) from each species was "swapped" and ligated into the flanking per material of the other species (Wheeler *et al*, ms submitted). The construct containing the Thr-Gly and associated encoding region from *D. simulans* resulted in transformants having a *D. simulans*-like song rhythm. Transformants with the reciprocal construct (ie Thr-Gly and associated region from *D. melanogaster*) had *D. melanogaster*-like songs.

Thus the Thr-Gly and associated encoding region appears to be important in regulating the period of the courtship song rhythm. In the three species whose song rhythm and Thr-Gly region have been analysed (*D. simulans*, *D. melanogaster* and *D. yakuba*), each had a different rhythm and a different number of Thr-Gly pairs, with an inverse relationship between period length and the number of pairs (Kyriacou & Hall, 1980; Yu *et al*, 1987b; Thackeray, 1989). The *D. melanogaster* strain from which the *per* gene was cloned and which was used in Wheeler *et al* (ms submitted) had 20 Thr-Gly pairs and a 55s rhythm. The *D. simulans* strain from which Wheeler *et al* (op cit) cloned the *per* gene had 24 Thr-Gly pairs and a 35s rhythm. Finally, the only strain of *D. yakuba* examined has 15 Thr-Gly pairs and an 80s rhythm. Could it be that the Thr-Gly repeat length is therefore critical for species specific song rhythms? To answer this, the song rhythms of 3 co-isogenic strains of *D. melanogaster*, each with a different number of Thr-Gly pairs, were examined. Any differences seen in period length between the strains would be due to the differing number of Thr-Gly pairs.

4.2 MATERIALS AND METHODS

4.2.1 Strains

The songs of 3 co-isogenic strains were recorded and analysed. Each strain had a different number of Thr-Gly repeats at the *per* locus. The analysis was performed "blind"; the strains were coded at Brandeis University in the USA and the code not revealed until the experiment was completed. Strain 7 was originally obtained from Oxford and encodes 20 Thr-Gly pairs in its gene. Strain 13 is a Canton-S strain (from M W Young, Rockefeller University) and has 23 Thr-Gly pairs. Strain 34 has 17 Thr-Gly pairs and was originally obtained from Chieti in Italy.

The 3 strains were co-isogenised at Brandeis University using

a backcrossing procedure whereby each strain was repeatedly backcrossed to an isogenic *y w* strain for 6 generations. per^+ females were mated with *y w* males and the heterozygous female progeny backcrossed to *y w* males. This was repeated for 6 generations. The resulting per^+/*y w* females were then crossed to per^+ sibling males. The homozygous per^+ female and hemizygous per^+ male progeny were extracted to form the co-isogenic strains.

4.2.2 Song recording and analysis

Song recording and analysis were carried out as described in Section 2.4, p32.

4.3 RESULTS

Tables 4.1-4.3 show the periods obtained from analysis with CLEAN, van den Berg and BMDP3R of strains 7, 13 and 34. The mean periods from the BMDP3R analysis are 54.5s, 59.4s and 56.4s for strains 7, 13 and 34 respectively - all close to the wild type value (Kyriacou & Hall, 1980). It is worth noting that slightly fewer songs from strain 7 give significant F-ratios from the BMDP3R analysis than the other two strains. Figures 4.1-4.3 show the BMDP3R, CLEAN and van den Berg analyses of one song from each strain. Note that the song of strain 34 no10 is a "climber" - the first peak of the spectrum gives a period of around 420s, but the 2nd peak value (around 52s) is taken as being the correct one.

A contingency test on the distribution of periods obtained from the CLEAN and van den Berg analyses (Table 4.4) shows that the song periods from the 3 strains are not significantly different to one another (χ^2=9.09 and 7.70, df=12 for the CLEAN and van den Berg analyses respectively). An analysis of variance performed on the periods obtained from the CLEAN and van den Berg analyses (see Tables 4.1-4.3) also shows no significant differences between the 3 strains (F=0.02; df=2,34 for CLEAN; F=0.37; df=2,34 for van den Berg). The results from all 3 strains were therefore combined, and the distribution of the periods from the CLEAN analysis is shown in Table 4.5 and Figure 4.4. The distributions of per^+ song periods (from Kyriacou & Hall, 1989) and the random song periods (see Section 2.4.3, p35) are also shown. A contingency test on the results from the CLEAN analysis (see Table 4.5) shows no significant difference between per^+ and the combined strain 7/13/34 results

Table 4.1 Song periods obtained from Strain 7 (20 Thr-Gly pairs) males by analysis with BMDP3R, CLEAN and van den Berg

FLY	BMDP3R secs (if sig)	F	df	CLEAN secs	van den Berg secs
1				71.1*	37.2*
2				59.0	60.5
3	61.1	4.4	3,26	62.2	61.1
4	57.2	3.3	3,22	58.9	57.0
5				31.0*	79.2*
6	57.8	3.7	3,29	56.4	105.2
9				31.1	31.4
10	56.1	4.2	3,21	56.0	56.3
11				59.0*	26.0*
12	48.0	3.3	3,23	47.0	48.2
13	46.5	7.0	3,20	46.7	46.7
14				56.0	57.1
15				28.0	76.9
mean	54.5			51.0	57.1
SEM	2.4			4.1	5.9

* periods obtained from the 2nd peak of the spectrogram

Table 4.2 Song periods obtained from Strain 13 (23 Thr-Gly pairs) by analysis with BMDP3R, CLEAN and van den Berg

FLY	BMDP3R secs (if sig)	F	df	CLEAN secs	van den Berg secs
2	57.8	5.5	3,28	58.2	57.9
4	68.7	4.3	3,27	68.9	68.9
5	55.5	4.8	3,20	53.9	55.4
6				41.3	25.2
7	65.3	2.9	4,27	24.7*	63.5
8	68.4	5.4	3,29	67.4	68.4
12	44.1	8.5	4,24	44.4	44.4*
14				23.5	64.5
15	70.4	3.4	3,26	70.6	70.3
16	35.5	3.3	3,30	27.8	35.7
17	69.0	6.7	3,12	66.7	67.2
mean	59.4			49.8	56.5
SEM	4.1			5.5	4.6

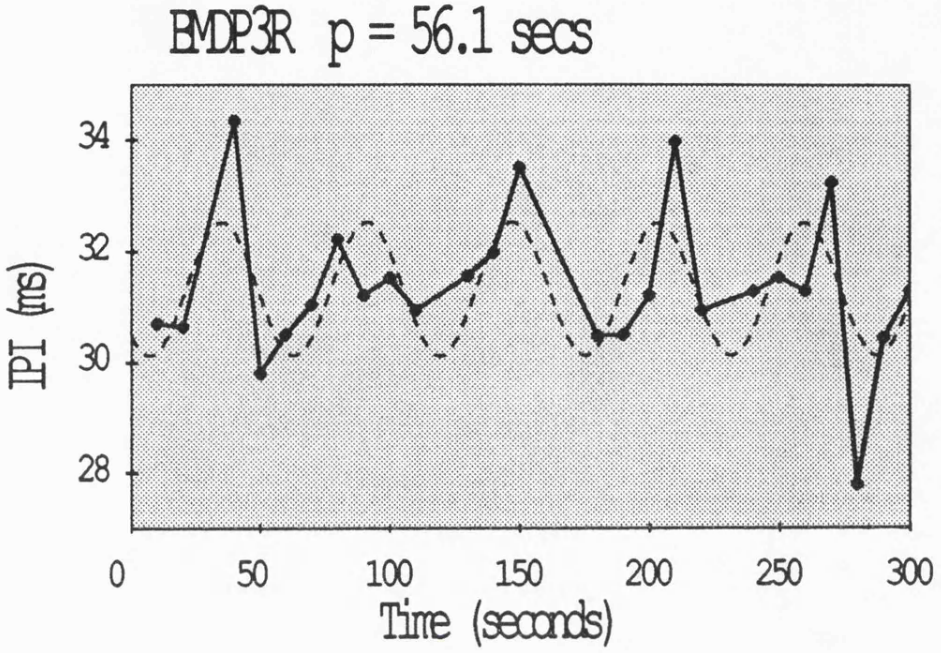
* period obtained from 2nd peak of the spectrogram

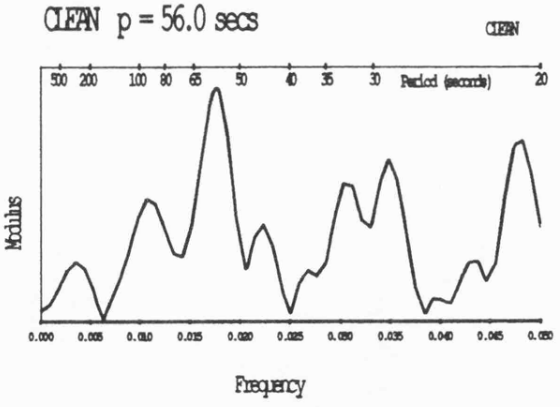
Table 4.3 Song periods obtained from Strain 34 (17 Thr-Gly pairs) by analysis with BMDP3R, CLEAN and van den Berg

FLY	BMDP3R secs (if sig)	F	df	CLEAN secs	van den Berg secs
2				21.5	33.5
3	77.7	5.7	3,31	77.6	78.0
4				23.0	23.4
5	58.6	5.2	4,24	60.0	60.2
6				34.3*	33.9*
7				31.0	31.0
8				85.3	90.1
10	51.4	14.8	4,27	51.7*	52.6*
11	52.3	11.5	4,28	51.7	53.3*
12	48.5	4.5	3,22	48.0	48.7
13	54.1	3.8	3,28	53.3	54.1
14	53.1	6.1	3,25	53.3	53.0
15	55.6	6.0	3,25	56.0	55.2
mean	56.4			49.8	51.3
SEM	3.2			5.3	5.1

* periods obtained from the 2nd peak of the spectrogram

Fig 4.1 BMDP3R, CLEAN and van den Berg analyses of Strain 7 no10





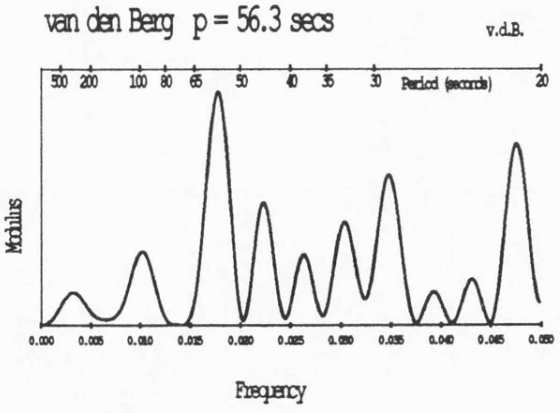
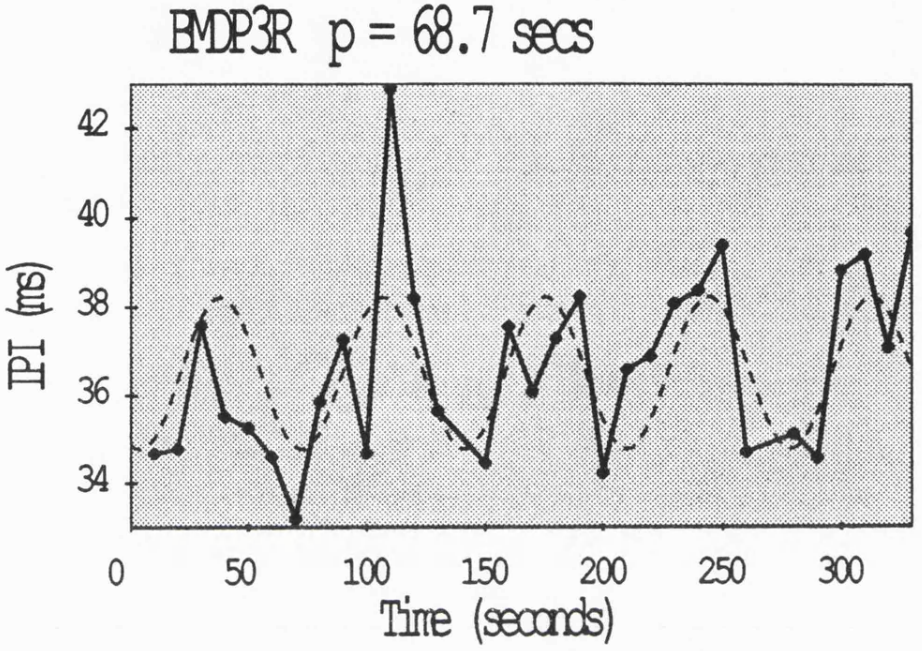
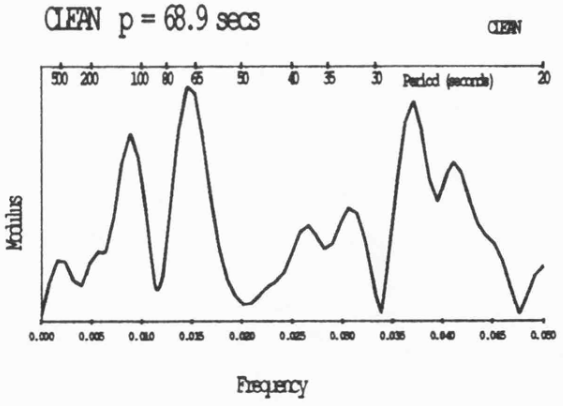


Fig 4.2 BMDP3R, CLEAN and van den Berg analyses of Strain 13 no4





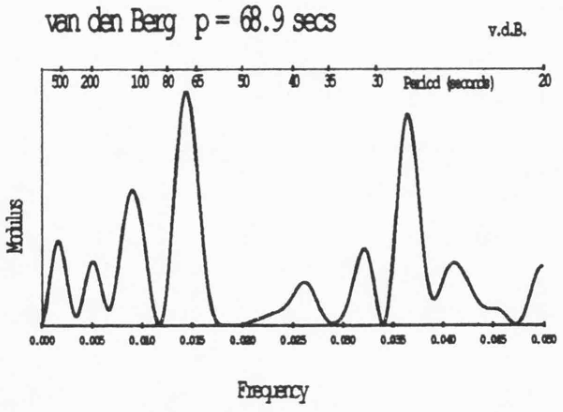
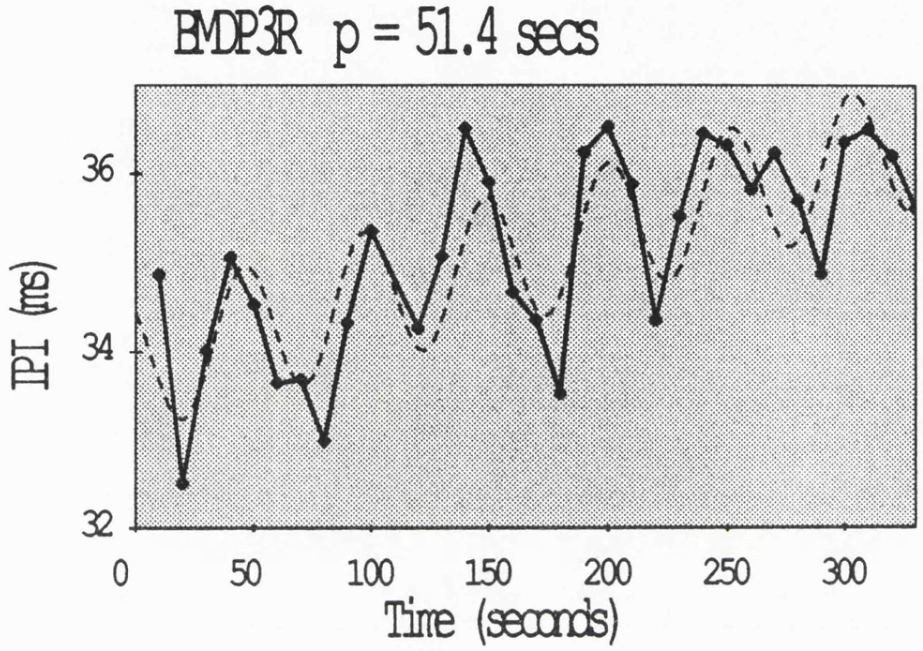
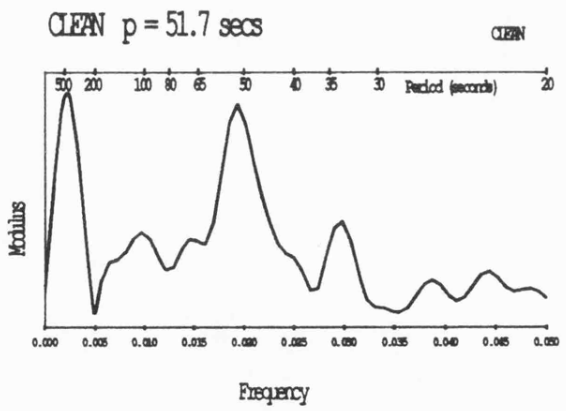


Fig 4.3 BMDP3R, CLEAN and van den Berg analyses of Strain 34 no10





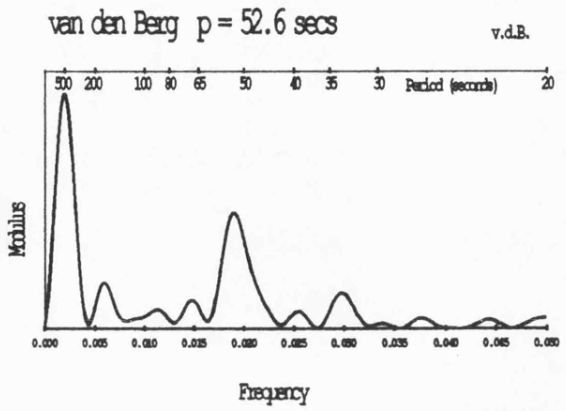


Table 4.4 Distribution of the song periods found in Strain 7, Strain 13 and Strain 34 with the CLEAN and van den Berg analyses

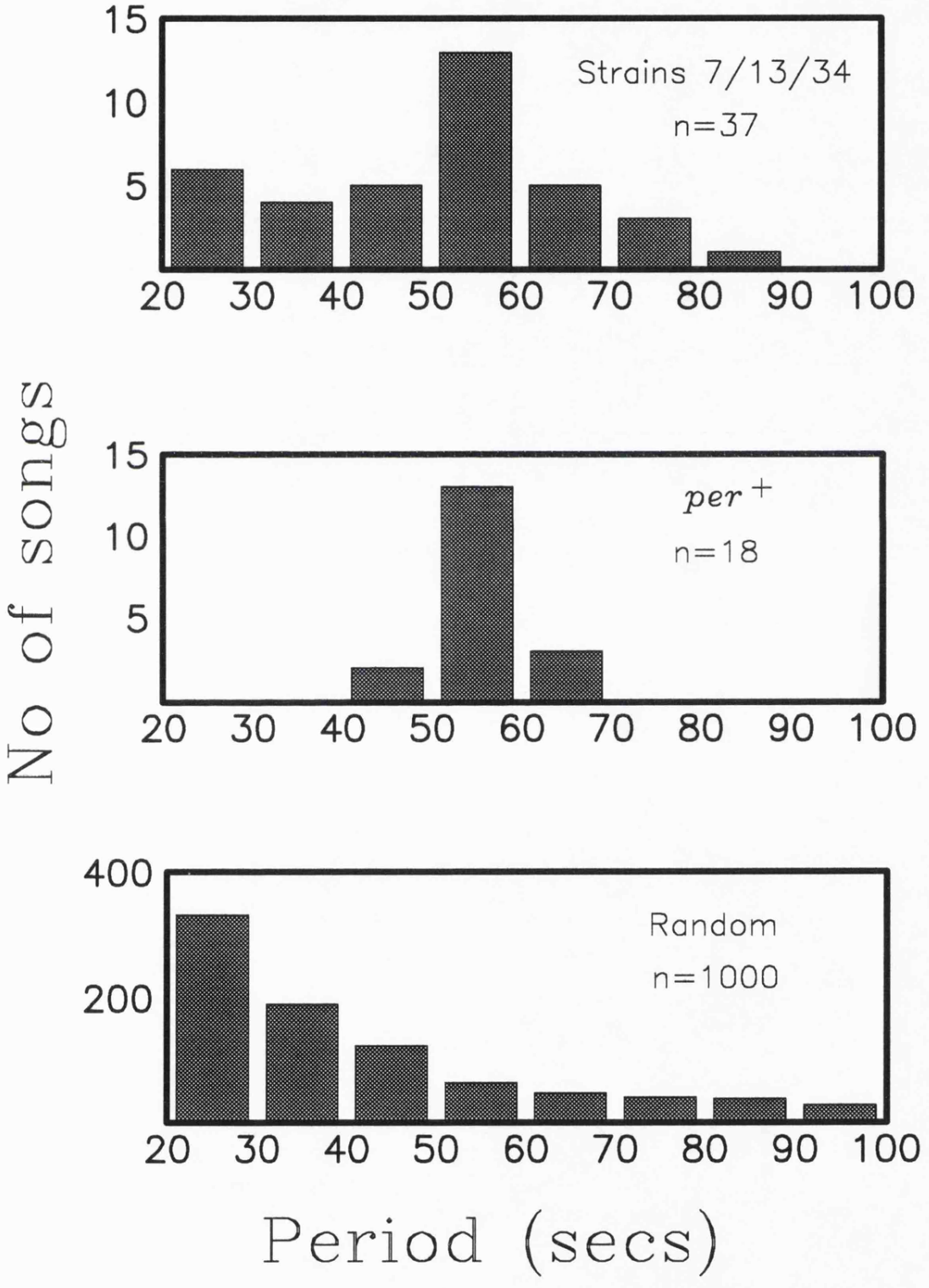
Strain	20–30	30–40	40–50	50–60	60–70	70–80	80+	Total
CLEAN								
Strain 7	1	2	2	6	1	1	0	13
Strain 13	3	0	2	2	3	1	0	11
Strain 34	2	2	1	5	1	1	1	13
van den Berg								
Strain 7	1	2	2	3	2	2	1	13
Strain 13	1	1	1	2	5	1	0	11
Strain 34	1	3	1	5	1	1	1	13

Table 4.5 Distribution of the song periods of Strains 7/13/34, per^+ and the random songs (CLEAN analysis)

Genotype	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100	100+	Total
7/13/34	6	4	5	13	5	3	1	0	0	37
*per^+	0	0	2	13	3	0	0	0	0	18
random	332	190	124	64	47	40	37	27	139	1000

* from Kyriacou & Hall, 1989

Fig 4.4 Periods of Strains 7/13/34, *per* $^{+}$ and random songs by analysis with CLEAN



(χ^2=10.47, df=6; see Table 4.5). Thus, the 3 strains all show periods in the wild type range.

It can be seen from Table 4.5 that 18 out of 37 periods fall in the 50-70s range (from the CLEAN distribution). The probability that any one random song will have a period in the 50-70s range is 0.111 (see Table 4.5). Using the binomial expansion, it is possible to calculate the probability that 18 or more songs would have periods in this range if the distribution of the songs followed that of the randomly generated songs. This probability is in the range of 1.2×10^{-8}. It is clear that the distribution of the song periods from strains 7/13/34 could not have been obtained by chance.

4.4 DISCUSSION

The three strains of *D. melanogaster* examined here do not show any differences in the periods of their song rhythms, which fall in the wild type range. Thus it does not appear that the number of Thr-Gly repeats alone can be responsible for the difference in song periods between species. This is confirmed by recent work done on 2 species of *D. simulans*, Haiti and Australia, which have 24 and 23 Thr-Gly repeats respectively. Both strains have song periods of around 35s, the normal *D. simulans* value (Wheeler *et al*, ms submitted).

Therefore it appears that the differences in song rhythm between species must be determined by amino acids elsewhere in the 700 base pair fragment which was swapped between *D. melanogaster* and *D. simulans* (Wheeler *et al*, ms submitted). This fragment can be divided into 3 intervals - the 180 base pairs upstream of the Thr-Gly repeat, the Thr-Gly repeat itself and the remaining 360 base pair region downstream to this. The amino acid sequence encoded by the 180 base pair upstream region is completely conserved between all strains of *D. melanogaster*, *D. simulans* and *D. yakuba* thus far examined, suggesting a selective constraint on this part of the protein (Thackeray, 1989; Wheeler *et al*, op cit). Thus attention is focussed on the region downstream of the Thr-Gly repeat.

Figure 4.5 shows the amino acid sequence of the Thr-Gly region of two strains of *D. simulans* (Haiti and Australia), three strains of *D. melanogaster* (Canton-S, Oregon-R and Chieti V) and *D. yakuba*. In addition to the differences in the number of Thr-Gly pairs, a number of amino acid substitutions can be seen. The three *D. melanogaster* strains

Fig 4.5 Amino acid sequence comparison of the song rhythm control region of per

```
D. sim (Haiti)                    ELDPPKTEPP  EPRGTCVSGA  SGPMSPVHEG  SGGSGSSGNF  TTASNIHMSS  VTNTSIAgtG

D. sim (Aus)  23 TG pairs                                                    -           -           tgtg
D. sim (Haiti)  24 TG pairs       gtgtgtgtgt  gtgtgtgtgt  gtgtgtgtgt  gtgtgtgtgt  gtgtgtgtGN  gtNSg----t
D. mel (C-S)  23 TG pairs                                                    -           -           tgtg
D. mel (O-R)  20 TG pairs                                                 -------        -           tgtg
D. mel (C-V)  17 TG pairs                                          ---    ----------     -           tgtg
D. yak  15 TG pairs                                                       ---------     ---------   tgtg

                                  *   *                                                     *
D. sim (Aus)                      TS SR-   T   I       S                                    D          P
D. sim (Haiti)                    TTSSR-GGSAA  VPPVTLTESL  LNKHNDEMEK  FMLKKHRESR  GRTGDKSKKS  ANDTLKMLEY
D. mel (C-S)                      AS SK-   A   I       S                                    E          T
D. mel (O-R)                      AS SK-   A   I       S                                    E          T
D. yak                            AS NYR   A   I       A                                    E          E

                                                                                          *
D. sim (Aus)                                                                              A
D. sim (Haiti)                    SGPGHGIKRG  GSHSWEGGAN  KPKQQLTLGT  DAILGAAGSA  GGAAGTGGVG  SGGAGVAGGG
D. mel (C-S)                                                                              V
D. mel (O-R)                                                                              V
D. yak                                                                                    V
```

* species specific amino acid changes (D. melanogaster relative to D. simulans)
---- amino acid deletion
gt GT repeats are in lower case for easy visualisation

are identical other than in the number of their Thr-Gly pairs, but there are 5 amino acid substitutions between the *D. simulans* strains. There are 4 species specific changes between *D. melanogaster* and *D. simulans* (marked with an asterisk in Fig 4.5). These are a threonine in *D. simulans* versus an alanine in *D. melanogaster*, an arginine versus a lysine, an aspartate versus a glutamate and an alanine versus a valine. The latter three substitutions are rather conservative changes. The change from an alanine to a threonine may result in an extra glycosylation or phosphorylation in the *D. simulans* protein. However, this threonine is one of many in the region and it is difficult to imagine that an extra threonine could make such a critical difference. Indeed the amino acid position immediately downstream has a serine in the Australia strain of *D. simulans*, but a threonine in the Haiti strain (see Figure 4.5), and yet the two strains have similar song rhythm periods (Wheeler *et al*, op cit).

However, three out of four of the amino acid substitutions discussed here cannot alone be responsible for differences in song rhythm between *Drosophila* species in general. At each of these three positions, the amino acid in *D. yakuba* is the same as in *D. melanogaster* (see Fig 4.5). In only one position (2nd from the left, marked with an asterisk in Figure 4.5) is the amino acid species specific to *D. melanogaster*, *D. simulans* and *D. yakuba*. This is not to say that only this amino acid is key to the control of species specific song rhythms. It is certainly possible that interactions between amino acids and possibly the Thr-Gly region are responsible for the characteristic song rhythm of each species. Each species may possess a specific combination of amino acids which leads to a characteristic courtship song rhythm.

The region around the Thr-Gly encoding repeat is also highly diverged in *D. virilis* and *D. pseudoobscura* (Colot *et al*, 1988). The *D. virilis* protein has only 2 tandem Thr-Gly repeats and the *D. pseudoobscura* protein has 7 Thr-Gly or Thr-Val repeats, interrupted by another sequence. Other regions of *per* sequence are highly conserved amongst species (Colot *et al*, 1988; Thackeray, 1989; Wheeler *et al*, op cit). It appears that the *per* gene may consist of several functional domains, with different domains contributing to different *per* functions. The highly variable region around the Thr-Gly encoding repeat is responsible for species specific differences in song rhythm; possibly highly conserved regions of *per* may control the locomotor activity and other rhythms which vary little between species. The song rhythm may act as a species recognition signal (Kyriacou & Hall, 1982) and thus should

vary between species. Consequently, the DNA region controlling this courtship characteristic should also have diverged between species.

In the three *D. melanogaster* strains discussed here, the number of Thr-Gly pairs has increased in increments of three (17 pairs in strain 34, 20 pairs in strain 7 and 23 in strain 13). This appears to be the result of an amplification of an 18 base pair nucleotide sequence. Two types of 6 base pair repeats also exist in the three strains. Amplification of a 6, an 18 and a 48 base pair nucleotide sequence has also occurred in the Haiti and Australia strains of *D. simulans*. The duplication or loss of such sequences could be driven by slippage mechanisms such as unequal crossover (Dover, 1989). Such mechanisms have the potential to produce multiple copies of a nucleotide sequence. However, in all strains of *D. melanogaster*, *D. simulans* and *D. yakuba* so far examined, the number of Thr-Gly pairs is between 15 and 24, perhaps suggesting an evolutionary constraint on the length of the repeat.

The importance of the Thr-Gly repeat in the function of the *per* protein is something of a conundrum. The similarity of the Thr-Gly repeat to a Ser-Gly repeat in the rat chondroitin sulphate proteoglycan is the basis for the hypothesis that *per* codes for a proteoglycan (Jackson et *al*, 1986; Reddy et *al*, 1986), which is supported by biochemical data (Reddy et al, 1986; Bargiello et al, 1987). It might be supposed, therefore, that the Thr-Gly region is vital for the function of *per*, yet it can be deleted without affecting the 24 hour locomotor activity rhythm (Yu et al, 1987b). In addition, glycosylation can be disrupted in mammals when Ser-Gly repeats are altered to Thr-Gly repeats (Hall & Kyriacou, 1990). However, in the Thr-Gly deleted construct, a few Thr-Gly pairs remain and there are several Ser-Gly pairs in the region (Jackson et *al*, 1986; Citri et *al*, 1987). These might be sufficient to maintain the proteoglycan-like character. It may be possible to remove or alter all the serines and threonines in this area, *in vitro*, in a Thr-Gly deleted gene, thus removing all potential glycosylation sites. If flies transformed with such a gene demonstrated circadian rhythmicity, then this would strongly suggest that the proteoglycan nature of *per* is irrelevant for circadian behaviour.

The *frequency* locus of *Neurospora crassa* controls the circadian rhythm found in conidiation and this gene has also been found to contain a modified Thr-Gly repeat region (McClung et *al*, 1989). It seems unlikely that such sequence similarity in two clock genes could have arisen by chance, suggesting that the Thr-Gly repeat must be of sufficient importance to clock function to be conserved throughout

evolution.

The 700 base pair region which includes the Thr-Gly encoding repeat is responsible for species specific differences in song rhythm (Wheeler et al, ms submitted). However, these differences appear to be due to amino acid substitutions downstream of the repeat and not to the number of Thr-Gly pairs. Slippage mechanisms may have been responsible for the amplification of the Thr-Gly encoding sequences; it is possible that slippage has produced a random number of Thr-Gly pairs and that the multiple copy phenomenon itself has no functional significance. The *D. virilis* protein has only two Thr-Gly tandem repeats and yet mediates a clock function. It is possible that only a few Thr-Gly pairs are necessary to maintain the proteoglycan characteristic (or other characteristic producing rhythmicity) of the *per* protein and that the "extra" repeats in other *Drosophila* species are merely accidents of DNA replication.

Chapter 5

Developmental rates of several clock mutants

5.1 INTRODUCTION

In addition to its effects on the locomotor activity rhythm of *Drosophila* adults, the *per* gene also exerts some control over the rhythm of pupal to adult eclosion. Adults tend to emerge from the pupae in a "gate" - a specific period around dawn (Saunders, 1982), producing an eclosion rhythm of about 24 hours. In constant darkness, the per^{s} and per^{L1} mutations shorten and lengthen this rhythm respectively, and it is apparently abolished in per^{01} (Konopka & Benzer, 1971). Thus the circadian clock would seem to act in the pupae as well as in the adult.

Interestingly, the *per* protein and transcript are observed at the embryonic, larval, pupal and adult stages (James *et al*, 1986; Bargiello *et al*, 1987; Saez & Young, 1988; Siwicki *et al*, 1988). However, the *per* protein is required only at the adult stage for the expression of the locomotor activity rhythm and thus does not seem to be necessary in the earlier stages in order to build clock structures (Ewer *et al*, 1988). It seems likely that *per* has some other function in these earlier stages. Timekeeping is of obvious importance during development and it is possible that biological clocks have a role to play (Snow & Tam, 1980). It was decided to investigate the effects on development time of the *per* mutations and of the *Clk6* and *andante* mutations. *Clk6* shortens the locomotor activity rhythm to around 22 hours, but has no effect on the song rhythm. The *andante* mutation lengthens the activity rhythm to around 26 hours and also seems to produce longer song cycles (see Chapter 3 for further details).

If an adult fly (except per^{01}) is ready to emerge from the pupa, it will "wait" until the next gate. However, under conditions of constant bright light, the eclosion rhythm is "damped out" and the gates disappear (Kyriacou *et al*, 1990a). This allows the development time of per^{+}, per^{s} and per^{L1} to be directly compared with per^{01}. The experiment was therefore carried out under such conditions.

5.2 MATERIALS AND METHODS

5.2.1 *per* strains

Co-isogenic strains of per^{+}, per^{s}, per^{L1} and per^{01} were created at Brandeis University by repeatedly backcrossing the strains to a *y w* strain for 6 generations. The resulting *per/y w* females were then crossed

to their per sibling males and the homozygous per progeny extracted to form the new, co-isogenic strains.

5.2.2 *andante* and *Clock*^{k06} strains

It was originally attempted to produce 3 co-isogenic strains of wild type, *andante* and *Clock*^{k06} (from now on referred to as *Clk6*). Unfortunately, this was unsuccessful due to the problem of equipment failure. Clearly, it was necessary to remove the effects of differing genetic backgrounds before comparing development time of the andante and *Clk6* mutants to that of the wild type. Figure 5.1 illustrates the strategy used to achieve this.

andante females (from J. Hall, Brandeis University) were crossed to wild type Brighton males (from L. Partridge, University of Edinburgh). The F1 progeny were allowed to interbreed to produce the F2 generation. The F2 males were then mated individually to FM7a females and then tested in the locomotor activity apparatus to ascertain whether they were wild type or andante. The FM7a/andante and FM7a/andante^{+} female progeny were backcrossed to their father, and the homozygous *andante* or *andante*$^{+}$ progeny extracted. The progeny from 3 *andante* and 3 *andante*$^{+}$ crosses were mixed together to form the 2 new strains.

The same procedure was repeated with a *Clk6 cho* strain, from J. Hall. In each pair of strains (ie *Clk6/Clk6*$^{+}$ and *andante/andante*$^{+}$) the genetic background is randomised, so that any genes which affect developmental time will be randomised across the wild type and mutant strains.

5.2.3 Experimental procedures

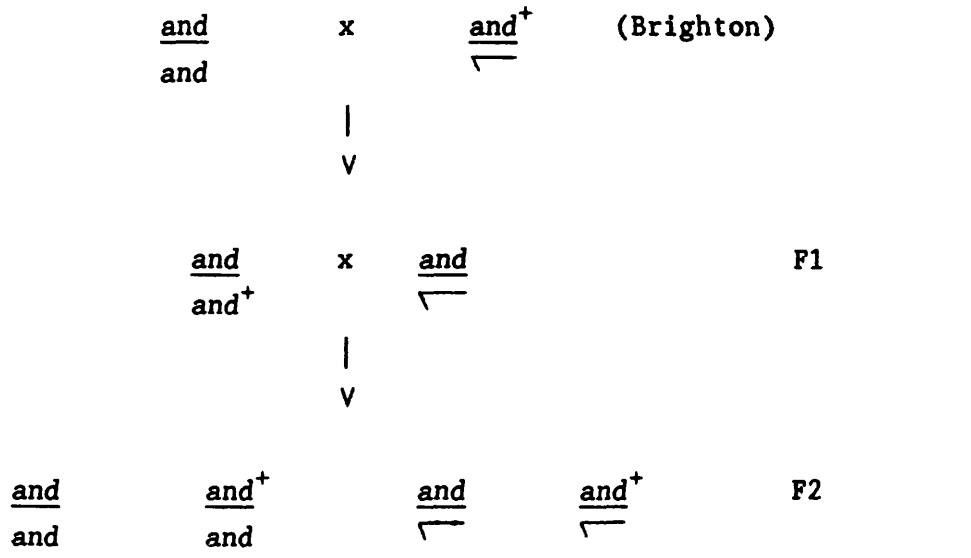
The experimental procedures are described in Section 2.5 (p36). Three sets of eggs were collected (at 11am, 1.30pm and 4.30pm) and examined for egg hatching. Another set was established from the 4.30pm collection and used to examine pupation and eclosion.

5.3 RESULTS

5.3.1 Egg hatching (egg to larva)

Egg hatching was scored for about a day and a half in order to

Figure 5.1 Procedure for randomisation of the genetic background in *andante* and *andante*$^{+}$



obtain a final score for the number of eggs which hatched. The percentage of eggs hatching was calculated from the final number of eggs hatching, rather than from the total number examined.

The results of egg hatching from the 4.30pm collection of all genotypes are illustrated in Figure 5.2. The Kolmogorov-Smirnov test (see Table 5.1) shows that *andante* eggs from the 11am collection develop significantly more slowly than *andante*$^{+}$ (χ^2=44.7; df=2). This also occurs in the eggs from the 1.30pm and 4.30pm collections (χ^2=32.29 & 16.80 for the 1.30pm and 4.30pm collections respectively; df=2).

Clk6 eggs similarly develop more slowly than *Clk6*$^{+}$ in the 11am collection (Kolmogorov-Smirnov test - χ^2=8.20; df=2). However, there are no significant differences in development time between *Clk6* and *Clk6*$^{+}$ in the 1.30pm and 4.30pm collections.

Insufficient *per*^{s} eggs were obtained from the 11am collection to make analysis worthwhile. In the 1.30pm collection, the Kolmogorov-Smirnov test shows no significant difference in the development rate of *per*^{s} and *per*$^{+}$ eggs (see Table 5.1). However, in the 4.30pm collection, *per*^{s} eggs develop significantly faster than *per*$^{+}$ (χ^2=56.53; df=2).

In the 11am collection *per*^{L1} eggs hatch more slowly than *per*$^{+}$ eggs (χ^2=9.21; df=2). However, there is no significant difference between development time of *per*^{L1} and *per*$^{+}$ eggs from the 1.30pm collection. Slower hatching of *per*^{L1} eggs versus *per*$^{+}$ eggs is again seen in the 4.30pm collection (χ^2=6.30; df=2 from the Kolmogorov-Smirnov test).

There are no significant differences in development time between *per*^{01} and *per*$^{+}$ eggs from either the 1.30pm or 4.30pm collections (see Table 5.1). Too few eggs were obtained from the 11am collection for a reliable analysis to be performed.

Table 5.2 shows the mean number (± SEM) of eggs per row (see Section 2.5.1, p36) which have hatched at specific time points. The means were calculated at the time points at which there were the largest differences between the genotypes under comparison, as in the Kolmogorov-Smirnov test. The means shown largely confirm the results of the Kolmogorov-Smirnov test, although there are a few discrepancies. For instance, in the 11am collection, the Kolmogorov-Smirnov test shows that *per*$^{+}$ develops faster than *per*^{L1}. However, the means of the 2 genotypes are very similar. The Kolmogorov-Smirnov test is based on the percentage of eggs hatching out of the total number which had hatched by the end of the experiment. The means, however, are based only on the actual number hatching, regardless of the viability.

In the *per*$^{+}$ genotype from the 11am collection, at time point 305

Table 5.1 Analysis of the development time of each genotype using the Kolmogorov-Smirnov test

Developmental stage		andante	*Clk6*	per^{s}	per^{L1}	per^{01}
Embryonic	11am	slow***	slow*	-	slow**	-
	1.30pm	slow***	NS	NS	NS	NS
	4.30pm	slow***	NS	fast***	slow*	NS
Embryonic + Larval		slow***	NS	NS	NS	fast***
Embryonic + Larval + Pupal		slow***	NS	fast*	slow***	fast***

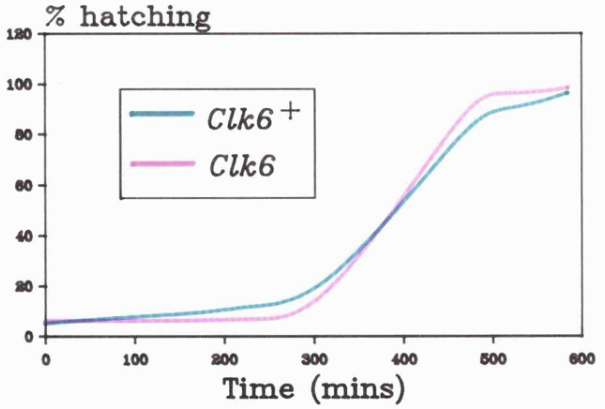
andante is compared to $andante^{+}$
Clk6 is compared to $Clk6^{+}$
per^{s}, per^{L1} & per^{01} are compared to per^{+}

NS - not significant

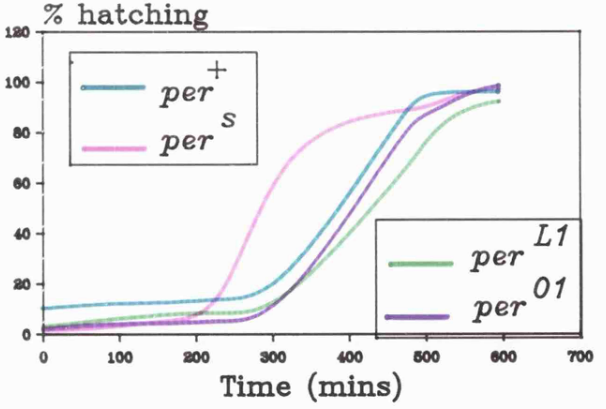
* $p<0.05$
** $p<0.01$
*** $p<0.001$

Fig 5.2 Percentage of eggs hatching across time
(from the 4.30pm collection)

Clk6



per



andante

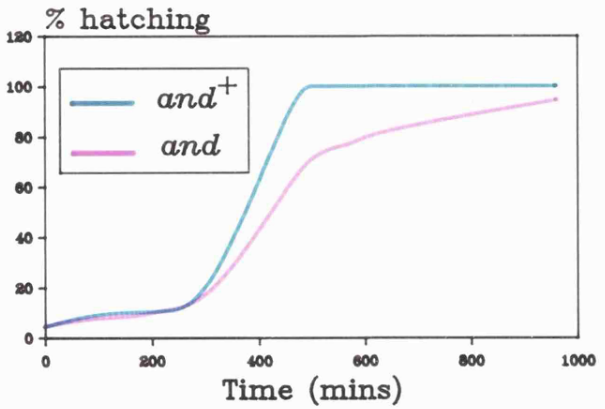


Table 5.2 A&B The mean number (± SEM) of individuals from each row of eggs* completing embryonic development at specific time points

A

	Time point (mins)	*andante*	*andante*$^{+}$	Time point (mins)	*Clk6*	*Clk6*$^{+}$
11am	230	0.92 ± 0.16	3.04 ± 0.19	120	0.69 ± 0.21	1.69 ± 0.37
1.30pm	195	1.00 ± 0.28	3.40 ± 0.21	90	0.30 ± 0.13	1.16 ± 0.18
4.30pm	470	1.86 ± 0.26	4.80 ± 0.09	475	4.30 ± 0.16	3.45 ± 0.24

B

	Time point (mins)	*per*$^{+}$	*per*^{s}	*per*^{L1}	*per*^{01}
11am	305	2.56 ± 0.19	-	2.15 ± 0.23	-
1.30pm	340	3.10 ± 0.34	3.38 ± 0.38		
	260	1.70 ± 0.35		1.60 ± 0.26	
	310	2.75 ± 0.33			4.28 ± 0.24
4.30pm	310	0.30 ± 0.13	3.75 ± 0.22		
	490	2.35 ± 0.27		3.35 ± 0.27	
	235	0.35 ± 0.13			0.25 ± 0.12

The means were calculated from the time points at which there were the largest differences between the genotypes under comparison. *per*$^{+}$ was compared to *per*^{s}, *per*^{L1} and *per*^{01}; the largest difference between each pair occurred at a different time point to the other pairs. The mean for each of these time points is shown for *per*$^{+}$; the mean for the appropriate corresponding time point is shown for the *per* mutants.

* There were 4 eggs per row in the 11am collection and 5 eggs per row in the 1.30 & 4.30pm collections.

minutes, 64 eggs have hatched out of a total of 100 (64%). However, this figure represents 87.7% of the number which actually completed the hatching process (data not shown). In the corresponding per^{L1} genotype, 45 out of a total of 80 eggs examined (56.3%) have hatched. This represents 62.5% of the number which actually completed hatching. Thus, a similar percentage of eggs hatched out of the total number examined (64% versus 56.3%). The means shown are based on this figure. However, a much lower proportion of the number to complete the hatching process had hatched from the per^{L1} genotype than from the per^{+} genotype (62.5% versus 87.7%). This is the figure on which the Kolmogorov-Smirnov test is based. Thus, differences in viability of genotypes (see Table 5.3) can lead to discrepancies between the mean number hatching per vial and the results from the Kolmogorov-Smirnov test.

Further discrepancies arise in the per^{+} versus per^{01} comparisons from the 1.30pm collection and the *Clk6* versus $Clk6^{+}$ & per^{+} versus per^{L1} comparisons from the 4.30pm collection. These can be explained in a similar fashion to above.

The viability (the percentage of eggs hatching by the end of the experiment out of the total number examined) of the eggs is shown in Table 5.3. The actual numbers of eggs hatching is shown in Appendix 3. In all collections, significantly fewer *andante* eggs complete the hatching process than $andante^{+}$ eggs, according to a chi-square contingency test (χ^2=11.53, 15.40 & 44.06 for the 11am, 1.30pm & 4.30pm collections respectively; df=1). *Clk6* and $Clk6^{+}$ have consistently similar viabilities to one another. Interestingly, per^{+} eggs have a considerably lower viablity than the eggs of the *per* mutants (χ^2=29.14 & 86.83 for the 1.30pm & 4.30pm collections respectively; df=3). This is particularly obvious in the 4.30pm collection, where the viability of per^{+} is only 50%.

Thus, per^{L1} is consistently slower (in 2 out of 3 collections) to develop than per^{+}. per^{s} appears to develop faster than per^{+}, at least in the eggs from the 4.30pm collection. However, there is no difference between per^{+} and the per^{01} mutant. Similarly, there is no consistent difference between *Clk6* and $Clk6^{+}$. *andante* develops at a slower rate than $andante^{+}$ in all three collections.

5.3.2 Larval pupation (larva to pupa)

Larval pupation was scored for 2 days to obtain a final number pupating. As in egg hatching, the percentage of larvae pupating was calculated from the final total which actually pupated, rather than from

Table 5.3 Viability of eggs, larvae and pupae of all genotypes

Developmental stage	*and*	*and*$^{+}$	*Clk6*	*Clk6*$^{+}$	*per*$^{+}$	*per*^{s}	*per*^{L1}	*per*^{01}
11am	69.2	88.5	94.2	88.5	73.0	-	84.7	-
Embryonic 1.30pm	73.5	94.0	97.0	98.0	67.0	93.2	90.0	92.9
4.30pm	56.2	96.0	90.4	81.0	50.0	94.0	93.0	88.0
Embryonic + Larval	31.0	79.6	66.0	65.2	31.5	53.5	52.5	57.5
Embryonic + Larval + Pupal	29.5	54.0	57.5	65.2	29.5	42.5	48.5	55.0

The numbers represent the percentages of eggs hatching, larvae pupating and adults emerging out of the total number examined.

the number actually set up.

A Kolmogorov-Smirnov test was again used to assess the statistical significance of differences in larval pupation rate between genotypes; the results are shown in Table 5.1. There are no significant differences between *Clk6* and *Clk6*$^{+}$ or between per^{+} and per^{s} or per^{L1}. However, *andante* is significantly slower than *andante*$^{+}$ and per^{01} has a much faster rate of pupation than per^{+} (χ^2=51.35 & 21.34 for *andante* & per^{01} respectively; df=2). The results are illustrated in Figure 5.3.

Table 5.4 illustrates the mean number of larvae pupating per vial (see Section 2.5.2, p37), at the time points at which there were the largest differences between genotypes. This occurred at time point 0 in the *Clk6* and *andante* flies. Unfortunately, the first group of larvae to hatch from these genotypes were missed. The results from Table 5.4 confirm those of the Kolmogorov-Smirnov test in the *andante*, *Clk6* and per^{01} genotypes (Table 5.1). There is a discrepancy in the per^{s} and per^{L1} genotypes; from the means both appear to pupate faster than per^{+}, although this effect is not seen from the Kolmogorov-Smirnov test. This apparent anomaly can be explained by differences in viability between the genotypes, as discussed in Section 5.3.1.

The viability of the larvae is shown in Table 5.3 and Appendix 3. The pattern of larval viability between genotypes is similar to that of the eggs. A contingency test shows no significant difference between the viabilities of *Clk6* and *Clk6*$^{+}$. Again, per^{+} is less viable than the *per* mutants (χ^2=32.88; df=3). Fewer *andante* larvae complete pupation than do *andante*$^{+}$ (χ^2=95.82; df=1). It is interesting to note that the survival rate from egg to larva is generally considerably higher than that of larva to pupa. It appears that many individuals are lost in the larval stage.

5.3.3 Adult eclosion (pupa to adult)

Table 5.1 shows the results of the Kolmogorov-Smirnov test applied to the adult eclosion rate. Eclosion was measured for 4 days and the percentages of adults eclosing were calculated from the final number to complete development. Once again, *andante* flies eclose more slowly than *andante*$^{+}$ (χ^2=50.11; df=2). There are no significant differences between *Clk6* and *Clk6*$^{+}$. Both per^{s} and per^{01} eclose more rapidly than per^{+} (χ^2=8.50 & 23.36 respectively; df=2); per^{L1} develops more slowly (χ^2=36.69; df=2). These results are illustrated in Figure 5.4.

The mean numbers of individuals eclosing per vial at specific time points are shown in Table 5.5. These results largely confirm those of

Fig 5.3 Percentage of larvae pupating across time

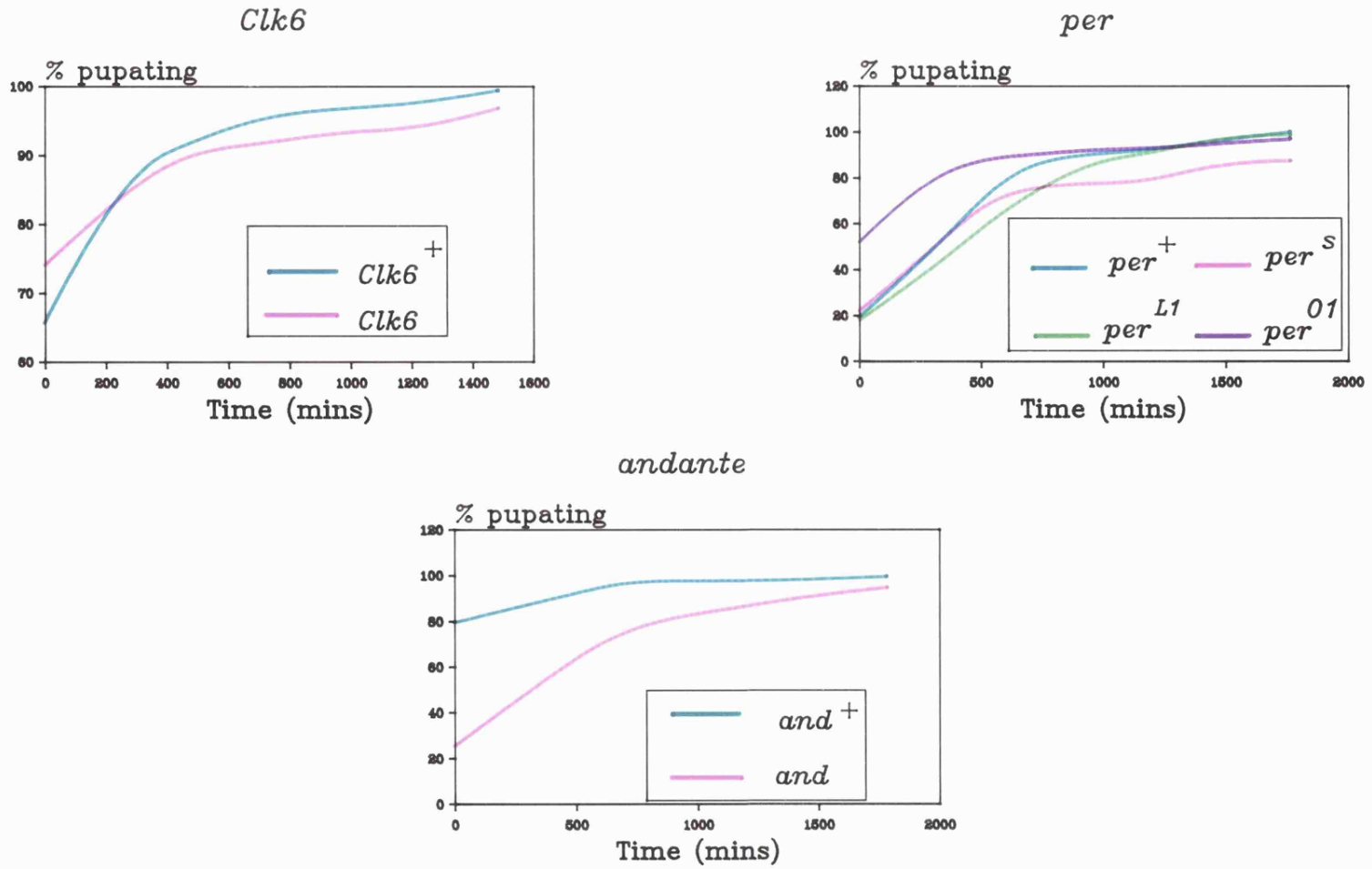


Table 5.4 A&B The mean number (± SEM) of individuals per vial* completing larval development at specific time points

A

Time point (mins)	andante	$andante^{+}$	Time point (mins)	*Clk6*	$Clk6^{+}$
0	0.80 ± 0.21	6.35 ± 0.53	0	4.90 ± 0.32	4.28 ± 0.44

B

Time point (mins)	per^{+}	per^{s}	per^{L1}	per^{01}
1205	2.90 ± 0.44	4.00 ± 0.33		
690	2.75 ± 0.44		3.85 ± 0.31	
260	1.35 ± 0.26			4.55 ± 0.45

Legend as Table 5.2

* 10 individuals per vial

Fig 5.4 Percentage of adults eclosing across time

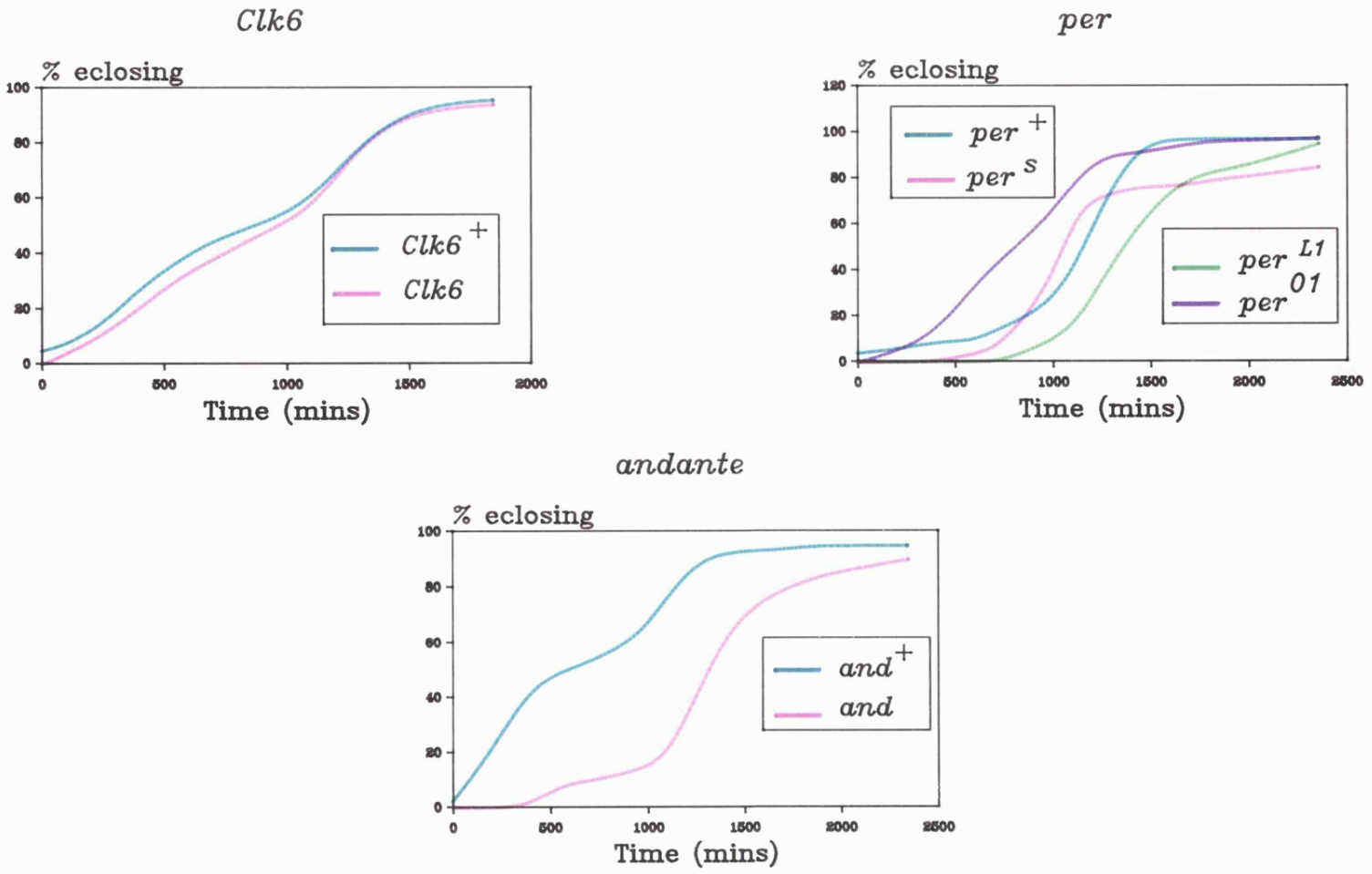


Table 5.5 A&B The mean number (± SEM) of individuals per vial* completing pupal development at specific time points

A

Time point (mins)	*andante*	*andante*$^{+}$	Time point (mins)	*Clk6*	*Clk6*$^{+}$
1100	2.45 ± 0.41	4.10 ± 0.53	370	1.05 ± 0.22	1.64 ± 0.31

B

Time point (mins)	*per*$^{+}$	*per*^{s}	*per*^{L1}	*per*^{01}
1125	1.25 ± 0.39	3.40 ± 0.44		
1455	2.80 ± 0.50		3.00 ± 0.38	
975	0.70 ± 0.21			3.45 ± 0.44

* 10 individuals per vial

Legend as Table 5.2

the Kolmogorov-Smirnov test, with the exception of the per^{+} versus per^{L1} comparison. The Kolmogorov-Smirnov test shows that per^{L1} is significantly slower to eclose than per^{+}, but at the time point shown, more per^{L1} flies per vial have actually eclosed than per^{+} flies. This, again, is due to viability differences between the 2 genotypes as explained in Section 5.3.1.

Table 5.3 shows the viability of the pupae of each genotype (see also Appendix 3). Again, *andante*$^{+}$ has a higher viability than *andante*, according to a chi-square contingency test (χ^2=24.68; df=1); *Clk6* and *Clk6*$^{+}$ have similar viabilities. Fewer per^{+} flies survive to adulthood than any of the *per* mutants (χ^2=28.73; df=3). *andante*$^{+}$ is the only genotype in which there is a large reduction in survival between the pupal and adult stage. In the other genotypes, the largest number of deaths occur between the larval and pupal stages.

5.4 DISCUSSION

The *andante* mutation appears to lengthen development time at all stages. This parallels its effects on the locomotor activity rhythm. However, it must be noted that in general mutants are less viable than their wild type counterparts and this can sometimes have the effect of lengthening development time. In fact, *andante* is allelic to *dusky*, which is a wing colour mutant (F R Jackson, pers comm to C P Kyriacou). Therefore perhaps not much significance should be attached to this result. The per^{L1} flies also develop more slowly than per^{+} at the embryonic and combined larval/pupal stages. Interestingly, the effect of per^{L1} compared to per^{+} is not so marked as the effect of *andante* compared to *andante*$^{+}$ even though the locomotor activity period is lengthened more in per^{L1} than in *andante* (28 hours versus 26 hours) compared to the wild type value of 24 hours.

As with the courtship song rhythm, the *Clk6* mutation does not appear to affect development time. This is interesting, as the mutation does exert some control over the eclosion rhythm (Dushay *et al*, 1990). *Clk6* flies emerge in gates which are about 22.5 hours apart. It seems, then, that the effects of the mutation on gating and on development time *per se* can be separated. There is no difference in the viability of *Clk6* and *Clk6*$^{+}$.

per^{s} seem to develop at a similar rate to per^{+} in the larval stage. In the pupal and embryonic stages, however, they develop more

quickly. The per^{01} individuals develop faster than per^{+} in the larval and pupal stages. This result reflects the short and ultradian periodicities found in the per^{s} and per^{01} mutants respectively. Surprisingly, per^{+} is less viable at all developmental stages than the three per mutants. This would suggest that the slower developmental rate of per^{L1} is not the result of a general reduction in fitness.

The results found here broadly agree with those found by Kyriacou et al (1990a) in a similar experiment using different co-isogenic strains of per. In that experiment, per^{s} was faster than per^{+} in the embryonic, larval and pupal stages. The per^{L1} flies took longer to develop than per^{+} in the larval and pupal stages, but not at the embryonic stage. per^{01} flies developed more quickly at the larval and pupal stages. The results from the two experiments are in the same direction throughout, but a difference in developmental time is seen more often in Kyriacou et al's experiment than in the results presented here. This could be due to the difference in genetic background between the flies used in the two experiments.

It seems likely that the shorter development time of per^{s} and per^{01} may contribute to an increased fitness in the two genotypes. This may be particularly true of per^{01} (which develops faster in the larval stage), as it might be expected to show an increased competitiveness in the larval scramble for food. Of course, any advantage from a more rapid development time may be negated by other effects, such as a lack of mating success or a poor adaptation to temporal environmental conditions. The relative fitness of the per^{+}, per^{s}, per^{L1} and per^{01} genotypes have been tested in a population cage. Each genotype was placed individually in a population cage with a large majority of FM7a flies. It was expected that the fittest per genotype would take over from the FM7a flies in the shortest time and that the least fit per genotype would take the longest time to take over. The experiment was not a success - the FM7a flies were very rapidly swamped by all the per genotypes (data in Appendix 2). There are plans to repeat the experiment in this laboratory, using a different methodology (R Costa & C P Kyriacou, pers comm).

The per gene seems to affect development time, although it may not affect all stages of development. The per transcript and protein are found in the embryonic, larval and pupal stages (James et al, 1986; Bargiello et al, 1987; Saez & Young, 1988; Siwicki et al, 1988). Saez & Young (1988) demonstrated the presence of per products in the pupal ring gland complex. This organ controls eclosion in the moth, Manduca sexta, via the release of eclosion hormone (Truman, 1985). The release of this

hormone is gated and seems to be under the control of a circadian clock. The high level of *per* expression in the ring gland complex may suggest that this organ itself provides a clock function (Saez & Young, 1988).

The *per* proteoglycan-gap junction hypothesis (Jackson *et al*, 1986; Reddy *et al*, 1986; Bargiello *et al*, 1987) may again be relevant in interpreting the developmental timing results. Gap junctions may be important during development in mediating the transfer of instructive molecules from cell to cell (Warner, 1985). During the progression of development, direct cell-to-cell communication pathways disappear between groups of cells with different developmental fates. The invertebrate embryo is progressively subdivided into developmental compartments whose boundaries are not crossed by the progeny of cells within compartments (Garcia-Bellido et al, 1976). When Lucifer Yellow dye is injected into the embryo of the mollusc *Lymnaea stagnalis* the dye passes freely from cell to cell in the early stages but at later stages the passage of the dye is prevented betwen compartments with different developmental fates (Serras & van den Biggelar, 1987).

The importance of gap junctions in development has been further illustrated by Warner (1985). By injecting antibodies to gap junction proteins into *Xenopus laevis* embryos, communication through gap junctions is prevented. Pattern formation is severely disrupted in non-communicating regions. If the *per* gene does exert its effect via gap junctional communication, then the more rapid communication seen in per^{s} cells (Bargiello *et al*, 1987) would explain the the faster development rate seen in this genotype. However, it is difficult to reconcile the faster development rate of per^{01} with the fact that intercellular communication is greatly reduced in per^{01} (Bargiello *et al*, 1987).

Timing mechanisms may control the developmental process via the cell division cycle (Snow & Tam, 1980). For instance, the appearance of the blastocyst cavity in the mouse embryo is not controlled by chronological age or by cell number (Smith & McClaren, 1977). The process is more likely to be related to nuclear events such as the number of nuclear divisions or DNA replications since fertilisation. However, the nucleus is not responsible for the timing of all developmental events. In *Xenopus* eggs, periodic changes in contractile activity are in phase with cleavage divisions, but can still persist in the absence of the nucleus (Hara *et al*, 1980). In lower eukaryotes, biochemical oscillations play an important role in the control of the cell cycle (Lloyd & Edwards, 1984). It is suggested that circadian rhythms may be derived from these very high frequency rhythms (Lloyd & Edwards, op cit). As yet, nothing is known

about the effect of the *per* gene on the cell cycle. A demonstration that *per* does affect the cell cycle might suggest a mechanism by which *per* could influence the timing of development.

Chapter 6

Selection for female song preferences

6.1 INTRODUCTION

The courtship song of the *Drosophila* male influences female mating propensity and mate choice (von Schilcher, 1976; Kyriacou & Hall, 1982). These studies have shown that both the IPI and the song rhythm appear to be important in allowing females to distinguish conspecific mates. Ewing (1988) has argued that song rhythm can have no importance in sexual selection, as courtships in the wild are probably too short to allow females to "count" IPI cycles. However, females may be aware of the rate of change of the IPI, which is dependent upon the period of the rhythm. Alternatively, individual females may be stimulated by one particular IPI value. By systematically varying his IPI, a male could stimulate a greater number of females than by producing a relatively constant IPI. The shorter the period of the rhythm, the more rapid will be the rate of change of the IPI. The species specific differences in song rhythm periods may be a reflection that females of some species require stimulation with fewer individual IPIs than females of other species.

Sexual selection by female choice is a controversial area within evolutionary biology. There is a plethora of examples in the literature (see Chapter 1) but little evidence that there is a genetic component in female choice. An exception to this is the work of Majerus and colleagues on the ladybird *Adalia bipunctata* (Majerus et al, 1982a; 1982b; 1986; O'Donald & Majerus, 1985). They showed that female ladybirds normally prefer melanic males and that the preference can be increased by selection over several generations. The preference is probably controlled by one or two major loci.

The experiment described here investigates the notion that *D. melanogaster* females demonstrate a preference for different IPI values and that the preference is inherited. Females mate most rapidly when presented with a song consisting of IPIs within her own species specific range (Kyriacou & Hall, 1982; 1986). It might therefore be expected that some females will prefer IPIs towards the bottom of that range and some will prefer IPIs towards the top of the range. A selection experiment has been performed on a population of flies in which the mean IPI of the male courtship song was about 35ms. One line of females was selected for a preference for a 30ms IPI and another line was selected for a preference for a 40ms IPI. If additive genetic variation for IPI preference exists in the base population, then it should be possible to increase the strength of selected preferences over several generations.

6.2 MATERIALS AND METHODS

6.2.1 Strains

In order to carry out a selection experiment successfully, it is necessary to begin with a heterogeneous population with as much genetic variability as possible. Two populations recently acquired from the wild and maintained in mass culture, North Carolina and Brighton (from L Partridge, University of Edinburgh), were crossed and allowed to randomly interbreed to the F2 generation. This population formed the base population for the selection procedure.

6.2.2 Experimental Procedure

Four songs of the base population were recorded and analysed and the mean IPI calculated for each song. The mean of the means was found to be 35.34ms (SE=0.551). Assuming that the mean IPI has some biological validity (von Schilcher, 1976; Kyriacou & Hall, 1982) then one would expect that females from this strain would express mating preferences for IPIs focussed broadly around this value. It was therefore decided to select with simulated songs with a constant IPI 5ms above and below the mean of the base population. Thus, the Hi line was selected with a constant 40ms IPI and the Lo line with a constant 30ms IPI.

The selection procedure was carried out as described in Section 2.6.1, p38. In Experiment 1, 6 generations of selection were carried out; the IPI preferences of selected females were investigated in generations 2, 3, 4 and 6. In Experiment 2, again 6 generations of selection were performed; the response to selection was measured in generations 1, 2, 3, 4 and 6.

6.3 RESULTS

6.3.1 Experiment 1

The flies were first tested for their response to selection in Generation 2, as described in Section 2.6.3, p40. The percentage of flies mating in each generation is shown in Figs 6.1-6.7. The Mann-Whitney test was used to assess the statistical significance of

differences in mating speed between flies which were stimulated with a 40ms IPI and those which were stimulated by a 30ms IPI. The z-scores from this test are shown in Table 6.1. Note that 25 z-scores are presented in this table. One would expect that at least one z-score would be significant at the 0.05 level simply by chance. Therefore it is also useful to consider the significance of each score at the 0.01 level; in this case, only one score remains significant.

It sometimes appears that there is a discrepancy between the percentage of flies mating (shown in Figures 6.1-6.13) and results from the Mann-Whitney test (see Tables 6.1 and 6.2). This can be explained by the fact that the Mann-Whitney test takes the individual mating speeds of the flies into account. It is possible that, from one selection line, 10 flies mate with both songs after 20 minutes, but that mating is much faster with one song than with the other. This would be revealed by the Mann-Whitney test but not by the percentage of flies mating as shown in the Figures.

In Generation 2 the HiA females mate faster with a 40ms IPI than with a 30ms IPI, although the result is not significant according to the Mann-Whitney test (see Table 6.1). In Generation 3, however, the opposite occurs and the HiA females mate faster with a 30ms IPI, although the result is again not significant. In Generations 4 and 6, these females mate equally well with both songs. These results are illustrated in Figure 6.1.

Figure 6.2 shows that LoA females "prefer" a 30ms IPI in Generation 2 (not significant according to Mann-Whitney), but mate equally well with both songs in Generations 3 and 4. No flies were tested in Generation 6. The ConA females mate equally well with both songs in Generation 2, but mate significantly faster with a 30ms IPI in Generation 3. In Generation 4, however, they seem to "prefer" a 40ms IPI and this result is also significant. Although in Generation 6 almost the same percentage of ConA females mate within 20 minutes with both songs (see Figure 6.3), the Mann-Whitney test shows that mating is faster with a 30ms IPI (not significant - see Table 6.1).

The HiB females also mate faster with a 40ms IPI in Generation 2 (not significant according to the Mann-Whitney test) but no preference for either song is seen in Generation 3. In Generation 4, they mate faster with a 30ms IPI, but the result is not significant. No difference in mating speed is seen between the two songs in Generation 6. Figure 6.4 illustrates the results fom the HiB females.

In Generation 3, the LoB females mate faster with a 40ms IPI

Table 6.1 z-scores from Mann-Whitney test at Generations 2, 3, 4 and 6 (Experiment 1)

Selection line	z-scores			
	G2	G3	G4	G6
HiA	-1.36	1.41	0.82	0.41@
LoA	1.06	-0.2	0.73	no result
ConA	-0.07	2.41*	-2.13*	1.71
HiB	-1.59	0.61	1.74	-0.04
LoB	no result	-1.94	0.47	-2.36@*
ConB	-0.92	no result	-0.34	-0.98
Base	1.89	2.70**	0.89	-2.34

negative score indicates faster mating with a 40ms IPI

* indicates $p<0.05$

** indicates $p<0.01$

@ indicates only 10 flies tested

Fig 6.1 Percentage of HiA flies mating at 20 mins in each generation (experiment 1)

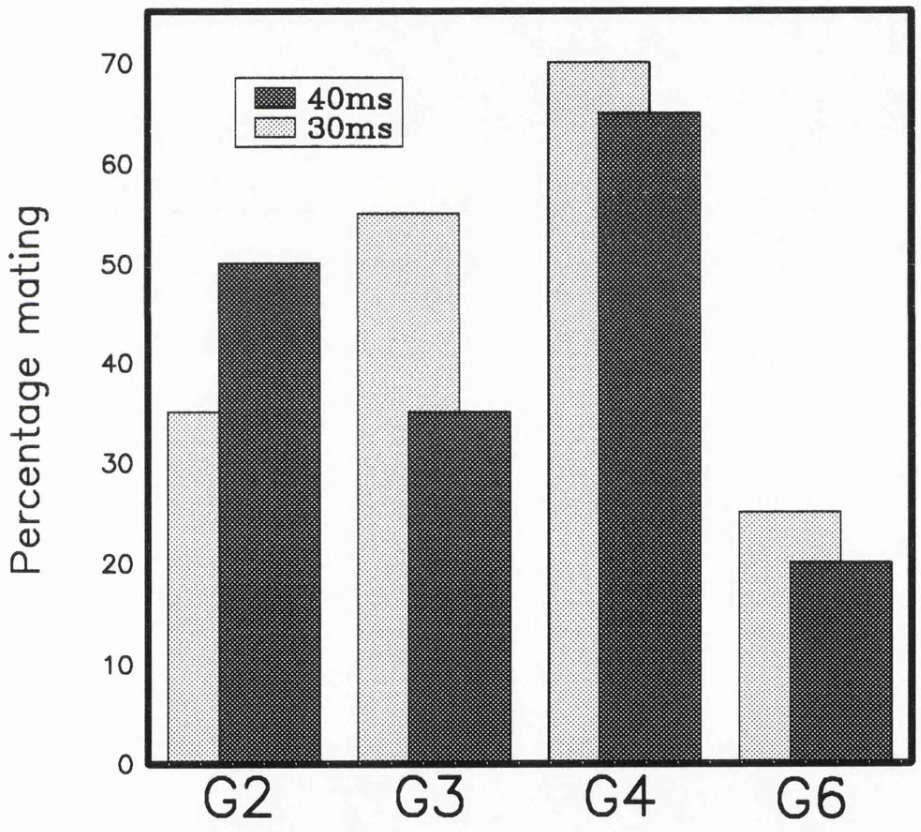


Fig 6.2 Percentage of LoA flies mating at 20 mins in each generation (experiment 1)

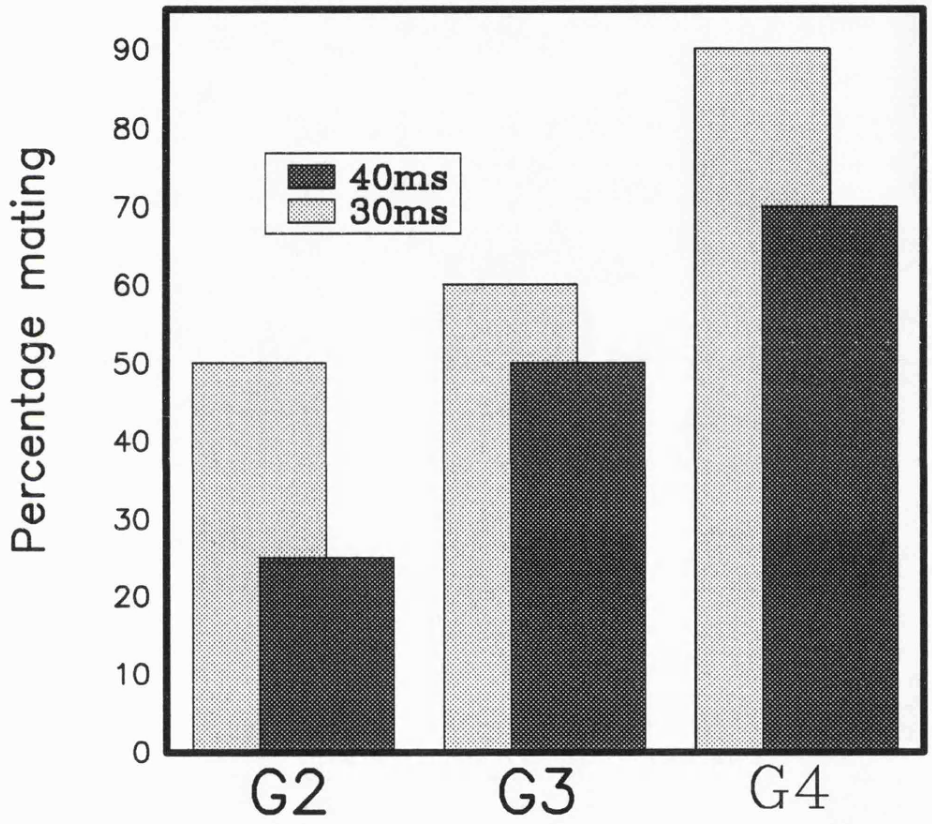


Fig 6.3 Percentage of ConA flies mating at 20 mins in each generation (experiment 1)

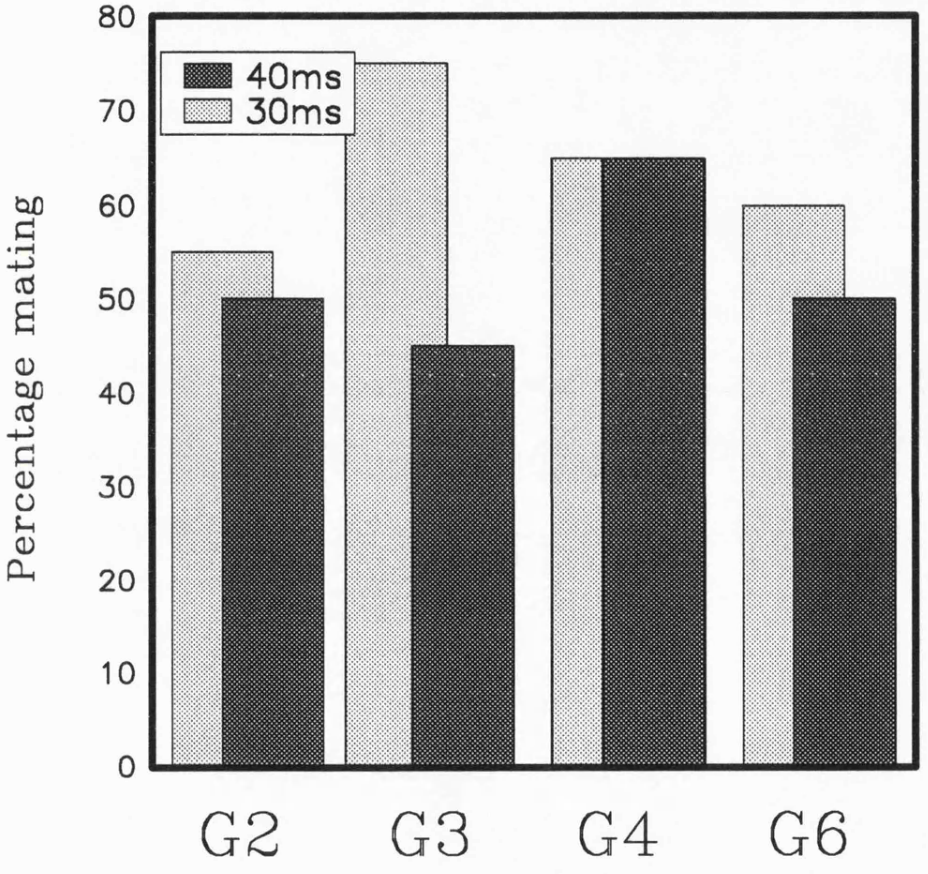


Fig 6.4 Percentage of HiB flies mating at 20 mins in each generation (experiment 1)

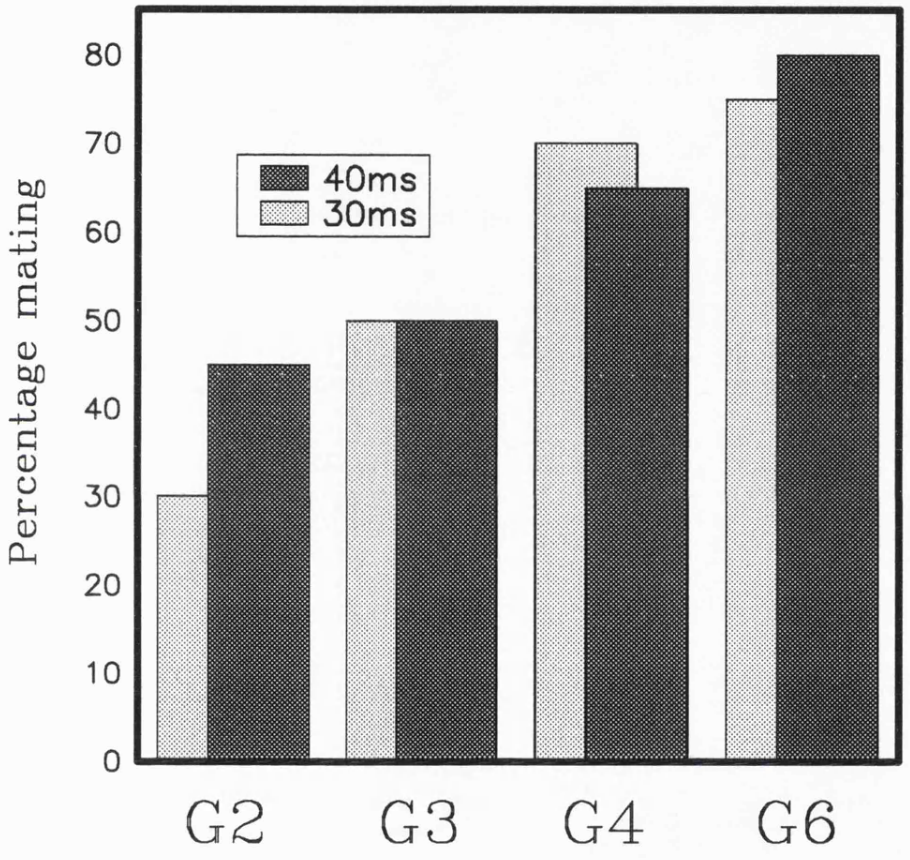


Fig 6.5 Percentage of LoB flies mating at 20 mins in each generation (experiment 1)

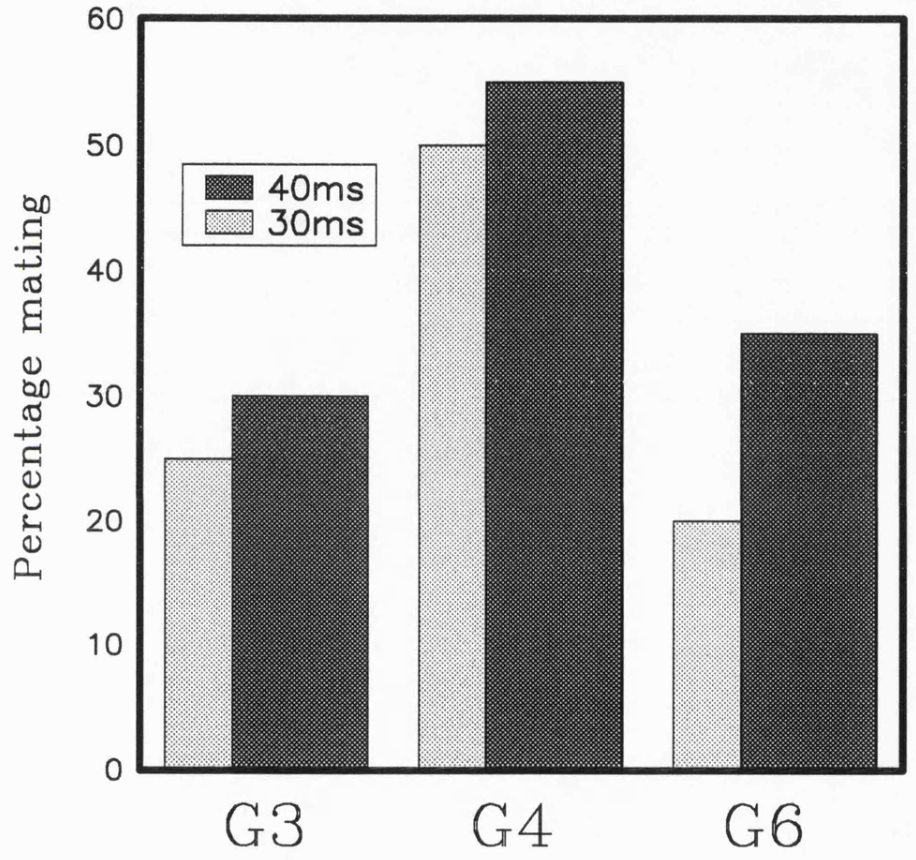


Fig 6.6 Percentage of ConB flies mating at 20 mins in each generation (experiment 1)

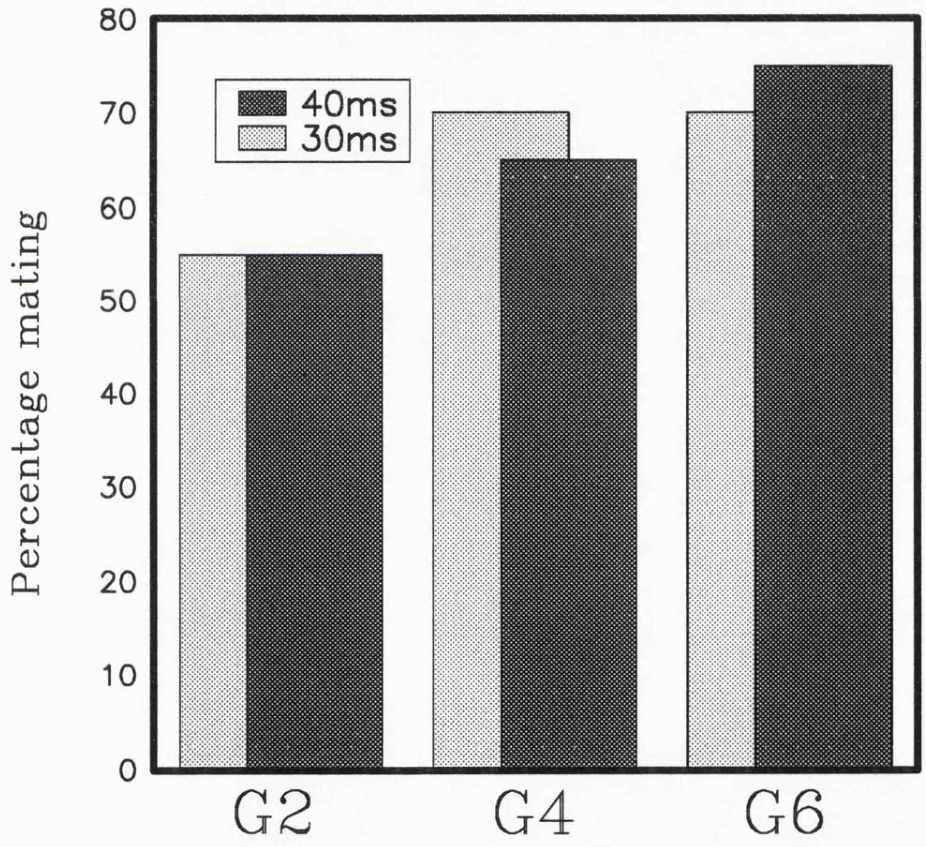
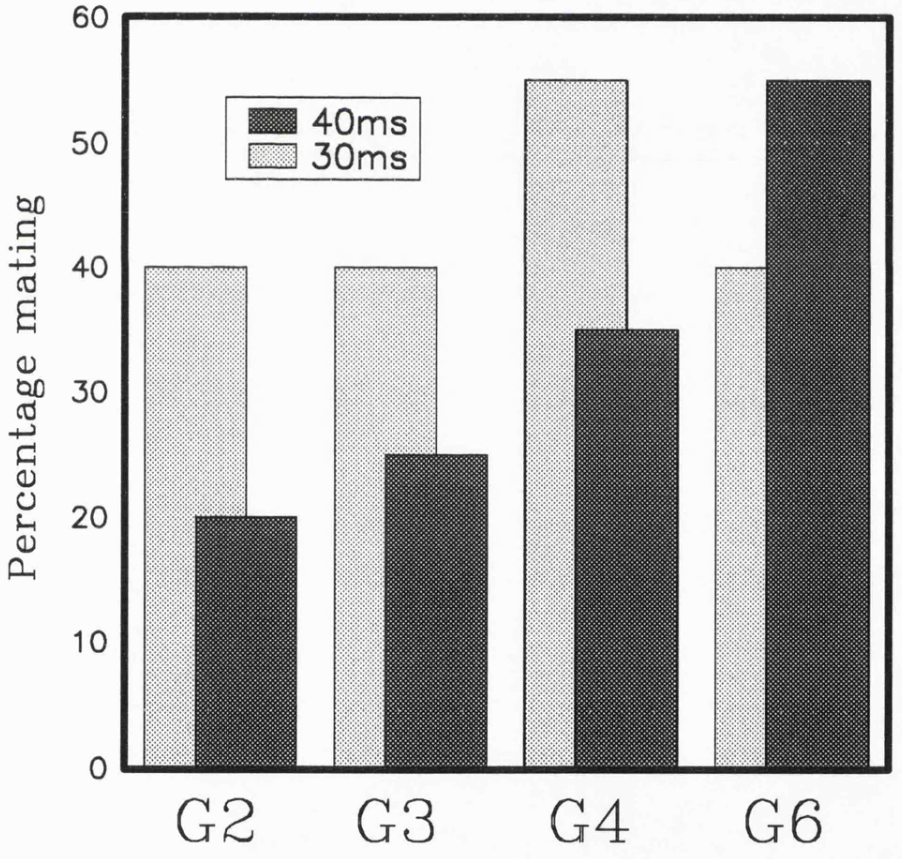


Fig 6.7 Percentage of Base flies mating at 20 mins in each generation (experiment 1)



and this result narrowly misses significance. There is no difference in mating speed between the two songs in Generation 4, but the LoB females again mate faster with a 40ms IPI in Generation 6 and this result is statistically significant according to the Mann-Whitney test. The results from the LoB females are shown in Figure 6.5. Figure 6.6 shows that ConB females mate equally well with both songs in Generations 2, 4 and 6. No result was obtained from Generation 3.

In Generations 2 and 3, the Base females mate faster with a 30ms IPI, and the result from Generation 3 is significant according to the Mann-Whitney test. In Generation 4, these females mate at approximately the same speed with both songs, although they demonstrate a non-significant preference for a 40ms IPI in Generation 6. These results are shown in Figure 6.7.

Some of the results from Generation 2 suggest that mating preferences for specific IPIs may have been selected, as the mating speeds with both songs were in the predicted directions. However, no clear pattern has emerged from the results of Generations 3, 4 and 6. It is obvious that overall mating speed becomes faster in the later generations, and this is illustrated in Figs 6.1-6.7. Only in the ConA flies does mating not appear to become faster, although in Generation 6 of the HiA line mating is very slow. Interestingly, mating speed also increases slightly in the Base line females, which were not subjected to any selection procedure. As described in Section 2.6.1 (p38), the first 7 females to mate during the selection procedure produced the next generation, therefore genes for fast mating in general would be selected as well as genes for mating preference. It is possible that the more subtle effect of mating preference has been masked by fast mating in general in the later generations.

For this reason the selection was restarted using a slightly different procedure which was designed to avoid selection for 'fast mating' genes (see Section 2.6.2, p39).

6.3.2 Experiment 2

The flies were tested for their response to selection in Generations 1, 2, 3, 4 and 6. The z-scores from the Mann-Whitney test are summarised in Table 6.2 and the percentage of flies mating are shown in Figs 6.8-6.13. The preferences of the Base population were not tested in this experiment, as the stock unfortunately died at a very early stage of the experiment. A larger scale test was performed in

Generation 6. Three replicates of the test procedure described in Section 2.6.3 (p40) were carried out in the HiA females; 4 replicates were carried out in the other lines. Between 16-20 females were tested in each replicate.

In Generation 1, more HiA females mate with a 40ms IPI, although the mating speeds are similar according to the Mann-Whitney test. In Generation 2, mating speeds are approximately equal with the two songs, although in Generation 3, the females significantly prefer a 30ms IPI. The HiA flies do not discriminate between a 30ms IPI and a 40ms IPI in Generation 4, but show a non-significant preference for a 40ms IPI in Generation 6. These results are illustrated in Figure 6.8.

LoA females mate faster with a 30ms IPI in Generations 1 and 2, although a very similar number actually mate within 20 minutes (see Figure 6.9). However, faster mating with a 40ms IPI is obtained in Generation 3. In Generations 4 and 6, the LoB females do not significantly discriminate between the two songs.

ConA females show a "preference" for a 30ms IPI in Generations 1 and 2 (statistically significant in Generation 2). However, in Generation 3, these females mate marginally quicker with a 40ms IPI. As seen in Figure 6.10 and Table 6.2, ConA females do not significantly discriminate between a 30ms and a 40ms IPI in Generations 4 and 6.

Figure 6.11 shows the percentages mating in the HiB females. In Generation 1, these flies mate significantly faster with a 40ms IPI, but the opposite is seen in Generation 3, where a non-significant preference for a 30ms IPI is seen. No discrimination between the two IPIs is seen in Generation 4, but a significant "preference" for a 40ms IPI is found in Generation 6.

In Generation 1, LoB females mate significantly faster with a 40ms IPI. However, mating is faster with a 30ms IPI in Generations 2 and 3, although the results are not significant. No discrimination between the two songs is seen in Generations 4 and 6. The results are illustrated in Figure 6.12.

ConB females mate faster with a 40ms IPI in Generation 1 and with a 30ms IPI in Generation 3, although these results are not significant. In Generation 4, these females show no discrimination between songs, but a non-significant "preference" for a 40ms IPI is seen in Generation 6.

It appears that the selection may have again succeeded in the early generations, although the results are less clear cut than in Experiment 1. Despite the fact that this experiment was designed to

Table 6.2 z-scores from Mann-Whitney test at Generations 1, 2, 3, 4 and 6 (Experiment 2)

Selection line	z-scores				
	G1	G2	G3	G4	G6
HiA	-0.64	0.75	3.15**	0.89	-1.10
LoA	1.41	2.17*	-3.18**	-0.40	-0.18
ConA	1.36	2.02*	-0.84	1.40	-0.43
HiB	-2.23*	no result	1.45	0.31	-2.1*
LoB	-2.00*	1.05	1.38	0.64	0.72
ConB	-1.25	no result	1.10	0.29	-1.57

negative score indicates faster mating with a 40ms IPI

* indicates $p<0.05$

** indicates $p<0.01$

Fig 6.8 Percentage of HiA flies mating at 20 mins at each generation (experiment 2)

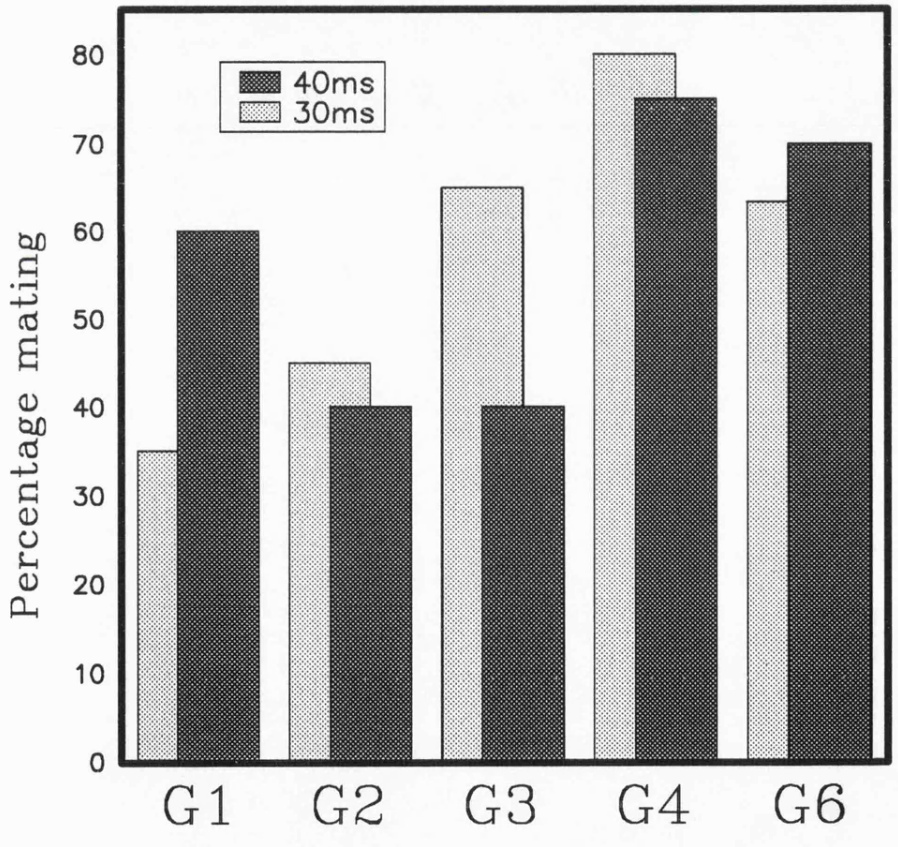


Fig 6.9 Percentage of LoA flies mating at 20 mins in each generation (experiment 2)

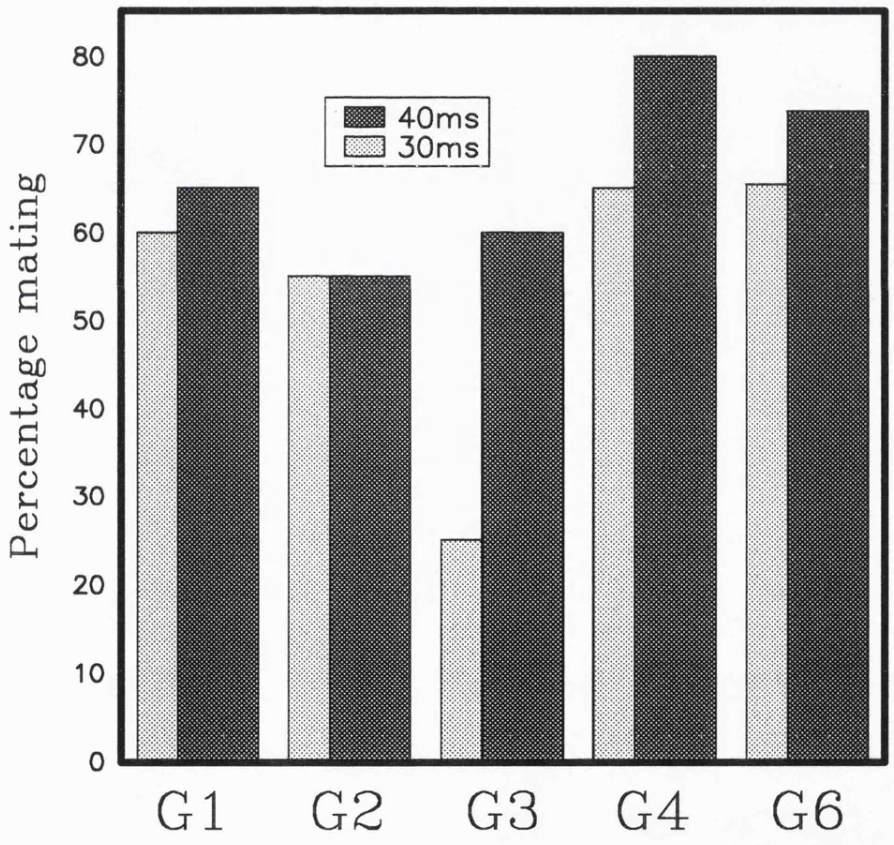


Fig 6.10 Percentage of ConA flies mating at 20 mins in each generation (experiment 2)

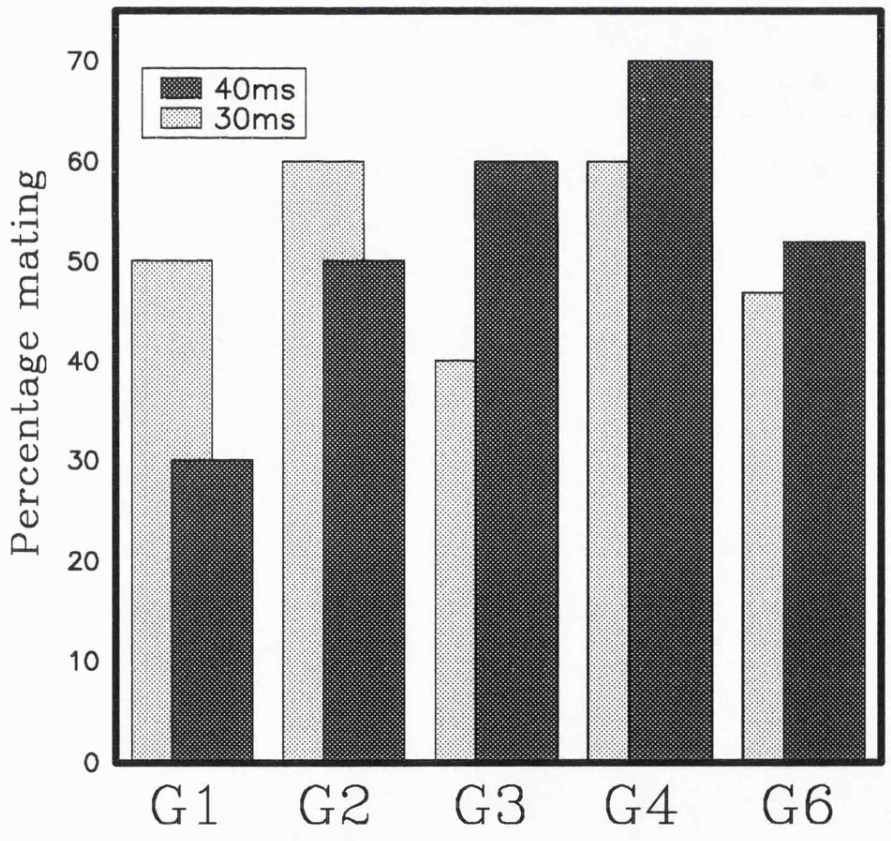


Fig 6.11 Percentage of HiB flies mating at 20 mins in each generation (experiment 2)

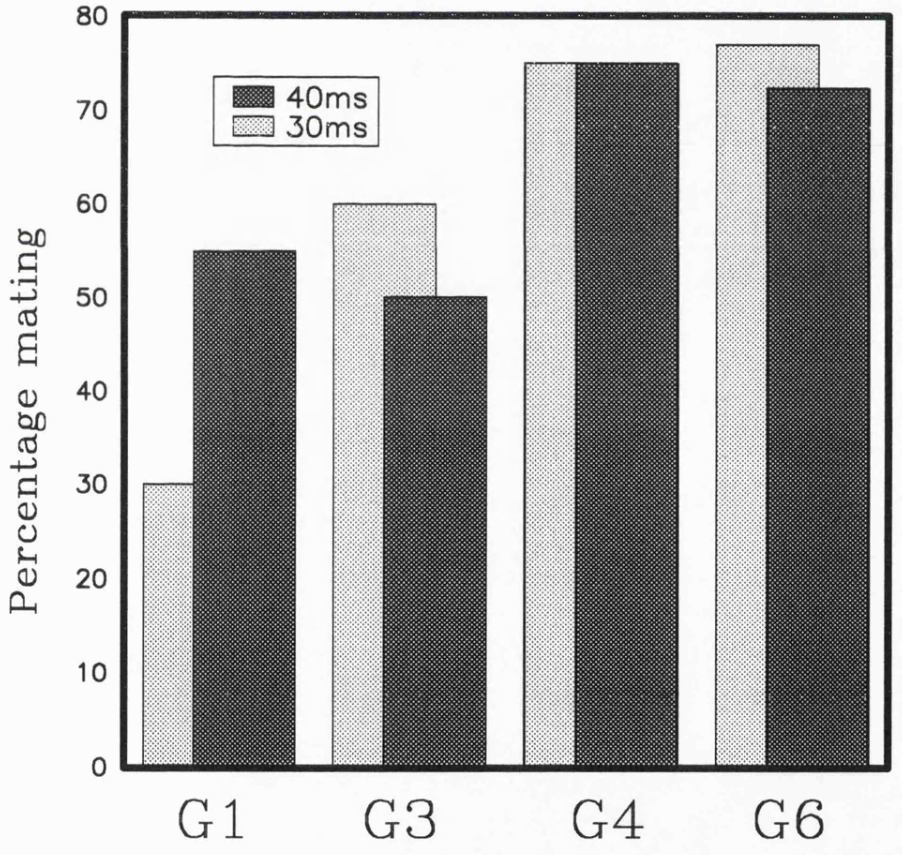


Fig 6.12 Percentage of LoB flies mating at 20 mins at each generation (experiment 2)

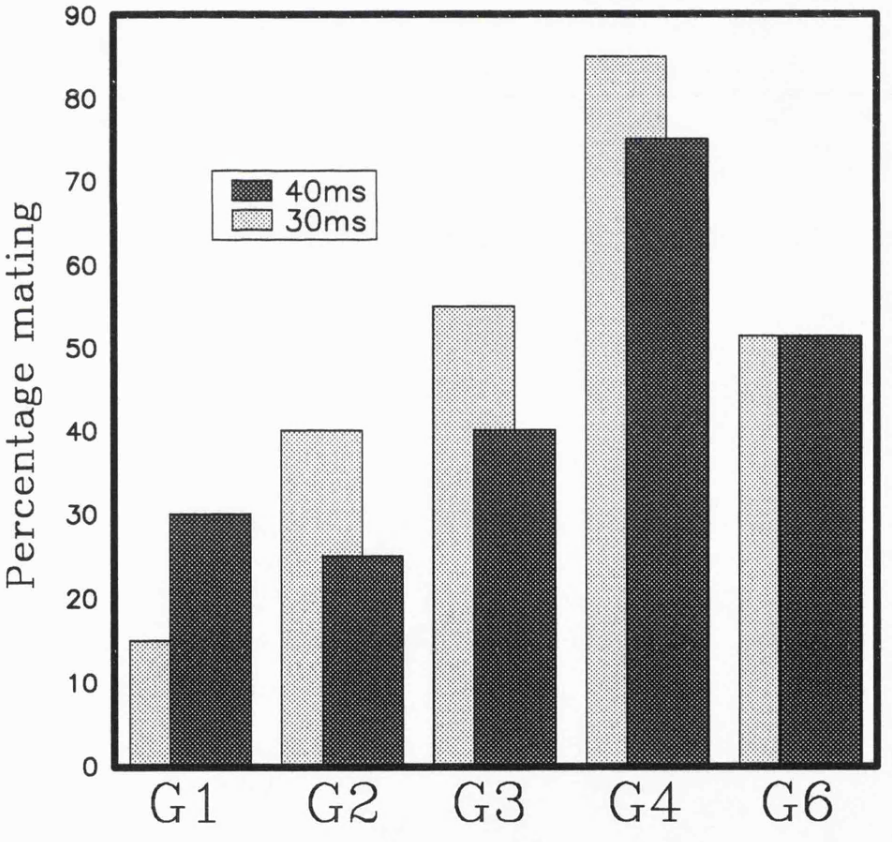
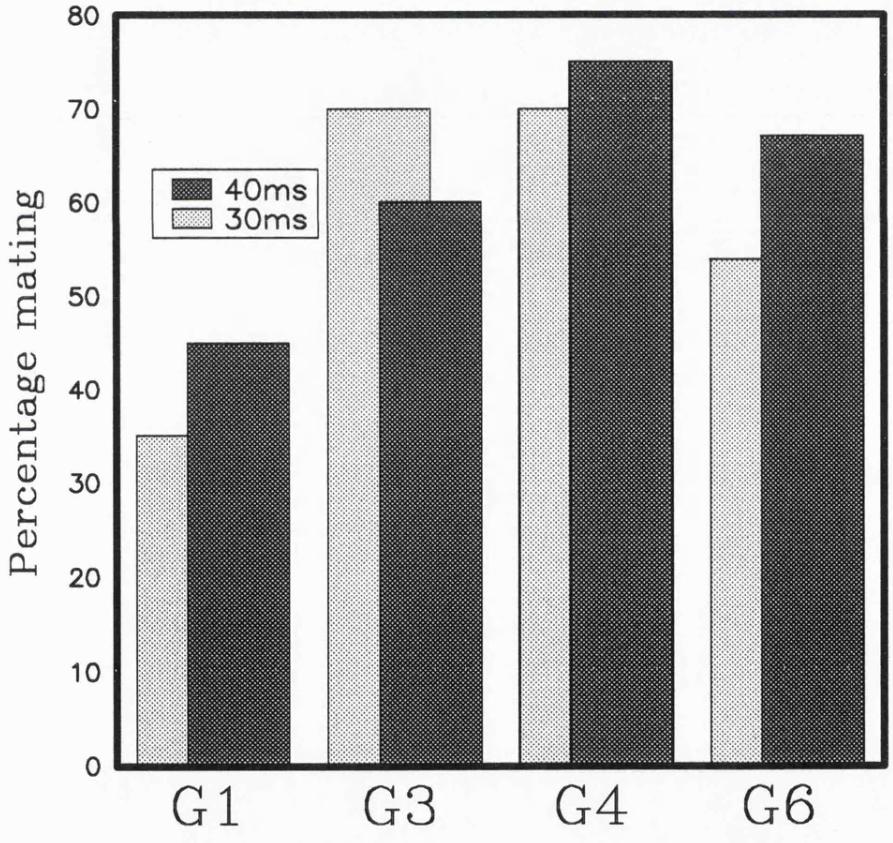


Fig 6.13 Percentage of ConB flies mating at 20 mins in each generation (experiment 2)



prevent the selection of "fast-mating genes", an increase in mating speed is seen in the HiA, HiB and LoB lines.

6.4 DISCUSSION

The selection experiment has attempted to establish 4 lines of flies, 2 selected for female preference for a 30ms IPI (Lo lines) and 2 selected for female preference for a 40ms IPI (Hi lines). If such a preference had been selected, then Hi line females would be expected to mate faster when stimulated with a 40ms IPI than when stimulated with a 30ms IPI. Conversely, Lo line females should mate faster with a 30ms IPI than with a 40ms IPI. The control lines should show no sustained preference for either IPI.

In Generation 2 of Experiment 1 all selected lines show the expected result, although the base population mates slightly faster with a 30ms IPI. Perhaps the base population has a natural preference for a 30ms IPI, but equally the result may have been obtained by chance, as only a small number of flies were tested. These results are encouraging, but unfortunately, the preferences established in the selection lines disappear in later generations. There is no sustained preference for either IPI in any of the lines and no conclusions can really be drawn from these data.

It is noticable that mating speed is much faster in later generations than in generation 2. The design of the experiment was such that the females which mate most rapidly are selected to form the next generation. It has been assumed that rapid mating implies a preference for the current song condition. However, it is almost certain that some females will mate rapidly regardless of song. It seems likely that genes for fast mating in general have been selected. Thus any IPI preference may have been masked by general fast mating in later generations - if all the flies are mating very rapidly then the preference may be swamped.

For this reason, the selection procedure was changed to avoid selecting for fast mating (see Section 2.6.2, p39). In Generation 1 of Experiment 2, both HiA and HiB females mate faster with a 40ms IPI than with a 30ms IPI. LoA females mate faster with a 30ms IPI. LoB females give an anomalous result, preferring a 40ms IPI. Of course, there is no reason to expect that the selection will work equally well in all lines. It appears, then, that the selection may again have succeeded in the

early generations. This would suggest that a major locus for IPI preference is involved.

In subsequent generations, however, this early result was again not sustained. It will be noticed that the difference between mating speeds at the two IPI values are rarely statistically significant, although they often appear quite obvious. This is because such small numbers have been used in the test procedure. Larger samples have been impossible to obtain due to the extremely labour intensive nature of the selection and stock maintenance procedures. In generation 6 the stock was built up so that a large number of flies could be tested. However, this did not have the desired effect of resurrecting the preferences seen in generation 1.

There are several major problems with the design of Experiment 2. It was intended to eliminate the problem of the selection of "fast-mating" genes, but has apparently failed to do so in the HiA, HiB and LoB lines. Any preferences in these lines are likely to be hidden by the effect of fast mating. It is evident that throwing out all females to mate in the first 6.5 minutes is insufficient to prevent selection for fast mating. In addition, allowing females to hear the song with which they are <u>not</u> being selected (ie playing a 30ms song to Hi line females before switching to a 40ms song - see Section 2.6.2, p39) may have caused problems. It is possible that some females may remember the "wrong" song and be stimulated to mate even when they no longer hear the song. Evidence from Kyriacou & Hall (1984) suggests that females' memory of songs decreases after about one minute. However, the ability to remember songs may vary from strain to strain; the effect of song on mating after a time delay was not tested in the strain used in this experiment.

The problem of selection for overall fast mating is associated with both Experiment 1 and Experiment 2, as is the problem of the small numbers of flies tested in each line. Subsequent experience (Chapters 7, 8 & 9) has shown that much variability exists between individual trials of mating experiments and that large numbers of flies must be tested if meaningful results are to be obtained. It was originally thought that testing two selection lines from each song condition over several generations would remove the problem of inter-trial variability. However, if the experiment is to be repeated, it would be preferable if larger numbers of flies could be tested.

The experiment has set out to investigate two theories - firstly that genetic variability for female IPI preference exists and

secondly that individual females are best stimulated by a particular IPI value. On reflection, it may have been preferable to have concentrated on the former theory. It is known that groups of females respond better to a rhythmic song than to one with a constant IPI (Kyriacou & Hall, 1982; 1986). Of course, this result could be a reflection of the fact that the rhythmic song would stimulate a larger number of females simply by varying the IPI. Alternatively, females may actually detect the rhythmic quality of a song and mate less well when hearing songs without this quality. There is some evidence that this may be the case from later experiments performed in this thesis. For this reason, it may be better to select with two rhythmic songs, one with a high mean IPI and one with a low mean IPI.

One of the major problems with the methodology of this experiment was the choice of strains used to form the Base population. Although the North Carolina and Brighton strains appear quite vigorous, when crossed there was a very high death rate. This meant that large numbers of flies had to be collected for experimental procedures to allow for many deaths. In addition, there was a large number of spontaneous mutations, mainly odd-shaped wings and yellow-ish body colour. It is possible that some kind of transposable element has been mobilized in this cross. It is not recommended that this combination of strains be used in further experiments.

If this experiment could be re-attempted and female IPI preferences selected, then this would be a great step forward in dissecting the genetic component of female mate choice. The work of Majerus and colleagues is the only well studied example of the genetics of female choice, but the ladybird is not an ideal model for genetical research. If genes for female mate preference could be isolated in *Drosophila*, then the whole armoury of genetic techniques available in this organism could be used to study these genes. It appears from the early response to selection that only a small number of major loci may be involved. It should be possible to map these loci and perhaps study them at the molecular level.

Another interesting question to arise from this experiment is how would males from each selection line sing? Alexander (1962) suggested that the courtship song of a male and the receptivity of the female to that song should evolve together. This could occur if the same genes controlled song production in the male and reception of the song in the female. This sort of "genetic coupling" may be seen in crickets (Hoy *et al*, 1977). Thus it might be expected that males from

the Hi lines may sing with a generally higher IPI than wild type, and males from the Lo lines with a generally lower IPI. This would be good evidence for the existence of genetic coupling, which at present is rather meagre.

The results from this experiment are tantalising, as they hint at the possibility of selection of genes for female mate choice in the early generations. However, in the experiments described here, the preferences were not sustained, probably due to difficulties with methodology. If these problems could be solved, then an investigation of this nature has the potential to answer several intriguing questions in the study of sexual selection. Given the large amount of time and effort necessary to perform this experiment, it was decided not to further pursue the investigation, as the probability of obtaining significant results seemed rather low. Instead, other questions of interest to sexual selection and female mate choice were investigated.

Chapter 7

Genetic coupling

Female song rhythm preferences

7.1 INTRODUCTION

Mating with a member of a different species has serious evolutionary consequences for an organism. Often, any offspring that are produced will be infertile. Thus it is essential that males and females are able to recognise conspecific mates in order that reproductive isolation between species be maintained. The coordination of the courtship signal production system in the male with the signal receptor system in the female will help to achieve this. If courtship communication is to evolve, then changes in one system must be accompanied by parallel changes in the other.

Alexander (1962) suggested that this could be achieved by genetic coupling, whereby signal and receptor systems share a common genetic basis. An alternative hypothesis (von Helversen & von Helversen, 1975) is that of co-evolution, in which signal and receptor systems are encoded by entirely different genes. Selective pressures would act independently on each system, eventually producing coordination between the two.

If genetic coupling does exist then it might be expected that, in *Drosophila*, any gene affecting the courtship song output by the male will also affect reception of the song by the female. It seems likely that a female would prefer the song produced by males from her own population over other types of song. With reference to the song rhythm and the *per* gene, it is possible that per^{+} females would prefer a 55s rhythm, per^{s} females a 40s rhythm and per^{L1} females an 80s rhythm. It is difficult to predict which song, if any, the per^{01} females would prefer.

The Threonine-Glycine repeat region of the *per* gene is particularly important in the control of the IPI cycles of the male courtship song (Yu *et al*, 1987b). Should genetic coupling exist with respect to the *per* gene, then it is possible that the Thr-Gly region may be also be important in the control of female song rhythm preferences

This experiment has investigated the song rhythm preferences of per^{+}, per^{s}, per^{L1} and per^{01} females, as well as the preferences of females transformed with a *per* gene from which the Thr-Gly encoding region had been deleted. As a control for the latter, females which had been transformed with the complete *per* gene were also investigated. A song simulator was used to generate three types of artificial song, with IPI cycles of 55s, 40s and 80s. These songs were played to each group of females and the mating speed with each song was taken as a measure of

female preference. Mating speed in silence was also examined.

7.2 MATERIALS AND METHODS

7.2.1 Strains

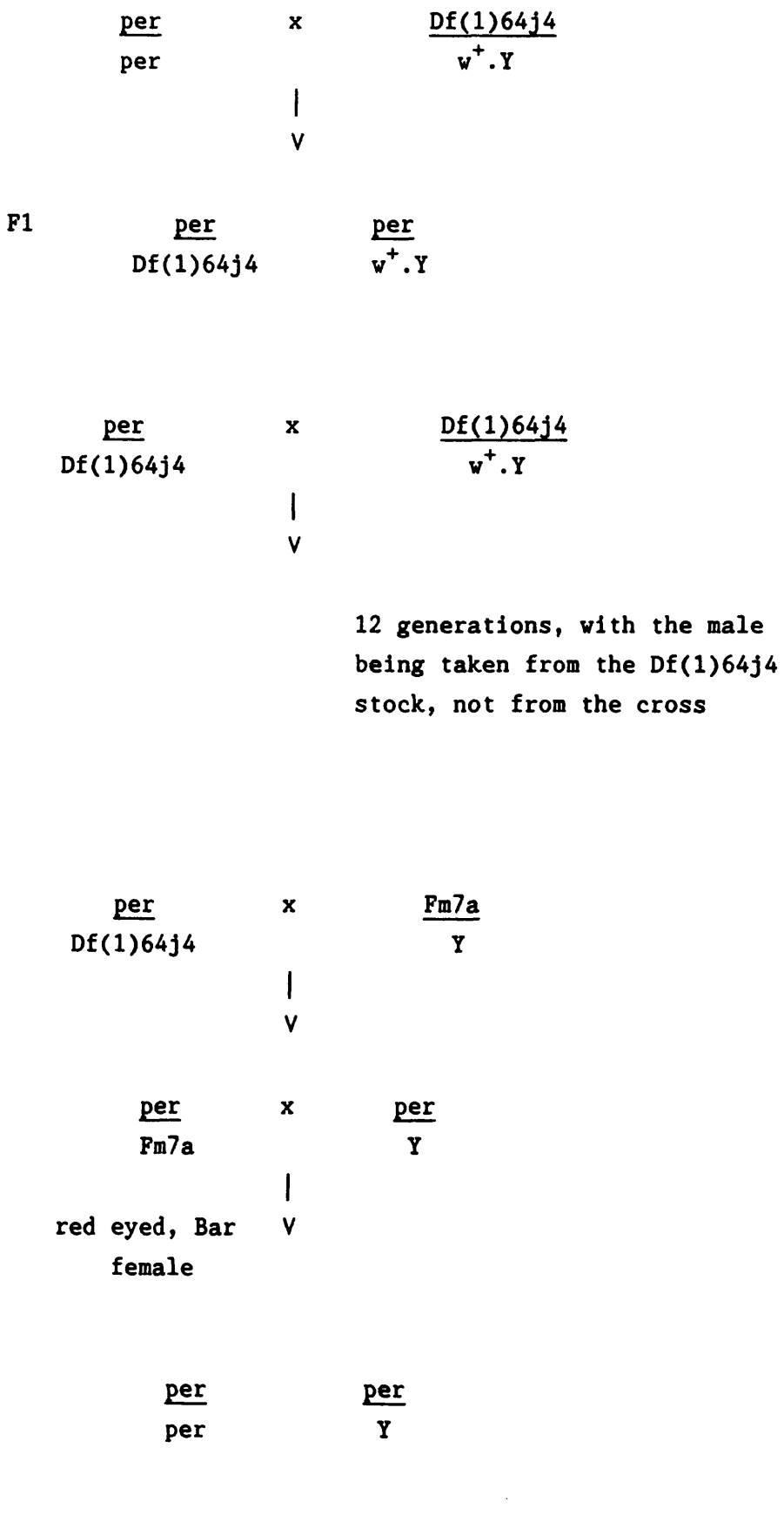
7.2.1.1 *per* strains

The original *per* mutations were induced by Ronald Konopka by treating *D. melanogaster* of the Canton-S strain with EMS (Konopka & Benzer, 1971). Subsequently, the three mutant alleles, per^{s}, per^{L1} and per^{01}, together with the wild type allele, per^{+}, were isogenised at Leicester using the Df(1)64j4 strain, which has a deletion uncovering the *per* locus. The males of this strain carry a translocation of a segment of the X chromosome on their Y chromosome (marked w^{+} - see Figure 7.1). The segment contains the cytological interval 2D1-2;3D3-4 (Smith & Konopka, 1981) and thus compensates for the missing material in the Df(1)64j4 chromosome. Figure 7.1 shows the isogenisation procedure. Virgin *per* females were crossed to Df(1)64j4 males and the female progeny backcrossed to males of the same strain. This backcross procedure was repeated for a further 12 generations. The resulting females were crossed to Fm7a males to remove the deficiency chromosome. The Fm7a/*per* female progeny were mated to the *per*/Y male progeny; homozygous *per* females and hemizygous *per* males were extracted to form the new *per* stock. Recombination would have occurred between the autosomes from the Df(1)64j4 strain and the Fm7a strain, resulting in randomisation of the autosomal variation across the 4 *per* strains.

7.2.1.2 Strains produced by P-element mediated transformation

The 13.2;2a strain was created at Brandeis University, USA, using P-element mediated transfomation (Hamblen *et al*, 1986). The vector used was the cp20.1 transformation vector, a ry^{+} vector (Yu *et al*, 1987). A 12.8 Kb fragment was inserted into this vector and the resulting construct used to transform a $per^{01}ry^{506}$ mutant using P-element transformation. Although the DNA fragment used was 12.8kb in length, it has subsequently been referred to as the 13.2kb fragment. Such terminology has been adopted in order to avoid confusion with a previous strain transformed with the same DNA fragment, but using a

Fig. 7.1 Procedure for isogenisation of the per strains



different vector. The 13.2kb fragment contains the complete transcription unit of the per gene (Yu et al,1987b).

A derivative of the 13.2kb fragment was constructed by an in-frame deletion of the Thr-Gly region. This removed the entire contiguous Thr-Gly repeat region, as well as some adjacent sequence at the 3′ end of the repeat. This deletion derivative, denoted ΔTG, was used to transform $per^{01}ry^{506}$ flies and the resulting strain was called Q-52C (see Yu et al, 1987b).

During P-element transformation, the cloned DNA is inserted ectopically into the genome. In the 13.2;2a strain, the DNA is located on chromosome 3, at polytene band 88-89 (J Hall, pers comm). In the Q-52C strain, the DNA is inserted on chromosome 3 at position 62A (J Hall, pers comm). Both these strains were made homozygous (ie 2 doses of the cloned DNA) before being used in the experiment. These strains were not originally homozygous for the per^{+} insert, with both males and females having either 2, 1 or no doses of the insert. Virgin females were thus mated individually to sibling males. The F1 progeny were allowed to interbreed to produce the F2 generation. The F2 progeny were examined and any lines in which ry^{506} offspring had been produced were discarded. Lines from which only wild type offspring had arisen should now be homozygous. These lines were maintained for several generations to make certain that no ry^{506} offspring were produced.

Homozygous strains of both 13.2;2a and Q-52C were tested in this experiment. In addition, a strain with only one dose of the per^{+} (13.2;2a) insert was examined. This strain was created by crossing the homozygous 13.2;2a strain described above with the original $per^{01}ry^{506}$ strain into which the DNA had been transformed. As a control, the preferences of the original $per^{0}ry^{506}$ strain were also investigated.

7.2.2 Experimental procedure

The experimental procedure was as described in Section 2.7.2 (p41).

7.3 RESULTS

7.3.1 Statistics

A Friedman (planned comparisons) test was used to assess the

significance of differences in mating speeds under different conditions ie with the 3 simulated songs and silence. This is a non-parametric test which involves ranking the total number of flies mating under each condition within one run of the experiment. Thus each song condition is ranked either 1, 2, 3 or 4. A total rank is obtained for each condition across the whole experiment and the total ranks compared (Hays, 1973).

A test which ranked the data within each run was chosen because of the large variation in the number of flies mating between runs. For instance, after 5 minutes of run 2 in the per^S experiment, 9 flies had mated with a 55s rhythm, 4 with a 40s rhythm, 7 with an 80s rhythm and 5 with silence - a total of 25 flies mating. In run 19 of the same experiment, 3 flies mated with a 55s rhythm, 1 with a 40s rhythm, 3 with an 80s rhythm and 0 with silence - a total of 7. In both experiments, flies mated most rapidly with a 55s and an 80s rhythm and these conditions were thus ranked 1 or 2. However, had the number of flies mating been ranked across the whole experiment, then the 40s rhythm condition of run 2 would have been ranked higher than the 55s or 80s rhythms of run 19 (4 flies mating as opposed to 3). It was important to avoid this, as the numbers mating in different runs of the experiment are not directly comparable due to possible environmental variations such as time of day of testing or background acoustic noise levels.

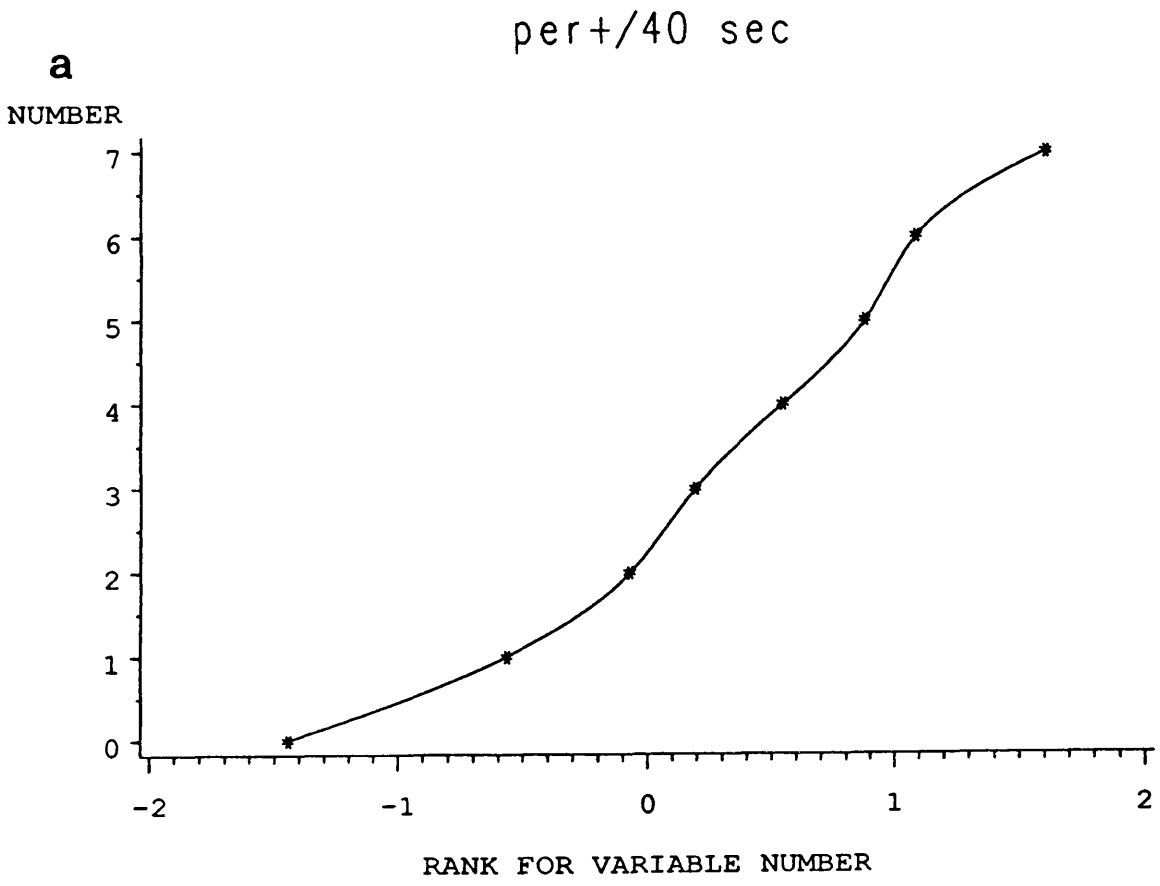
In addition to the reasons mentioned above, a non-parametric method of analysis was chosen because the data obtained from these experiments are not normally distributed. Thus parametric tests, such as an analysis of variance, would be inappropriate.

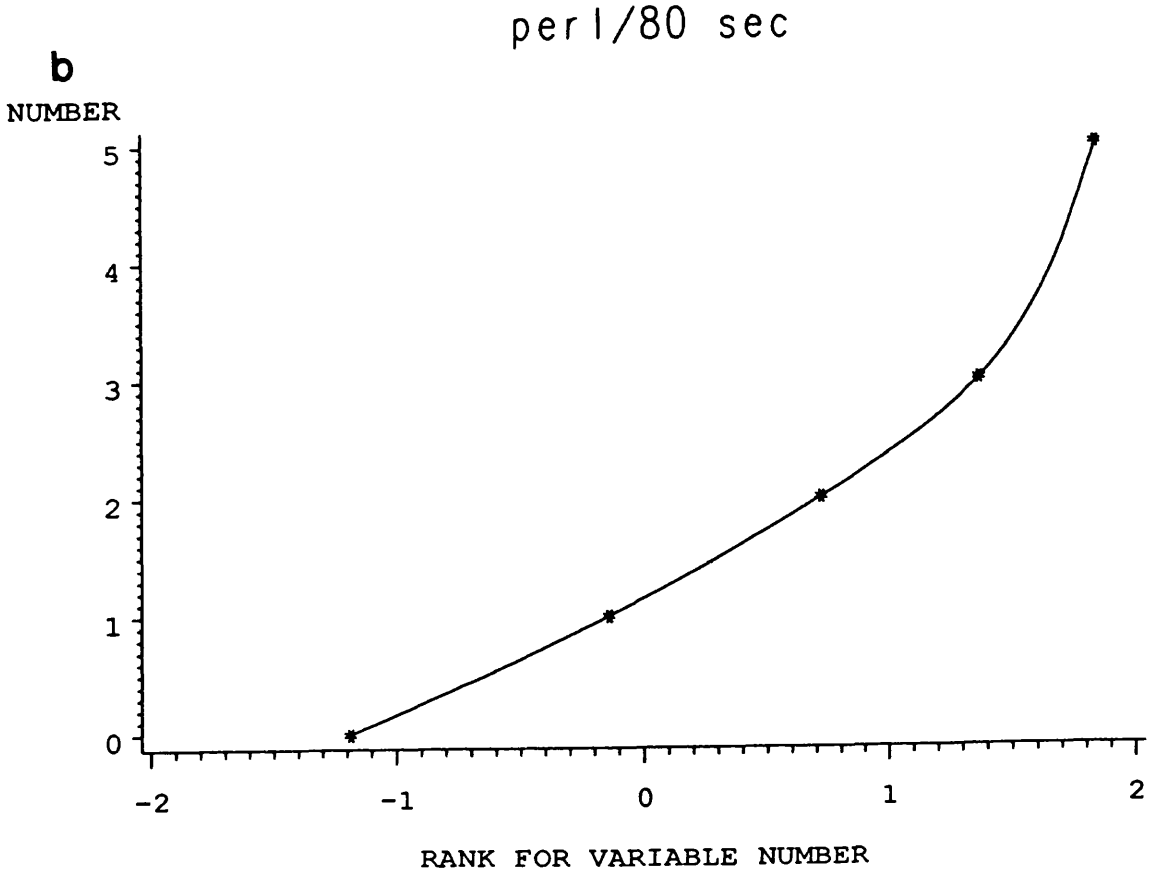
Non-normality of the data was established using the PROC RANKS procedure of the SAS computer package (SAS Users Manual, 1985). The program calculates the normal scores of each genotype/song class (eg per^+ flies with a 55s song rhythm) from the standardised normal distribution (mean=0, sd=1), using a sample size equal to that of the actual data set. For example, the per^+/55s class has 19 data values (ie 19 runs of the experiment) so the normal scores would be calculated using a sample size of 19. The program calculates the normal scores using the formula:

$$y = \frac{\psi(r_i - 3/8)}{(n+1)/4}$$

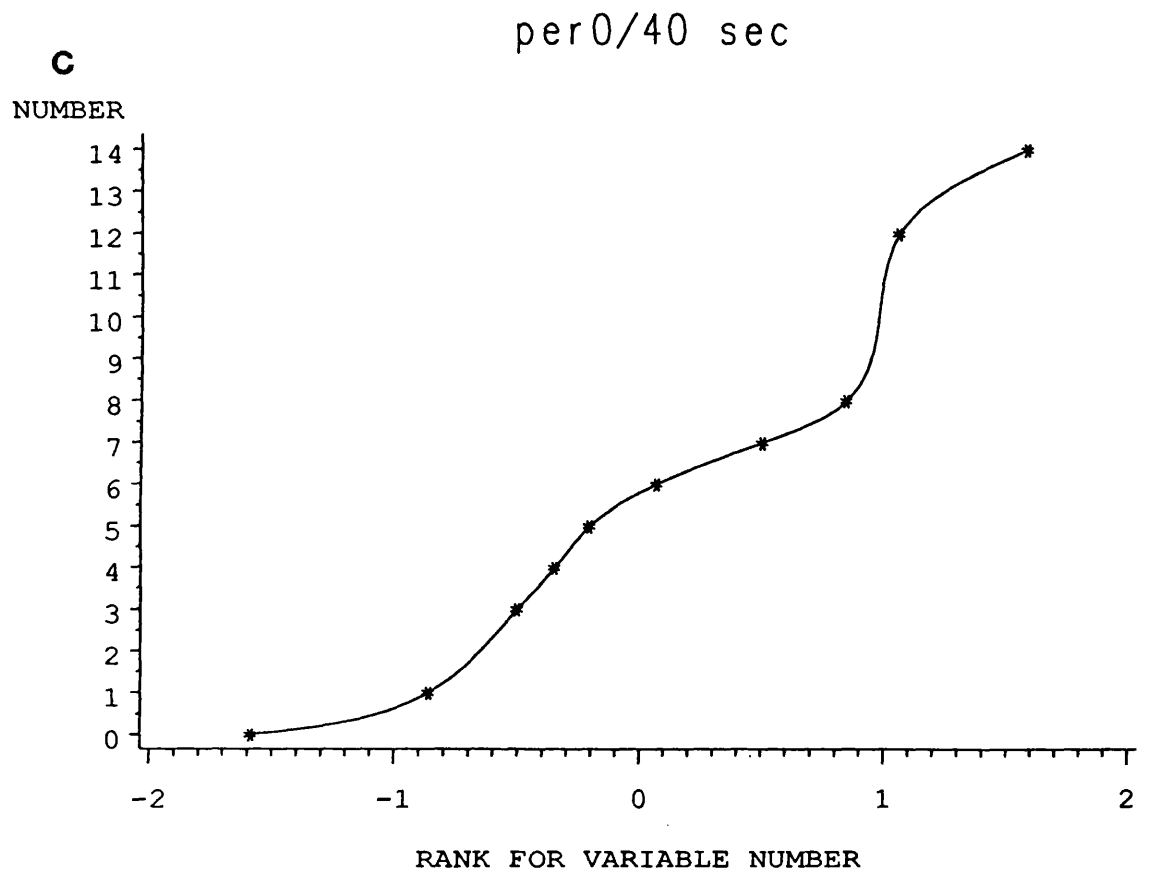
ψ = inverse normal cumulative frequency
r_i = rank
n = no. of observations

Fig 7.2 Illustration of normality or non-normality of the data using normal scores

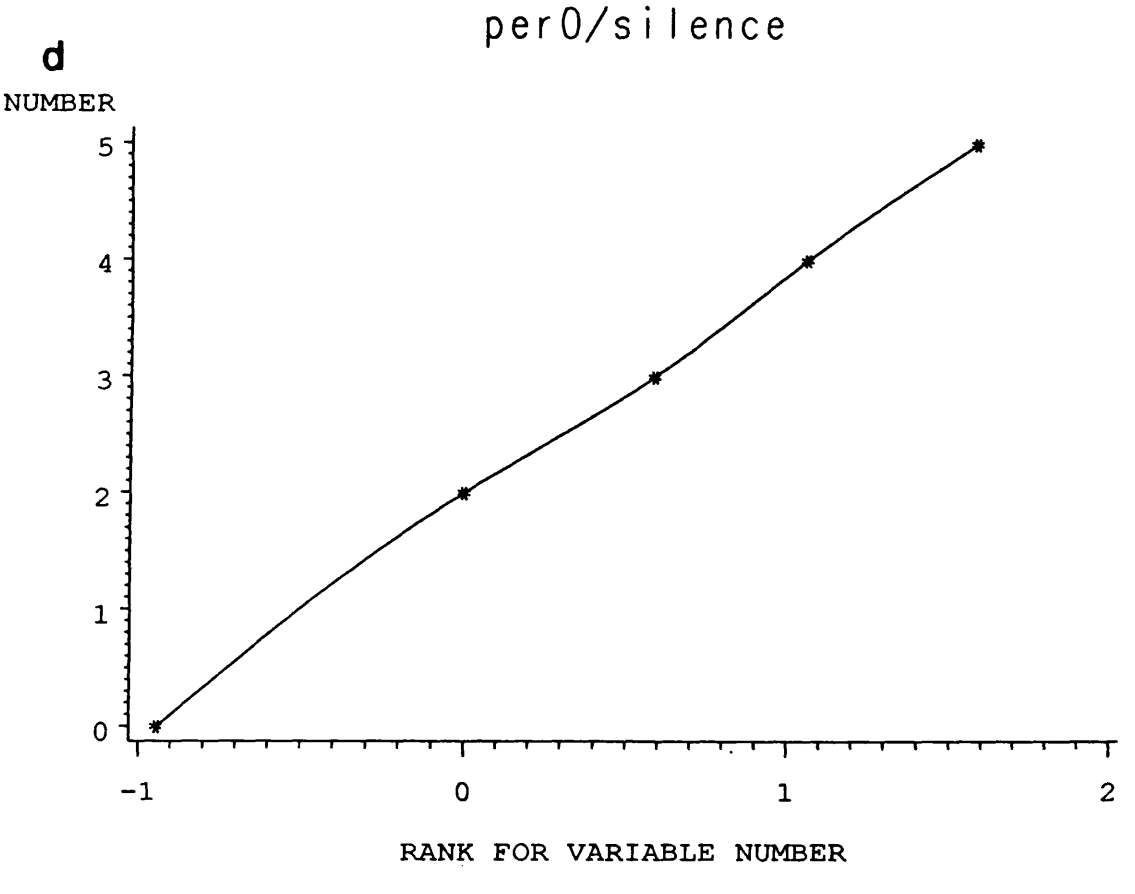




c


per0/40 sec
NUMBER
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-2
-1
0
1
2
RANK FOR VARIABLE NUMBER

d


per0/silence
NUMBER
5
4
3
2
1
0
-1
0
1
2
RANK FOR VARIABLE NUMBER

What are normal scores? Consider the per^+/55s rhythm example. 19 values could be sampled at random from the standardised normal distribution and ranked according to size. This could be repeated *ad infinitum* and the mean of the values ranked 1st calculated, the mean of the values ranked 2nd calculated etc. The means which would be expected from this procedure are the normal scores.

Once the normal scores have been calculated, the program assigns each normal score to an actual data value from the genotype/song class under investigation. The highest normal score is assigned to the highest data value and the lowest is assigned to the lowest data value etc. A graph of the normal scores against the actual data values to which they have been assigned is then produced. If the data under investigation is normally distributed, then a straight line passing through the origin is produced.

Such graphs were produced for each genotype/song class ie 32 graphs (8 genotypes x 4 songs). Figure 7.2a-c shows a sample of these graphs, from which it is clear by inspection that the number of flies mating in the classes illustrated are not normally distributed. Figure 7.2d shows a genotype/song class which came close to being normally distributed; only 6 out of 32 classes could be considered to be close to a normal distribution. Thus it is clear that parametric tests would not be suitable for analysis of these data.

7.3.2 Co-isogenic *per* strains

Table 7.1 shows the total number of flies mating by the 5th minute within the whole experiment. Table 7.2 summarises the differences in mean ranks and the confidence limits from pairwise comparisons of the number of flies mating at 5 minutes in each condition; it also indicates their significance according to the Friedman test described above. Figure 7.3 shows the mean number mating at 5 minutes.

In the per^+ females, mating is significantly faster with a 55s and an 80s rhythm than with a 40s rhythm or silence. The females do not appear to discriminate between a 55s and an 80s rhythm or between a 40s rhythm and silence. 19 runs of the experiment were performed.

A similar result is obtained with per^s females, although there is less discrimination in favour of an 80s rhythm. Mating is significantly faster with a 55s rhythm than with a 40s rhythm or

Table 7.1 Total number of flies mating at 5 minutes with each condition

Genotype	55 secs	40 secs	80 secs	Silence	Total tested
per^{+}	98	52	93	47	1900
per^{s}	123	86	104	78	2000
per^{L1}	41	29	25	20	1900
per^{01}	113	102	126	34	1800

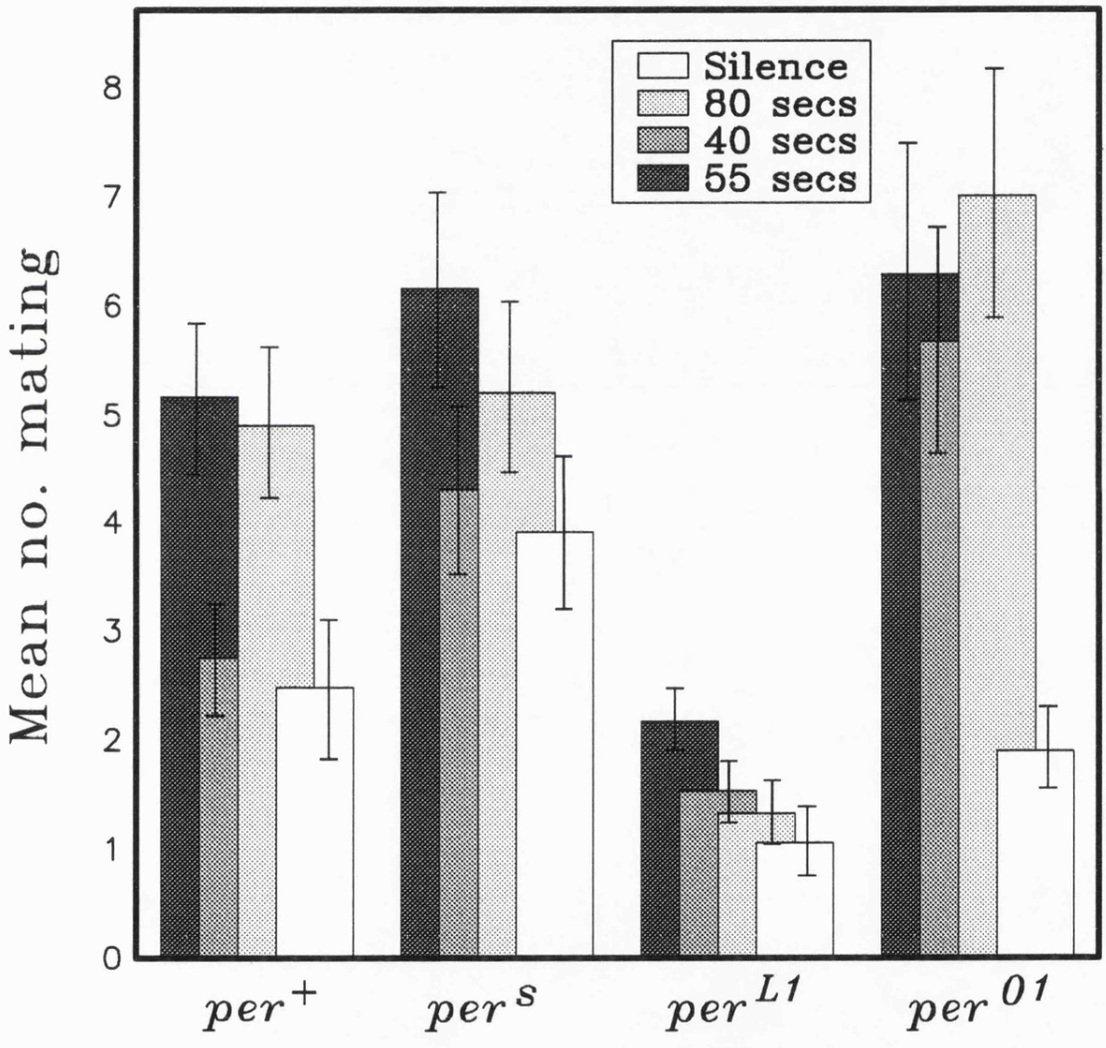
.....

Table 7.2 Differences between mean ranks and 95% confidence limits from Friedman tests (pairwise comparisons)

Genotype	Confidence limits	55/40	55/80	55/sil	40/80	40/sil	80/sil
per^{+}	1.075	1.526*	0.395	1.974*	1.132*	0.447	1.579*
per^{s}	1.048	1.300*	0.900	1.150*	0.450	0.150	0.250
per^{L1}	1.075	0.816	0.921	1.000*	0.105	0.184	0.079
per^{01}	1.105	0.333	0.083	1.695*	0.417	1.361*	1.778*

* indicates $p<0.05$

Fig 7.3 Mean number of flies mating at 5 mins $\pm$ S.E.M.



silence. However there is no significant difference between mating speeds with an 80s rhythm and with any other condition. Thus, mating speed with an 80s rhythm is midway between that with a 55s rhythm and a 40s rhythm or silence. Again, females do not discriminate between a 40s rhythm and silence. 20 runs were carried out with the per^{s} strain.

The results from the per^{L1} females should be viewed with some caution, as very few flies mated (see Table 7.1 and Fig 7.3). 19 runs of the experiment were performed. The only significant difference between mating speeds was that between silence and a 55s rhythm. Figure 7.3, however, demonstrates that females do seem to discriminate in favour of a 55s rhythm over other conditions.

In marked contrast to the above results, per^{01} females do not appear to favour a 55s rhythm. In fact, they do not discriminate between any of the song conditions, although mating speed is significantly faster with the songs than with silence. 18 runs of the experiment were carried out.

In summmary, females with a functional *per* gene (per^{+}, per^{s} & per^{L1}) seem to mate faster with a 55s or 80s rhythm than with a 40s rhythm or with silence. Flies with a non-functional *per* gene (per^{01}) do not discriminate between the 3 song rhythms tested, although they mate markedly faster with any song tested than with silence.

Table 7.3 shows the number of flies mating in the morning and afternoon under each song condition. Runs of the experiment which began before 11.45am were classed as morning runs; those which began after this time were classed as afternoon runs.

It is possible that the flies' response to different song conditions would vary acccording to the time of day. A χ^2 contingency test was performed independently for each genotype on the number of flies mating with each song condition in the morning and afternoon (see Table 7.3A). The test shows that the patterns of mating in the morning and the afternoon are not significantly different (χ^2 = 1.57, 0.19, 3.17, 1.02 for per^{+}, per^{s}, per^{L1} and per^{01} respectively; df=3). That is, the response of the flies to the different song conditions is consistent regardless of the time of day.

A further χ^2 contingency test was carried out on the total number of flies mating across the 4 song conditions within each genotype. The number of flies mating or not mating in the morning was compared to the number mating or not mating in the afternoon (see Table 7.3B). The test shows that the mating speeds of the per^{s} and per^{L1} flies do not differ in the morning and the afternoon (χ^2 = 1.96 & 0.08

Table 7.3 A & B Number of flies mating at 5 minutes in the morning and afternoon

A

Genotype		55s	40s	80s	Silence	Total tested
per^{+}	AM	63	32	63	27	1400
	PM	35	20	30	20	500
per^{s}	AM	97	66	80	61	1500
	PM	26	20	24	17	500
per^{L1}	AM	27	22	21	16	1400
	PM	14	7	4	4	500
per^{01}	AM	54	42	58	15	1000
	PM	59	60	68	19	800

.....

B

Genotype		Total no. mating (all songs)	Total no. not mating (all songs)	Total tested
per^{+}	AM	185	1215	1400
	PM	105	395	500
per^{s}	AM	304	1196	1500
	PM	87	413	500
per^{L1}	AM	86	1314	1400
	PM	29	471	500
per^{01}	AM	169	831	1000
	PM	206	594	800

respectively; df=1). However, both per^{+} and per^{01} females mate faster in the afternoon than in the morning (χ^2 = 17.27 & 21.11 respectively; df=1).

7.3.3 13.2;2a, Q-52C and $per^{01}ry^{506}$ strains

Table 7.4 shows the total number of flies mating in the experiment and Table 7.5 summarises the differences in mean ranks and the confidence limits from pairwise comparisons of the number mating in each condition, using a Friedman test. Figure 7.4 shows the mean number mating at 5 minutes.

None of the females examined here discriminated between any of the 3 songs tested, although all mated considerably faster with any song than with silence. The difference in mating speed between a 55s rhythm and silence very narrowly missed significance at the 5% level in the 13.2;2a (1 dose) females and between a 40s rhythm and silence in the $per^{01}ry^{506}$ females. 18, 14, 10 and 10 runs of the experiment were performed for the 13.2;2a (2 doses), 13.2;2a (1 dose), Q-52C and $per^{01}ry^{506}$ females respectively.

It is clear that the transformed strains more closely resembled per^{01} strains than a wild type strain. Thus it appears that transformation with per^{+} DNA is not capable of restoring wild type behaviour, with respect to song rhythm discrimination, to per^{01} females.

Table 7.6 shows the number of flies mating in the morning and the afternoon under each song condition. A χ^2 contingency test (see Table 7.6A) shows that, as previously, the patterns of mating do not differ according to the time of day (χ^2 = 2.65, 6.80, 3.64 & 0.68 for the 13.2;2a (2 doses), 13.2;2a (1 dose), Q-52C and $per^{01}ry^{506}$ flies respectively; df=3). However, a further χ^2 contingency test (see Table 7.6B) on the total numbers mating within each genotype across the 4 song conditions shows that the 13.2;2a (2 doses) females mate faster in the afternoon (χ^2 = 17.41; df=1). In contrast, 13.2;2a (1 dose) females mate faster in the morning (χ^2 = 4.69; df=1). The Q-52C and $per^{01}ry^{506}$ females mate at a similar speed in both the morning and the afternoon.

7.4 DISCUSSION

This experiment shows that females with a functional *per* gene (per^{+} and per^{s}) discriminate between different song rhythms. They

Table 7.4 Total number of flies mating at 5 minutes in each condition

Genotype	55 secs	40 secs	80 secs	Silence	Total tested
13.2;2a 2 doses	220	200	212	106	1800
13.2;2a 1 dose	126	127	130	87	1400
Q-52C	93	96	91	56	1000
$per^{01}ry^{506}$	87	80	86	30	1000

.....

Table 7.5 Differences between mean ranks and 95% confidence limits from Friedman tests (pairwise comparisons)

Genotype	Confidence limits	55/40	55/80	55/sil	40/80	40/sil	80/sil
13.2;2a 2 doses	1.105	0.417	0.111	1.806*	0.528	1.389*	1.917*
13.2;2a 1 dose	1.253	0.286	0.393	1.250	0.107	1.536*	1.643*
Q-52C	1.482	0.100	0.100	1.600*	0.200	1.700*	1.500*
$per^{01}ry^{506}$	1.482	0.750	0.250	2.000*	0.500	1.250	1.750*

* indicates $p<0.05$

Fig 7.4 Mean number of flies mating at 5 mins ± S.E.M.

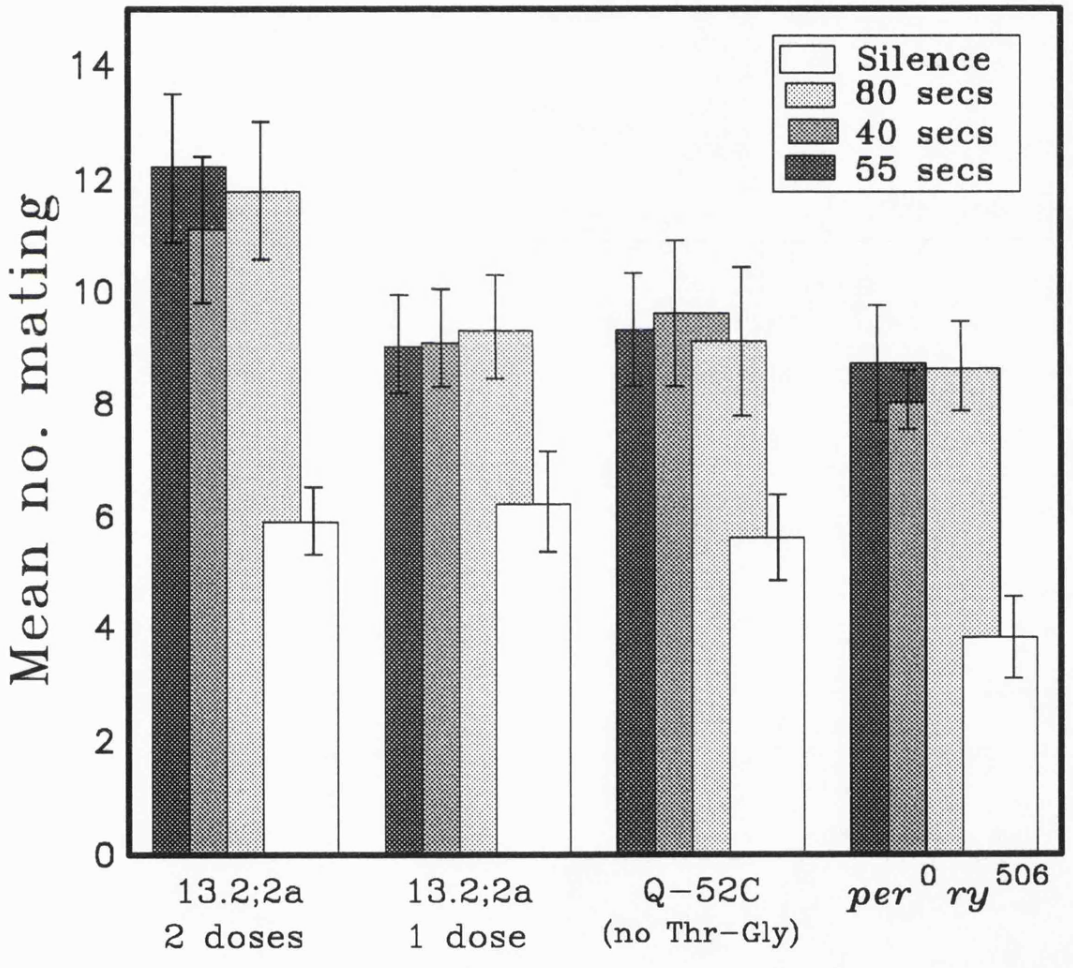


Table 7.6 A & B Number of flies mating at 5 minutes in the morning and the afternoon

A

Genotype		55 secs	40 secs	80 secs	Sil	Total tested
13.2;2a (2 doses)	AM	152	128	138	76	1300
	PM	68	72	74	30	500
13.2;2a (1 dose)	AM	97	96	88	72	1000
	PM	29	31	42	15	400
Q-52C	AM	58	55	52	26	600
	PM	35	41	39	30	400
$per^{01}ry^{506}$	AM	55	46	53	24	600
	PM	32	34	33	14	400

B

Genotype		Total no. mating (all songs)	Total no. not mating (all songs)	Total tested
13.2;2a (2 doses)	AM	494	806	1300
	PM	244	256	500
13.2;2a (1 dose)	AM	353	647	1000
	PM	117	283	400
Q-52C	AM	191	409	600
	PM	145	255	400
$per^{01}ry^{506}$	AM	178	422	600
	PM	113	287	400

demonstrate a firm preference for a wild type, 55s song rhythm or an 80s rhythm over a 40s rhythm. However the 80s preference is less marked in per^{s} females and absent in per^{L1}. Very few per^{L1} females mate, so any conclusions about this genotype must be tentative. The per^{01} females, which lack a functional *per* gene, do not seem to discriminate between any of the IPI cycles, although they mate faster with all three songs than with silence. This shows that the per^{01} females are capable of hearing and processing song information.

The result with per^{01} females seems to provide some evidence for the genetic coupling theory. The *per* gene appears to contribute towards female recognition of song rhythm, as well as production of song rhythm by the male. However, the genetic coupling theory generally holds that a mutation would produce corresponding changes in both signal and receptor systems (eg Doherty & Hoy, 1985). Therefore per^{s} females would be expected to prefer a 40s rhythm and per^{L1} females an 80s rhythm. This is clearly not the case. Thus it must be recognised that in a genetically coupled system it is possible that some mutations can cause a change in the signal but not in the receptor or vice versa. This could be disadvantageous from an evolutionary viewpoint, as males and females from the same population could be unable to recognise one another's courtship signals. Presumably, such mutations would be strongly selected against and contribute little to the evolution of courtship communication. Alternatively, there could be selection on other genes to restore coordination between signal and receptor systems - co-evolution. For instance, in the per^{s} strain, there could be selection in the female for genes other than *per* to produce a preference for a 40s song rhythm. Thus, genetic coupling and co-evolution could operate simultaneously.

It would be interesting to set up a per^{s} strain in which there was a large amount of background genetic variation and to measure song rhythm preferences of the females over many generations. The possible development of a preference for a 40s rhythm would probably be due to co-evolution, and of course it might be possible to map and characterize the gene or genes responsible. Alternatively, it is possible that selection could occur in the per^{s} males to produce a song rhythm of 55s in response to existing female preferences. This seems unlikely, as the per^{s} strains examined in Kyriacou & Hall (1980) had existed for the previous 10 years without any selection for the production of a 55s song rhythm. The song rhythm preferences of a strain of per^{s} which had been allowed to "drift" in laboratory populations have been previously

investigated in this laboratory (N Smith & M Hill, unpublished). It appears from the results that these per^s females do not discriminate between different song rhythms. However, the experimental procedure used in this investigation was poor and the results are probably unreliable. An attempt was made to repeat the experiment (data not shown) but despite several attempts this was a failure as very few matings were obtained from this strain. It was not possible to draw any conclusions from the results of this attempted replication.

It is interesting that per^+ females mate almost as well with an 80s rhythm as with a wild type 55s rhythm. It appears that per^+ females may be discriminating against a 40s IPI cycle, rather than favouring a 55s cycle. *D. simulans* males produce a courtship song with hum and pulse components which are similar to those of *D. melanogaster* (Kyriacou & Hall, 1980; Cowling & Burnet, 1981). However, the IPIs oscillate around a mean of 40ms-80ms, with a period of 35s-40s (Kyriacou & Hall, 1980; 1986). It seems likely that the rhythm and the IPI are the parameters most important in allowing *D. melanogaster* and *D. simulans* females to distinguish the songs of conspecific males (Kyriacou & Hall, 1982; 1986). It is possible that per^+ females are reacting against a component of the song of another species (40s - *D. simulans*) rather than choosing the song of a conspecific (55s - *D. melanogaster*).

An 80s song rhythm is characteristic of another *Drosophila* species, *D. yakuba* (Thackeray, 1989), so it may be wondered why per^+ females do not also discriminate against an 80s rhythm. However, this species has a rather different courtship song to *D. simulans* and *D. melanogaster*. It is completely lacking in the hum song element (Cowling & Burnet, 1981) and has two distinct pulse song types, both with a mean IPI of about 100ms-110ms (Thackeray, 1989). Perhaps, then, *D. melanogaster* females do not require to select against an 80s rhythm as the IPIs of *D. yakuba* males are so different from those of *D. melanogaster*.

In relation to the above, D. melanogaster and D. *simulans* are human commensals and are thus sympatric in most parts of the world (Lachaise et *al*, 1988). This further emphasises the need for effective pre-mating barriers between the two species, in which female choice is probably of great importance (Kyriacou & Hall, 1982, 1986). However, *D. yakuba* is found only in Africa (Lachaise et *al*, 1988) which means that *D. yakuba* and *D. melanogaster* are sympatric only over a small area of *D. melanogaster's* range. Thus for most populations of *D. melanogaster*, geographical isolation will prevent mating with *D. yakuba*, and therefore

there will probably be no selection for D. melanogaster females to discriminate against an 80s song rhythm. The *D. melanogaster per* strains tested here were derived from an Oregon-R strain, originally obtained from Ohio, USA. It would be interesting to examine the song rhythm preferences of a strain of *D. melanogaster* females collected from an area also populated by *D. yakuba*. It is possible that such females may have been selected to discriminate between a 55s and an 80s song rhythm.

The per^{+} and per^{s} females mate equally well with silence as with a 40s IPI cycle. Thus a 40s cycle does not appear to act as a stimulus to mating in these flies. However, a 40s rhythm does appear to encourage mating in per^{01} females, as they mate much better with this song than with silence. Perhaps the *per* gene allows females to distinguish between "desirable" stimuli to mating (55s and 80s rhythms) and "undesirable" stimuli (40s rhythm). Clearly, the effect of an undesirable stimulus (40s) is the same as no stimulus (silence) in per^{+} and per^{s} females. per^{01} females do not appear to have the ability to distinguish between desirable and undesirable stimuli, as their mating propensity is reduced only in the silence condition.

From the above results it seems likely that the *per* gene does contribute towards female mate choice. However, it remains a possibility that the preference for a 55s rhythm is controlled by a gene or genes which is very closely linked to the *per* gene and resistant to the isogenisation procedure. If this was the case, selection could have occured in the per^{01} strain to abolish song rhythm preferences. Restoration of the 55s rhythm preference in per^{01} flies transformed with per^{+} DNA would conclusively demonstrate the existence of genetic coupling. However, it has not been possible to achieve this - the 13.2;2a and Q-52C females do not distinguish between the three song rhythms. The 13.2;2a, Q-52C and $per^{01}ry^{506}$ strains all demonstrate per^{01}-like behaviour, in that they mate less well with silence than with a 40s rhythm.

It is possible that the inserted per^{+} DNA may have disrupted another gene important in the signal/receptor system, thereby abolishing the 55s preference in the 13.2;2a (2 doses) females. For this reason the song rhythm preferences of females with only one autosomal dose of per^{+} DNA (13.2;2a (1 dose)) were also examined. If the per^{+} insert had disrupted another gene, these females should still have one intact copy of that gene. However, these females also behaved in a per^{01}-like fashion, so this does not appear to be the explanation for the failure

to restore song rhythm preferences. Alternatively, the positioning of the per^+ DNA on chromosome 3 instead of in its normal place on the X-chromosome may have disrupted interactions of *per* with modifier genes important in establishing the preference. This may indicate that the *per* gene could be part of a supergene complex similar to that seen controlling shell colours in the snail (Cain & Sheppard, 1954). Unfortunately, without a suitable control, it is impossible to establish whether or not the Thr-Gly region contributes to recognition of song rhythm by the female as well as production of song rhythm by the male.

The experiment was not designed to allow the accurate comparison of mating speeds between strains as each strain was examined separately and were therefore subject to slight changes in environmental conditions. Bearing this in mind, it is still interesting to note that per^{01}, 13.2;2a, Q-52C and $per^{01}ry^{506}$ strains generally mate more rapidly than the per^+, per^s and per^{L1} strains. It is possible that these females which lack a functional *per* gene in the correct X chromosomal location are generally less discriminating and are hence more eager to mate.

This experiment provides good evidence for genetic coupling with respect to the *per* gene. It is unfortunate that it has not been possible to restore the 55s preference in per^{01} flies transformed with per^+ DNA, especially in light of the results from Wheeler *et al* (ms submitted). These workers showed that species-specific differences in song rhythm between *D. melanogaster* and *D. simulans* are controlled by the Thr-Gly region of the *per* gene (see Section 4.1, p58). It would have been most interesting to have been able to investigate the effect on female preferences of transforming *D. melanogaster* flies with the *D. simulans per* gene or a chimaeric *D. melanogaster/D. simulans per* gene. Would such females have shown the preference for a 40s song rhythm which is typical of *D. simulans* females (Kyriacou & Hall, 1982)? Unfortunately, given the poor response of the 13.2;2a transformed females, any results would be difficult to interpret.

Chapter 8

Possible mechanisms influencing female song rhythm preferences

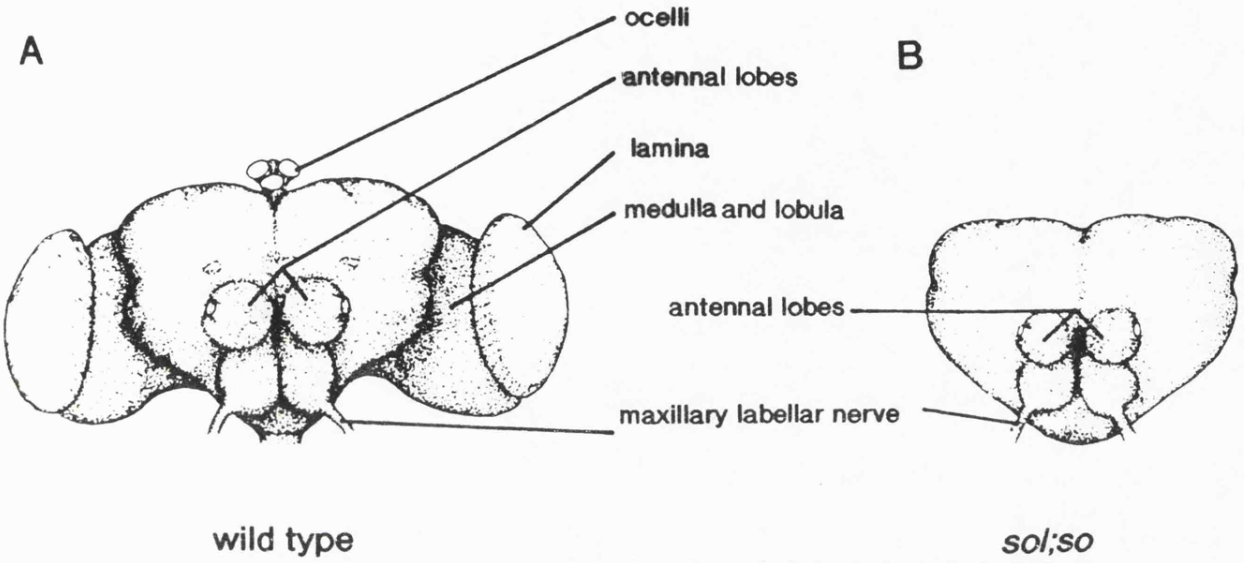
8.1 INTRODUCTION

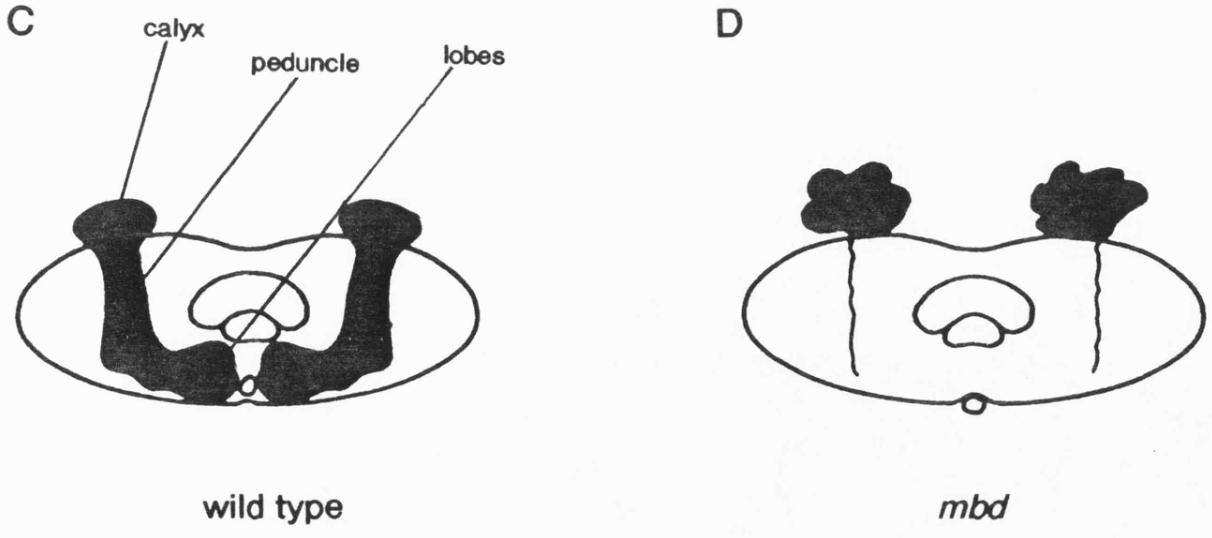
The results discussed in Chapter 7 suggest that the *per* gene contributes towards the expression of female song rhythm preferences in *Drosophila*. However, nothing is known about the putative physiological mechanism controlling female song rhythm preferences (eg a clock-type mechanism?) or its possible anatomical location. The song recognition mechanism of female grasshoppers is located in the brain, although the song production mechanism is in the thorax (Bauer & von Helversen, 1987). There is much evidence in Orthopterans that the mechanism controlling the locomotor activity rhythm is located in the optic lobes of the brain (eg Saunders, 1982) and several optic lobe mutants of *Drosophila* show slightly aberrant circadian activity rhythms (Helfrich, 1986).

It was decided to examine the song rhythm preferences of three brain mutants, *mushroom-body-deranged (mbd)*, *small optic lobes;sine oculis (sol;so)* and *optomotor-blind (omb)*. The mushroom bodies are two large bundles in the median protocerebrum, consisting each of a calyx, a peduncle and two lobes (Heisenberg, 1980). The thin parallel fibres (Kenyon cells) of the peduncle extend from back to front through the whole brain (Fig 8.1C). The mushroom bodies are part of the antennal pathway and are sensitive to olfactory stimulation; *mbd* mutants are deficient in olfactory learning (Heisenberg et al, 1985). Electrical stimulation near the mushroom bodies can elicit courtship behaviour in crickets and grasshoppers (Wadepuhl & Huber, 1979) and genetic mosaics in *Drosophila* have shown that tissue in this region is required to be male for courtship to occur (Hall, 1979). However, *mbd* males are not deficient in courtship behaviour (Heisenberg, 1980). The *mbd* mutant has severe defects in its mushroom bodies; only a few fibres of the peduncle are present, no lobes are detected and the calyces are enlarged (Heisenberg, 1980; see Fig 8.1D).

The optic lobes of the brain are made up of the lobula and lobula plate, the medulla and the lamina (Fischbach & Heisenberg, 1984; see Fig 8.1A). They are connected to the compound eye by the optic nerve. The *omb* mutant lacks the giant neurones of the lobula plate (Fischbach & Heisenberg, 1984), whilst the additive effects of the *sol* and *so* mutations (in the *sol;so* double mutant) reduce the optic lobes to 5% of their wild type size (Fischbach & Technau, 1984 - see Figure 8.1B). Thus the *so;so* mutants have a much more severe defect than the *omb* mutants.

Fig 8.1 Comparison of the wild type brain to the brains of the *mbd* and *sol;so* mutants





A & B From Helfrich (1986)

C & D From Heisenberg (1980)

Constant bright light is known to damp-out biological rhythms in several insect species (Saunders, 1982), including the locomotor activity rhythm of *Drosophila* (Zerr et al, ms submitted). The contribution of *per* to female song rhythm preferences suggests that the preferences may be mediated by a clock-type mechanism. If this is so, then it is possible that the mechanism may not function in females which have been kept in constant bright light. Therefore, the song preferences of per^{+} females raised in this fashion were investigated.

8.2 MATERIALS AND METHODS

8.2.1 Strains

The 3 brain mutants used were *optomotor-blind*^{H31} (*omb*^{H31}, referred to as *omb*), the double mutant *small optic lobes;sine oculis* (*sol;so*) and *mushroom body deranged*^{KS65} (*mbd*^{KS65}, referred to as *mbd*). All 3 mutants were kindly supplied by M Heisenberg.

The per^{+} flies used to investigate the effect of bright light on female song preferences were as described in Section 7.2.1.1 (p84).

8.2.2 Experimental Procedure

The experimental procedure was as described in Section 2.8 (p42).

8.3 RESULTS

A Friedman test (planned comparisons) was used to assess the statistical significance of differences in mating speed between song conditions (see Section 7.3.1, p85).

Mating was very poor in the *mbd* females, as seen in Table 8.1 and Figure 8.2. 15 runs of the experiment were performed. Table 8.2 shows the differences in mean ranks and the confidence limits from the Friedman test, demonstrating that there are no significant differences between mating speeds with a 55s rhythm, a 40s rhythm or silence. Thus, the *mbd* females do not appear to discriminate between song rhythms, or between song and silence. However, as so few flies mated, any conclusions about this genotype must be tentative.

Table 8.1 Total number of flies mating at 5 minutes

Genotype	55 secs	40 secs	Silence	Total tested
mbd	22	26	25	1125
sol;so	164	174	123	1050
omb	153	153	117	1200
per^+ (bright light)	136	132	72	1350

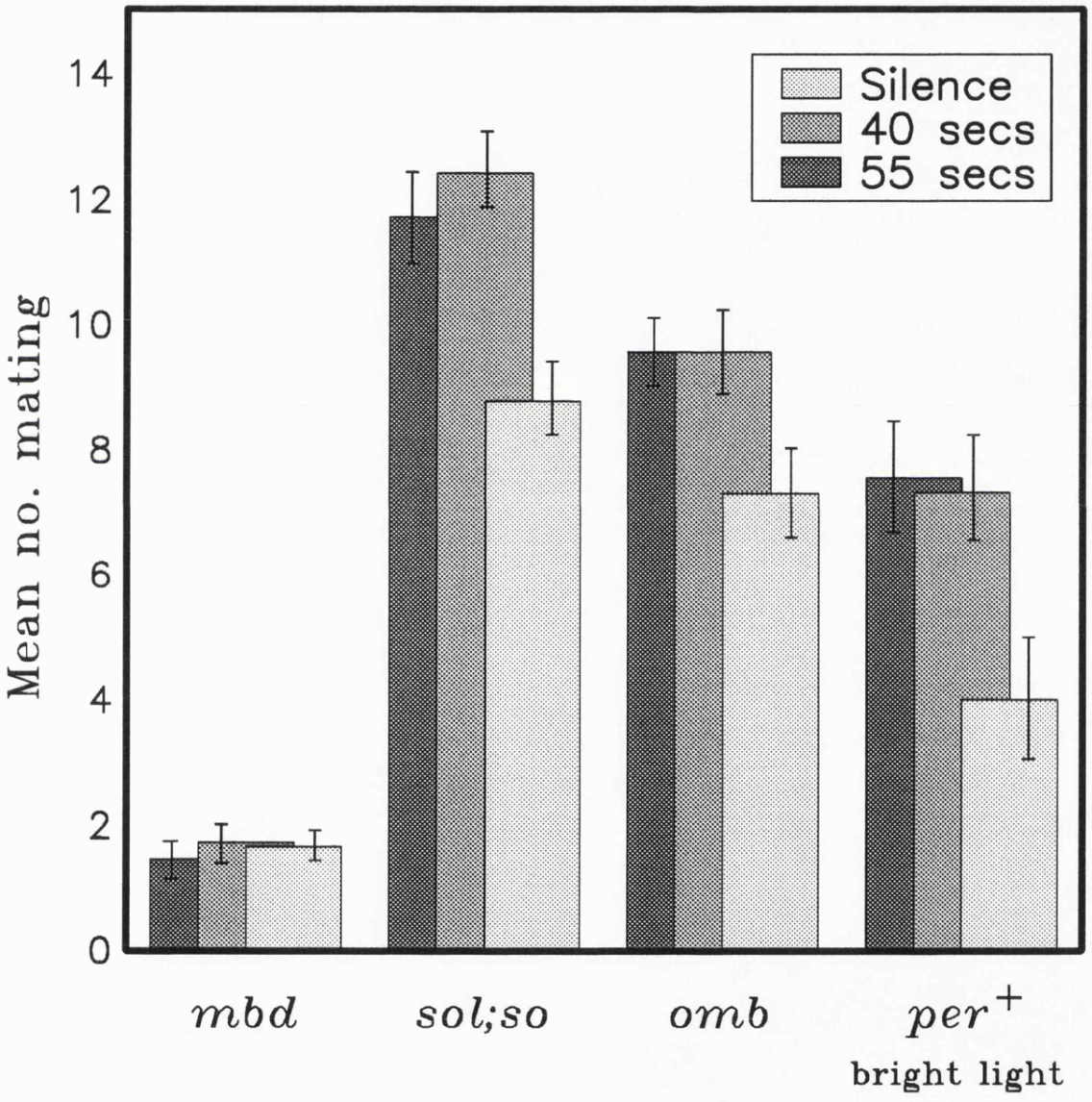
.....

Table 8.2 Differences between mean ranks and 95% confidence limits from Friedman test (pairwise comparisons)

Genotype	Confidence limits	55/40	55/sil	40/sil
mbd	0.855	0.033	0.233	0.267
sol;so	0.885	0.179	1.036*	1.214*
omb	0.828	0.094	1.031*	0.938*
per^+ (bright light)	0.780	0.111	1.028*	1.139*

* indicates $p<0.05$

Fig 8.2 Mean number of flies mating after 5 minutes ± S.E.M.



In both the *sol;so* and *omb* females there are no significant differences between mating speeds with a 55s or a 40s rhythm. However, these females do discriminate between song and silence, mating much more slowly with silence. 14 runs of the experiment were performed with the *sol;so* females and 16 with the *omb* females.

18 runs were performed with the per^+ females raised in constant bright light. These females also failed to discriminate between a 55s rhythm and a 40s rhythm, but preferred both songs to silence. In contrast, the same strain raised in a normal light/dark cycle showed a strong preference for a 55s rhythm, but did not discriminate between a 40s rhythm and silence (see Section 7.3.2, p87).

8.4 DISCUSSION

It is impossible to draw any reliable conclusions from the *mbd* females, as so few matings were obtained. However, it is noteworthy that these females constitute the only group that mate as well with silence as with song. Neurons connecting the mushroom bodies to the antennal lobes in crickets are capable of responding to acoustic stimuli and hence the mushroom bodies may be part of the auditory pathway in this organism (Schildberger, 1981). Thus, it is possible that the *mbd* mutants cannot hear or process song information correctly.

The *sol;so* and *omb* females behave in a similar fashion to the per^{01} flies described in Chapter 7 ie they do not discriminate between a 55s and a 40s rhythm, but mate better with both songs than with silence. It appears that disrupting the optic lobes of the brain has the same effect on female song rhythm preferences as abolishing *per* function. This perhaps suggests that the mechanism for female song preferences is located in the optic lobes. Indeed, antibody staining to the *per* protein is found in the glial cells of the optic lobes (Siwicki *et al*, 1988). Interestingly, immunoreactive staining of the sol^+ protein has a similar pattern to that of the per^+ protein (S Delaney, pers comm).

Anti-*per* antibody staining is absent from glial cells (including those in the optic lobes) of flies transformed with the 13.2kb per^+ DNA fragment (Zerr *et al*, ms submitted). Results described in Chapter 7 show that 13.2;2a (per^+) transformants do not demonstrate a preference for a 55s song rhythm. This may provide further evidence for the location of the song rhythm preference mechanism in the optic lobes. However, it must be noted that the transformants tested for *per* staining

by Zerr et al (the 13.2;34 strain) have the 13.2kb per^+ DNA inserted into chromosome 2, whilst the 13.2;2a transformants described in Chapter 7 have the same DNA inserted into chromosome 3.

Despite this evidence, the putative optic lobe location for the song rhythm preference mechanism must be viewed with some caution. The *omb* mutant has a much less severe defect in the optic lobes than the *sol;so* double mutant (Fischbach & Technau, 1984; Fischbach & Heisenberg, 1984). Wild type locomotor activity rhythms are found in *omb* flies, but *sol;so* mutants show complex rhythmicity, with several periodicities occurring simultaneously (Helfrich, 1986). Thus the behavioural abnormality is correlated with the severity of the anatomical defect. It was anticipated that a similar correlation may be seen in the expression of female song rhythm preferences, with *omb* females being more similar to wild type than are *sol;so* females. However, this clearly does not occur. It is possible that any gross brain abnormalities could abolish female preferences and that the effect is not specifically due to the defects in the optic lobes.

It has been suggested that a common neuronal network is a necessary prerequisite for genetic coupling ie that the neuronal mechanism controlling female song reception must be the same as the mechanism controlling male song production (Doherty & Hoy, 1985; Bauer & von Helversen, 1987). This is clearly untrue, as genes may have pleiotropic effects on more than one neural network. Such pleiotropy is already demonstrated by the *per* gene, as the song rhythm is controlled from the thorax and the activity rhythm from the brain (Hall, 1984). Chapter 7 provides some evidence for genetic coupling with respect to the *per* gene and the courtship song rhythm. A demonstration of the control of female song rhythm preferences from the optic lobes of the brain would show that common neuronal networks for song production and reception are not necessary for the existence of genetic coupling.

The per^{01}-like behaviour of females raised in constant bright light may indicate that song rhythm preferences are controlled by a clock-type mechanism. Rhythmic behaviour in many insects is damped out in conditions of bright light, and the rhythms of locomotor activity and eclosion are abolished in D. melanogaster under this regime (Saunders, 1982; Zerr et al, ms submitted). Flies kept in constant light show very weak immunoreactive staining of the per protein in the brain (Zerr et al, op cit), so it seems that the *per* gene may be suppressed in the brain region under such conditions. Perhaps surprisingly, then, the song rhythms of per^+ males raised in constant bright light are normal

(Kyriacou & Hall, 1980). It would be interesting to examine *per* staining in the thorax of flies kept in constant bright light, as the *per* gene mediates song rhythmicity from the thoracic ganglia (Hall, 1984). Perhaps *per* expression and function in the thorax is not abolished in conditions of constant light.

The results from Chapter 7 strongly implicate *per* in the control of female song rhythm preferences. Given that both song preferences and *per* immunoreactive staining in the brain are abolished under conditions of constant bright light, it seems likely that song preferences are mediated from the brain. The fact that such preferences are absent in *omb* and *sol;so* double mutants may suggest that the optic lobes of the brain are particularly involved. However, this latter conclusion must remain tentative until further evidence is provided. The demonstration that 13.2;2a transformants show neither wild type song preferences nor wild type per^{+} immunoreactive staining in the optic lobes further implicates these anatomical structures as the site of control of female song preferences.

Chapter 9

Effect of temperature on male song production and female song reception

9.1 INTRODUCTION

Temperature compensation is required for accurate time measurement. In biological systems, the rate of biochemical processes is more than doubled with a $10^{o}C$ rise in temperature. If no mechanism existed to compensate for this effect, then endogenous clocks would run "fast" or "slow" according to the temperature of their surroundings.

This is illustrated by the eclosion rhythm of *D. pseudoobscura*. The effect of temperature changes on this rhythm are very slight indeed, as it varies by only about half-an-hour at temperatures of between 16-21^{o}C (Pittendrigh, 1954). The rhythm of conidiation in *Neurospora crassa* is also temperature compensated, and the compensation appears to be genetically controlled (Feldman, 1971). However, in the *Neurospora* rhythm mutant, *frq-9*, the clock speeds up considerably when the temperature is raised, demonstrating a loss of temperature compensation in this mutant (Loros & Feldman, 1986).

Although the locomotor activity rhythm of wild type *D. melanogaster* is also temperature compensated, the per^{S} and per^{L1} mutants demonstrate a degree of temperature sensitivity in this behaviour (Konopka *et al*, 1989). Periodicities of the per^{L1} mutant vary from 25-29 hours at temperatures of between 15-26^{o}C, with the rhythms becoming longer at higher temperatures. The per^{S} mutants exhibit a lesser degree of temperature sensitivity, with periodicities of the locomotor activity rhythm becoming shorter as the temperature increases. Thus it appears that in both *Drosophila* and *Neurospora*, genes controlling overt rhythmicity also contribute towards temperature compensation.

The rhythm in the courtship song of per^{+} males is also temperature compensated (Kyriacou & Hall, 1980), although the mean value around which the IPIs oscillate is subject to large fluctuations according to the ambient temperature (Shorey, 1962). For instance, at 18^{o}C, the mean IPI of *D. melanogaster* has a value of around 48ms, similar to that of *D. simulans* (Cowling & Burnet, 1981). It is clear that, at low temperatures, the maintenence of species isolation could be at risk, as the IPI is one of the courtship song characters that *Drosophila* females use to distinguish conspecific mates (Kyriacou & Hall, 1982). At low temperatures, a *D. simulans* female could mistake a *D. melanogaster* male singing with a mean IPI of 48ms for a member of her own species.

Clearly, *Drosophila* do not normally live under laboratory

conditions of controlled temperature. Therefore, for courtship communication to be effective in the wild, it is important that the effects of temperature on male song production be paralleled by its effects on female song reception. This idea has been termed "temperature coupling" by Gerhardt (1978). Temperature coupling has been demonstrated in several species of cricket, grasshopper and tree frog (Walker, 1957; Doherty, 1985; von Helversen, 1979; Gerhardt, 1978). The pulse repitition rate in the song of the cricket, *Oecanthus quadripunctatus*, increases with temperature. Using playback of natural and simulated songs, Walker (1957) showed that warm females respond best to the songs of warm males and cold females to the songs of cold males.

The experiments described in this Chapter investigate the effects of temperature on male song production and female song reception. The songs of per^{S} and per^{L1} males are examined at 18°C, to ascertain whether the temperature sensitivity of the activity rhythms of these mutants will be paralleled in the song rhythms. In addition, the effect of low temperature on female reception of IPI cycles in the courtship song is investigated, to determine whether this temporally programmed behaviour is sensitive to changes in temperature. The temperature coupling theory would predict that females kept at a certain temperature would respond best to courtship songs with an overall IPI value typical of that temperature. To test whether temperature coupling with respect to IPI occurs, females are tested at different temperatures with songs cycling around different mean IPIs. Finally, to complete this series of studies, the change in the mean IPI of the songs of *D. simulans* at 18°C is also examined.

9.2 MATERIALS AND METHODS

9.2.1 Strains

All *per* strains were as described in Section 7.2.1.1 (p84). The same per^{+} strain was used to test both female IPI and song rhythm preferences. The Georgetown strain (from B Burnet) was used for song recordings of *D. simulans*.

9.3 RESULTS

9.3.1 Effect of low temperature on male song rhythm production

The experimental procedure is described in Section 2.9.1 (p42). Table 9.1 shows the periods of the songs from the per^+ males recorded at low temperatures. 7 out of 8 songs show a significant rhythm with the BMDP3R analysis, with most of the periods falling into the 50-65s range. Note that per^+ no291 has a period of 55.0s on analysis with CLEAN, but a period of 93.0s with the van den Berg analysis. The BMDP3R analysis of this song gives two significant periods of 56.6s and 92.6s. A marginally higher F-ratio is obtained with the latter period (see Table 9.1). The analysis of per^+ no303 with BMDP3R, CLEAN and van den Berg is illustrated in Figure 9.1. Table 9.2 shows the distribution of the periods obtained from a van den Berg analysis of songs from per^+ males recorded at low temperatures and at 25°C (25°C data from Kyriacou *et al*, 1990b). A χ^2 contingency test demonstrates that the 2 distributions are not significantly different (χ^2=12.17; df=6). This confirms the result of Kyriacou & Hall (1980) which showed that the song rhythms of per^+ flies are temperature compensated. The distribution of the song periods from the CLEAN analysis is very similar to that of the van den Berg analysis.

Table 9.3 shows the song periods of per^s males recorded at low temperatures. Only one song has a significant rhythm with BMDP3R, and this is illustrated in Figure 9.2. The distribution of the periods obtained from the van den Berg analysis is shown in Table 9.2. A χ^2 contingency test shows that the distribution of the song periods of per^s males recorded at 18°C is not significantly different from that of per^s males recorded at 25°C (χ^2=1.81; df=3). The distribution of the song periods from the CLEAN analysis is again very similar to that of the van den Berg analysis.

The distribution of the song periods (van den Berg analysis) of per^{L1} males recorded at low temperature is also shown in Table 9.2. Again, a χ^2 contingency test shows shows no significant difference between the distributions of the periods of the songs recorded at low temperature and at 25°C (χ^2=7.35; df=6). Table 9.4 shows the periods obtained by analysis with BMDP3R, CLEAN and van den Berg. 6 out of 8 songs are significant according to BMDP3R, with all but one having a period of over 70s. The song of per^{L1} no370 is illustrated in Figure 9.3. The distribution of the song periods from the CLEAN analysis is

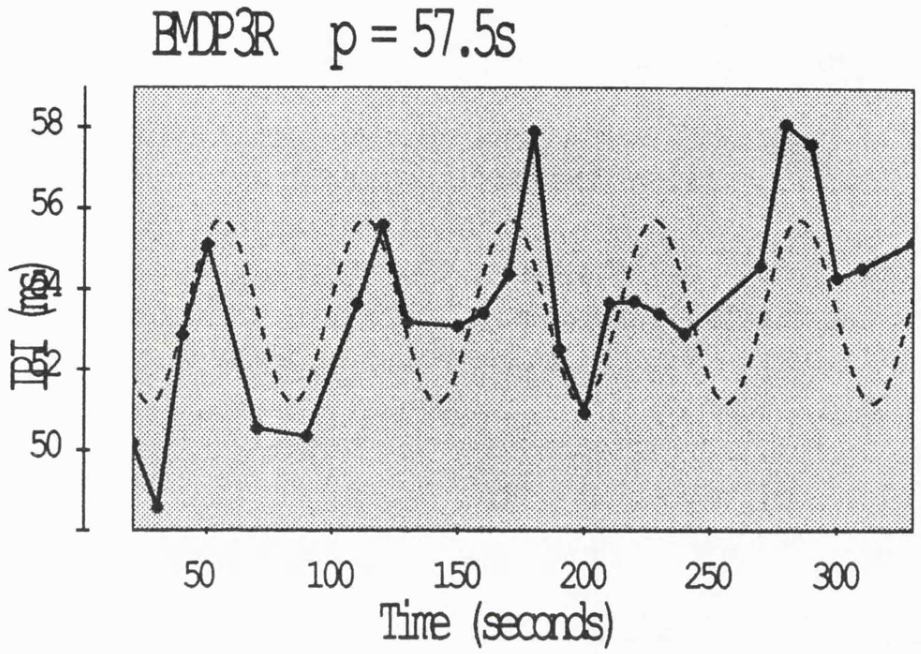
Table 9.1 BMDP3R, CLEAN and van den Berg analyses of per^+ songs recorded at low temperatures

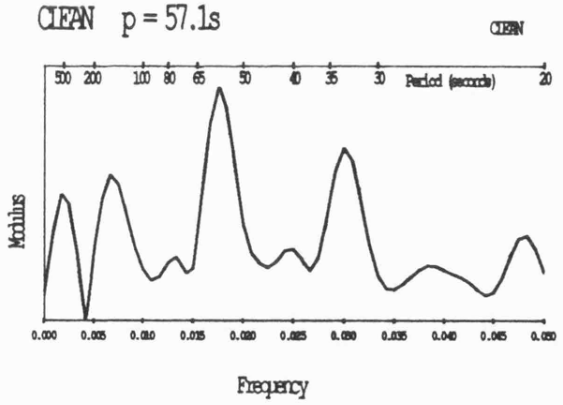
FLY	BMDP3R secs (if sig)	F	df	CLEAN secs	van den Berg secs
288	65.8	13.57	4,28	69.1@	129.0@
290	33.7	3.60	3,28	33.3	33.8
291	92.6	5.33	4,23	55.0	93.0
	or				
	56.6	4.60	5,22		
292	58.0	3.25	3,21	58.9	58.8
294	51.2	3.72	5,19	51.2*	51.9*
301				30.6	86.0
302	59.8	3.51	3,25	60.9	60.2
303	57.5	5.43	3,21	57.1	57.4
mean	59.8 or 54.7				
SEM	6.68 or 3.86				

* period obtained from the 2nd peak of the spectrogram

@ period obtained from the 3rd peak of the spectrogram

Fig 9.1 BMDP3R, CLEAN and van den Berg analyses of *per+* no303





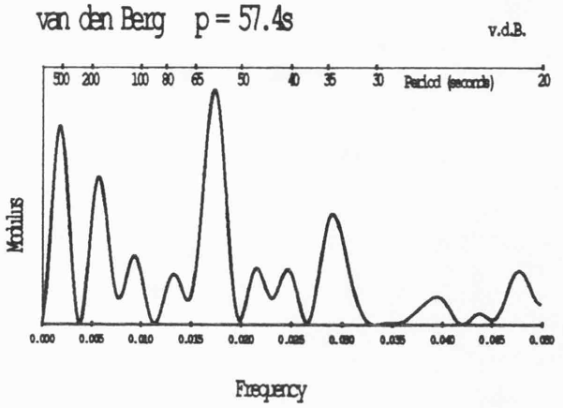


Table 9.2 Distribution of periods from a van den Berg analysis of per^{+}, per^{s} and per^{L1} songs recorded at low temperature and at 25°C

	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100	100-110	110-120	120+	N
Low temperature												
per^{+}	0	1	0	3	1	0	1	1	0	0	1	8
per^{s}	0	2	4	1	1	0	0	0	0	0	0	8
per^{L1}	0	0	0	1	0	2	2	1	1	1	2	10
25°C												
per^{+}*	0	0	4	16	4	0	1	0	0	0	0	25
per^{s}*	0	3	8	1	0	0	0	0	0	0	0	12
per^{L1}*	0	0	0	0	1	4	4	2	1	0	0	12

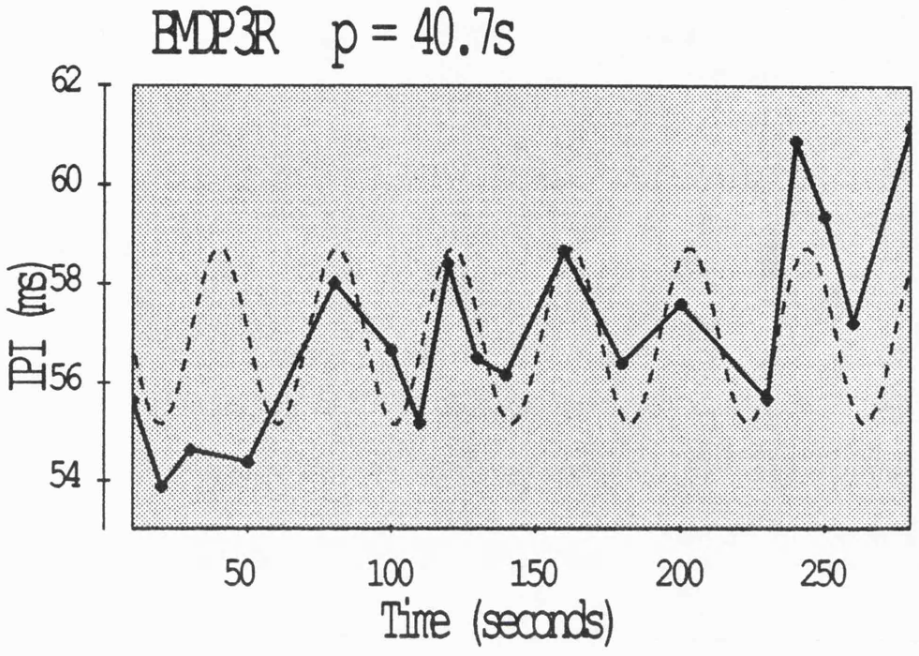
* From Kyriacou et al (1990b)

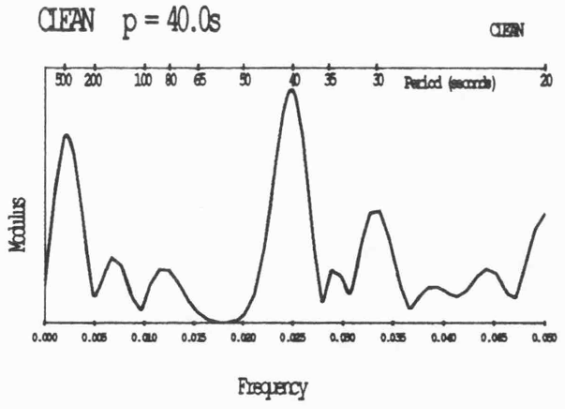
Table 9.3 BMDP3R, CLEAN and van den Berg analyses of per^s songs recorded at low temperature

FLY	BMDP3R secs (if sig)	F	df	CLEAN secs	van den Berg secs
443				32.3*	41.7*
446				40.0	40.7
447				61.3	62.0
448	40.7	3.44	3,14	40.0	40.8*
449				54.5	55.9*
450				28.4*	31.3*
451				32.6*	32.9*
452				25.6	40.2

* period obtained from the 2nd peak of the spectrogram

Fig 9.2 BMDP3R, CLEAN and van den Berg analyses of *pers* no448





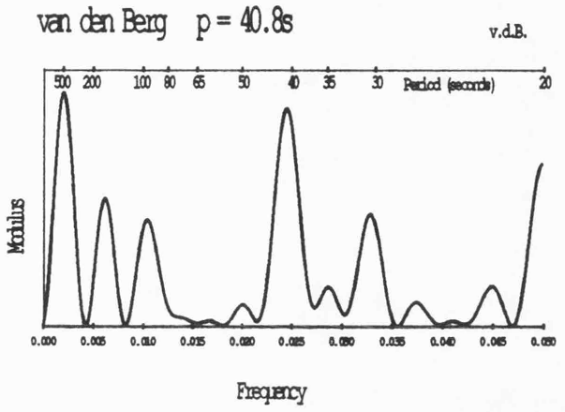
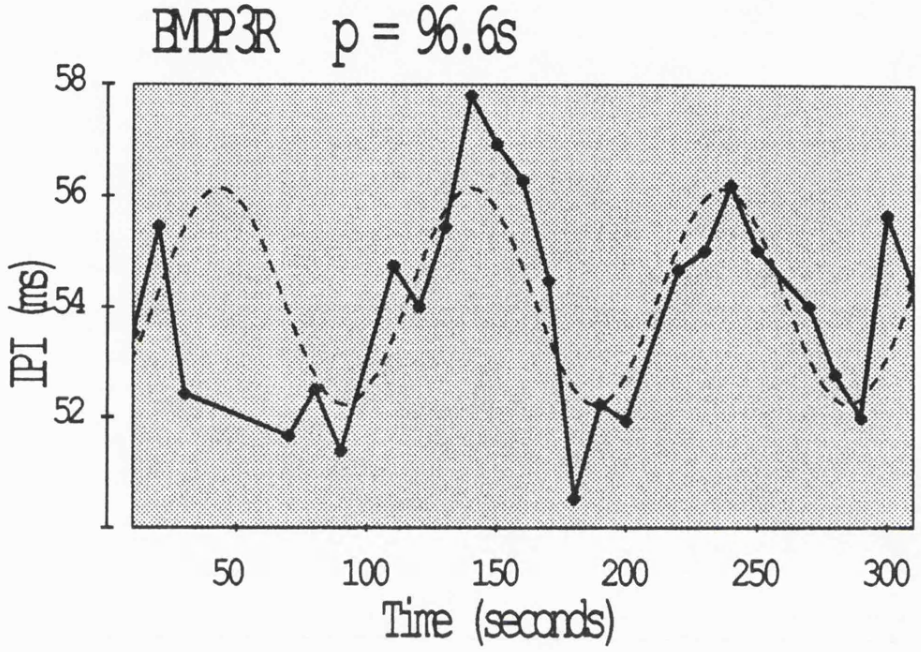


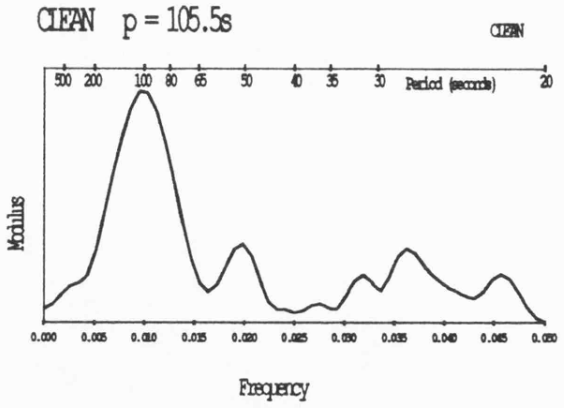
Table 9.4 BMDP3R, CLEAN and van den Berg analyses of per^{L1} songs recorded at low temperature

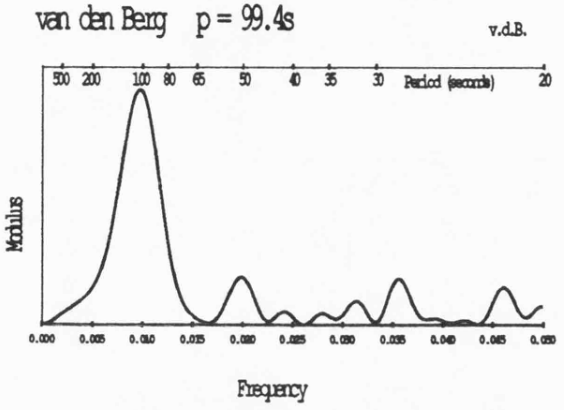
FLY	BMDP3R secs (if sig)	F	df	CLEAN secs	van den Berg secs
369	54.2	4.33	3,19	53.3	54.1
370	96.6	8.05	3,21	105.5	99.4
371	81.0	3.28	3,17	80.0*	81.6
372	76.8	5.53	3,18	75.0	76.6
373				22.6	114.3
374	85.1	3.68	3,18	86.2	83.4
375	140.7	8.83	4,30	132.0*	137.9*
376	128.8	6.26	3,32	130.9	133.3*
377				98.5*	94.2*
378	106.2	8.07	4,30	97.1*	108.1*
mean	96.2				
SEM	10.03				

* period obtained from the 2nd peak of the spectrogram

Fig 9.3 BMDP3R, CLEAN and van den Berg analyses of *perL1* no370







very similar to that of the van den Berg analysis.

In addition, a 2 way analysis of variance was performed on the song periods from a CLEAN analysis of per^{+}, per^{s} and per^{L1} males recorded at low temperatures and at 25°C. The data for the songs recorded at 25°C was obtained from Kyriacou & Hall (1989 - data not shown). The 2 way ANOVA showed a highly significant difference between genotypes ($F=50.01$; $df=2,62$). However, there was no significant difference between the periods obtained at low temperature versus those obtained at 25°C ($F=0.01$; $df=1,62$). In addition, there was no significant interaction between the genotype and temperature terms ($F=0.39$; $df=2,62$). These results would suggest that neither per^{+}, per^{s} nor per^{L1} show any temperature sensitivity with respect to their courtship song rhythms.

9.3.2 Effect of low temperature on female song rhythm preferences

The song rhythm preferences of per^{+} females were investigated at a temperature of 18°C. The females were tested with 2 simulated songs (mean IPI=35ms, with a cycle of 55s or 40s) and with silence. The experimental procedure was as described in Section 2.7.2 (p41), except that the experiment was performed at 18°C. 18 runs of the experiment were performed ie a total of 1350 flies were tested.

A Friedman test (planned comparisons) shows that, at 18°C, mating speed with a 55s rhythm is significantly faster than that with a 40s rhythm or with silence. However, there is no significant difference between the mating speeds obtained with a 40s rhythm compared to silence. The differences in mean ranks and the 95% confidence limits are shown in Table 9.5 and the results are illustrated in Figure 9.4.

Fig 9.4 Mean number of flies mating after 5 minutes ± S.E.M.

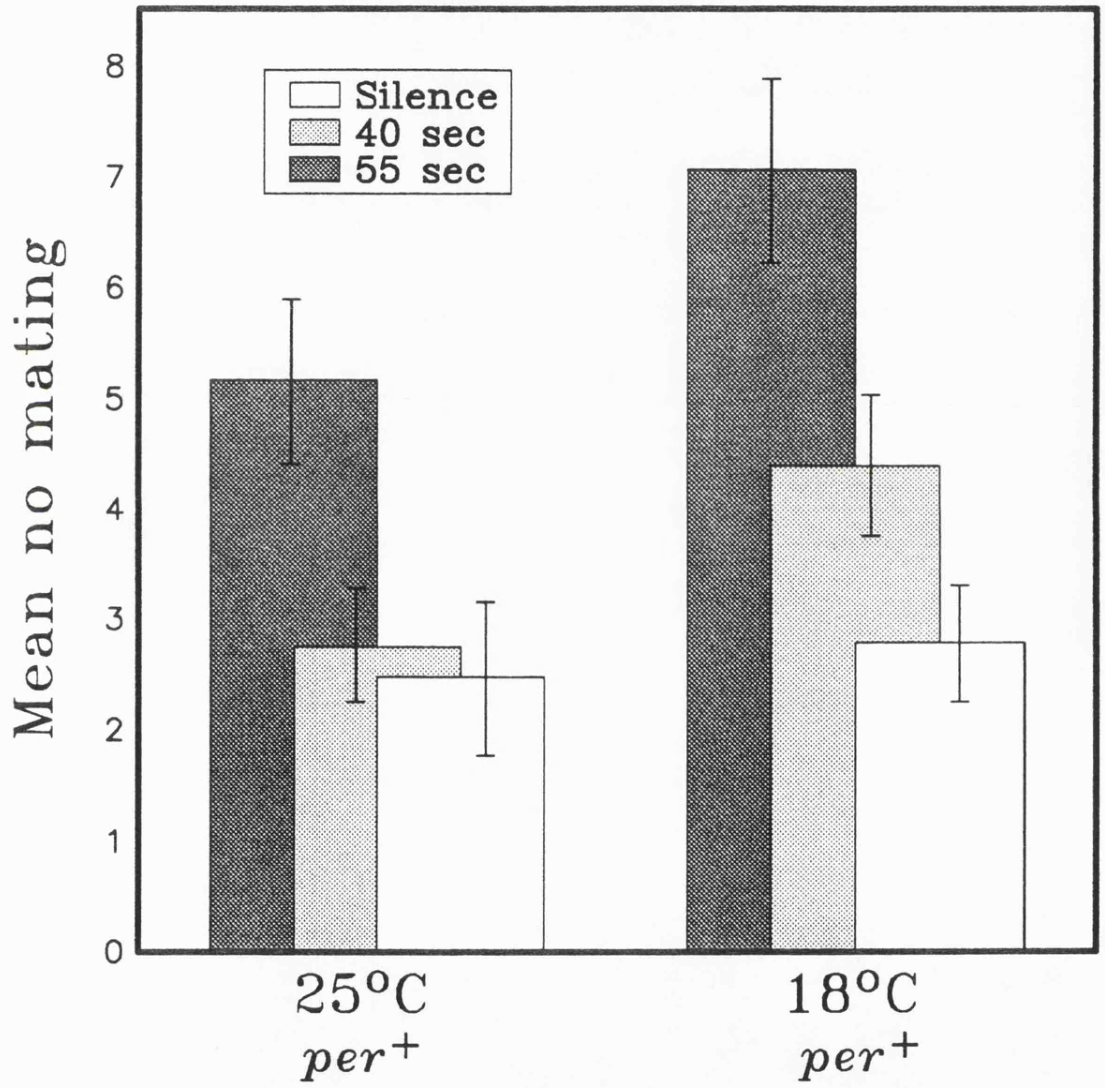


Table 9.5 Differences in mean ranks and 95% confidence limits from Friedman test (pairwise comparisons)

Genotype	Confidence limits	55/40	55/sil	40/sil
per^+	0.780	1.083*	1.417*	0.333

* indicates $p<0.05$

The song preferences demonstrated here are very similar to those seen in the per^+ females at 25°C (Section 7.3.1, p85), so it appears that changes in temperature do not affect female song rhythm preferences.

9.3.3 Effect of temperature on female IPI preferences

9.3.3.1 Method & Statistics

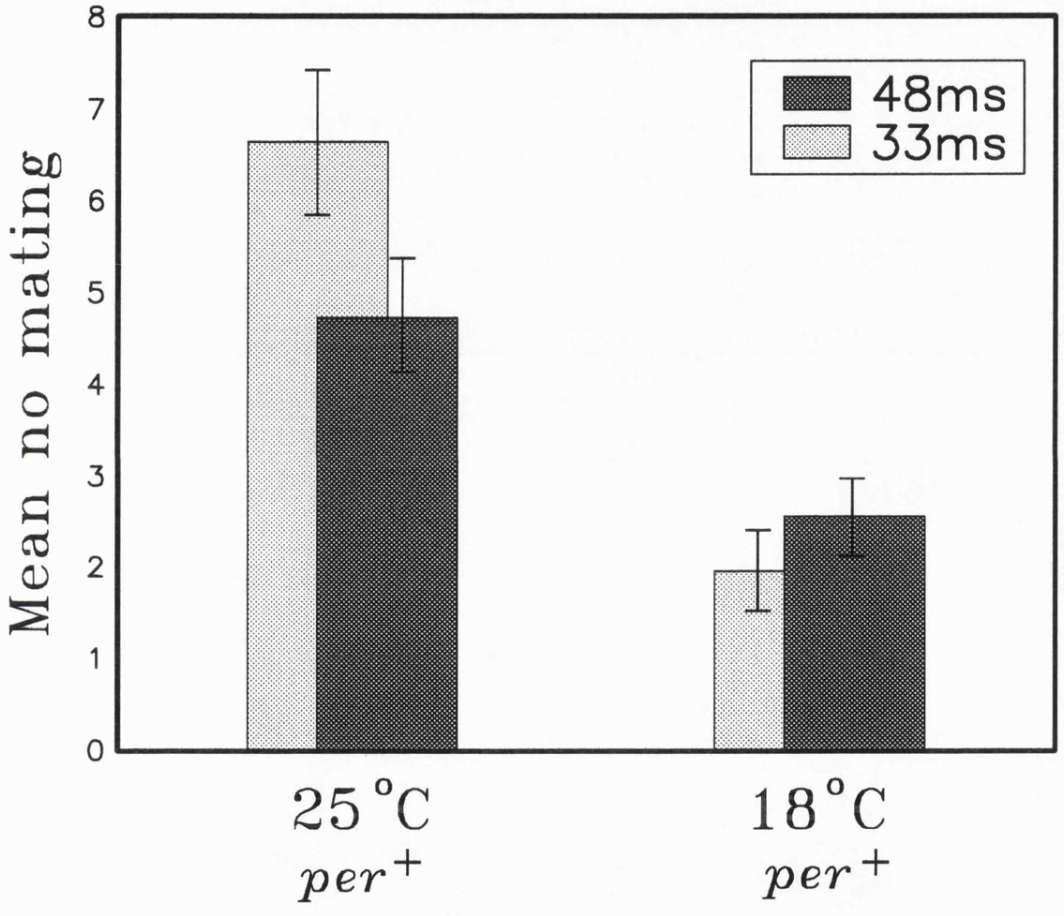
The method is fully described in Section 2.9.3 (p43). Briefly, the song preferences of per^+ females were tested at 18°C and 25°C. Two simulated songs were used - one with a mean IPI of 33ms (corresponding to male song at 25°C) and one with a mean IPI of 48ms (corresponding to male song at 18°C). Both songs cycled with a period of 55s.

A Friedman test was again employed in the analysis of these data (see Section 7.3.1, p85). As there were only two conditions in each run (and hence only one comparison to be made), there was no need to use the planned comparisons method and thus the standard Friedman test was employed (Hays, 1972). A χ^2 value, corrected for tied ranks, was obtained from this test.

9.3.3.2 Female IPI preferences

Table 9.6 shows the number of flies mating with both songs

Fig 9.5 Mean number of flies mating after 5 minutes $\pm$ S.E.M.



(cycling with a 55s period around a mean IPI of 33ms or 48ms) at 18°C and 25°C. The Friedman test demonstrates that, at 25°C, females mate significantly faster with a mean IPI of 33ms than with a mean IPI of 48ms (χ^2 = 4.96; df = 1). However, at 18°C, females do not demonstrate a preference for either song (χ^2 = 0.826; df = 1), although they mate very slightly faster with a 48ms IPI than with a 33ms IPI.

Table 9.6 Number of flies mating at 5 minutes with songs with a mean IPI of 33ms or 48ms, at 18°C and 25°C

Temperature	No mating		No tested
	33ms	48ms	
25°C	166	118	1250
18°C	39	51	800

It is clear that, as expected, the flies mate considerably faster at 25°C than at 18°C, regardless of song. This is illustrated in Figure 9.5.

9.3.4 Effect of temperature on IPI production of *D. simulans*

When analysing a *Drosophila* courtship song, it is necessary to set "cut-off" values for the length of an IPI, above and below which it will not be included in the analysis (see Section 2.4.1, p32). In *D. melanogaster*, the lower and upper cut-off values are set at 15ms and 65ms respectively for a song with a mean IPI of 35ms. The cut-off values alter according to the mean IPI of the individual song under analysis. This method was arrived at after extensive analysis of many *D. melanogaster* songs, taking into account the mean and the distribution of the IPIs (see Kyriacou & Hall, 1989). This compares with an upper cut-off value of 75ms used by Cowling & Burnet (1981) in every

Table 9.7 Mean IPIs and SEMs from analysis of *D simulans* courtship songs using 3 different cut-off values, at temperatures of 18°C and 25°C.

	Fly	Mean IPIs (ms) at different cut-off values		
		100ms	**155ms**	**195ms**
	1	56.45	60.91	65.39
	2	60.63	70.77	76.70
18°C	3	87.04	100.30	105.19
	4	72.96	78.25	83.20
	5	67.94	82.09	84.77
		mean=69.00	mean=78.46	mean=83.05
		SE=5.34	SE=6.55	SE=6.50
	6	57.15	60.09	60.92
25°C	7	54.18	58.87	60.71
	8	53.83	58.70	62.26
	9	50.09	56.32	60.44
		mean=53.81	mean=58.50	mean=61.08
		SE=1.45	SE=0.79	SE=0.41

courtship.

It is not sensible to set the cut-off values of *D. simulans* songs in the same way as with *D. melanogaster* songs. This is because the songs from the 2 species have different IPI distributions; the distribution of *D simulans* is more skewed in the direction of the long IPIs than is the distribution of *D melanogaster* (Cowling & Burnet, 1981). Therefore, it was decided to retain the lower cut-off value at 15ms and to analyse each song using 3 different upper cut-off values of 100ms, 155ms and 195ms.

The mean IPIs of the songs are shown in Table 9.7. Using a cut-off of 100ms, the mean of the IPI means is 69.00ms (SEM=5.34) at 18°C and 53.81ms (SEM=1.45) at 25°C. With a cut-off of 155ms, the mean of the means is 78.46 (SEM=6.55) at 18°C and 58.50 (SEM=0.79) at 25°C. With the highest cut-off value of 195ms, the mean of the means is 83.05ms (SEM=6.50) at 18°C and 61.08ms (SEM=0.41) at 25°C. Note that the mean IPI values are more widely distributed at 18°C than at 25°C. With a cut-off value of 100ms, the coefficient of variation of the means is 0.173 at 18°C and 0.054 at 25°C. It is also clear that, as in *D melanogaster*, the mean IPI of *D simulans* increases with a decrease in temperature.

9.4 DISCUSSION

In biological systems, a rise in temperature of 10°C can result in the doubling of the rate of biochemical processes. Clearly, this could have a severely disruptive effect on biological rhythms, as time-keeping mechanisms would run "fast" or "slow" depending upon temperature. However, a general feature of biological clocks is that they show temperature compensation ie the period of the rhythm is not temperature dependent (eg Moore-Ede et al, 1982; Saunders, 1982). Both the courtship song rhythm and the locomotor activity rhythm of wild type *Drosophila* show temperature compensation (Kyriacou & Hall, 1980; Konopka et al, 1989).

However, some mutations which alter overt rhythmicity also disturb temperature compensation. In the *frq-9* mutant of *Neurospora crassa* the period of the conidiation rhythm ranges from about 30 hours at 18°C to about 15 hours at 30°C (Loros & Feldman, 1986). Thus, at lower temperatures, the underlying biochemical processes appear to be slower, as expected, resulting in a slower rate of change and hence in a

longer rhythm. Similarly, in the per^S mutant of *Drosophila*, the period of the locomotor activity rhythm is lengthened with a decrease in temperature (Konopka *et al*, 1989). Surprisingly, however, the period is shortened in per^{L1} mutants with a decrease in temperature ie the clock runs faster (Konopka *et al*, 1989). From the results presented here, however, it is clear that the song rhythms of per^S and per^{L1} mutants are temperature compensated. Thus if the *per* gene does indeed contribute towards temperature compensation, then its action would appear to be specific to some oscillators but not to others.

These results give few clues as to how the *per* gene could mediate its effect on temperature compensation. It has been suggested that *per* controls overt rhythmicity by coupling together a population of ultradian oscillators (Dowse & Ringo, 1987), possibly via a mechanism of intercellular communication (Bargiello *et al*, 1987). It is possible that a decrease in temperature could result in a reduced rate of intercellular communication and hence in a "looser" coupling and longer period. This could be compensated for in per^+ flies, but not in per^S or per^{L1} mutants. However, this model cannot explain why the period of the locomotor activity rhythm in per^{L1} mutants is shortened. In addition, it is hard to see why coupling should be affected in the pacemaker controlling locomotor activity but not in the pacemaker controlling courtship song.

It appears that the song rhythm preferences of wild type *D. melanogaster* females do not alter at lower temperatures. At 18^oC, per^+ females display the same preference for a 55s rhythm as they do at 25^oC. Thus, both song rhythm production by males and song rhythm reception by females appear to be temperature compensated. The *per* gene is known to affect both male song rhythm production and female reception (Kyriacou & Hall, 1980; Chapter 7, this thesis). Mosaic studies have shown that song rhythm production in males is controlled via the thoracic nervous system (Hall, 1984), whereas the results from Chapter 8 suggest that the optic lobes of the brain may be involved in the control of female song rhythm preferences. Thus temperature compensation appears to occur in at least two neural networks.

For effective courtship communication to take place, it is essential that the effects of temperature on male song production be matched by similar effects on female song reception ie "temperature coupling" (Gerhardt, 1978). Temperature coupling with respect to song rhythm has not been specifically demonstrated here, as song rhythm does not alter with temperature in wild type flies (Kyriacou & Hall, 1980).

Hence, female song rhythm reception would not be expected to change according to temperature. However, the IPI of the male courtship song does alter with temperature, becoming longer as the temperature decreases (Shorey, 1962). Hence, IPI production by males and IPI reception by females may be expected to demonstrate temperature coupling.

At 25°C, the *D. melanogaster* males tested here produce an IPI which fluctuates rhythmically around a mean of 33ms. At 18°C, they produce a mean IPI of 48ms. Females of the same strain prefer a song with a mean IPI of 33ms at 25°C, but mate equally well with both songs at 18°C. This seems rather puzzling at first glance. One very simple explanation would be that females are not able to discriminate between different IPIs at low temperatures. However, they clearly do discriminate between different IPI cycles (55s versus 40s), so this seems to be unlikely.

It is possible to speculate that a type of temperature coupling may be occurring in this system. At 25°C, females may "choose" not to mate when stimulated with a song with a 48ms mean IPI. This is reminiscent of the results of Chapter 7, where females appear to discriminate against a song cycle of 40s. However, at 18°C, the females tested here no longer demonstrate an aversion to the song with a 48ms IPI. Thus, the mechanisms controlling IPI production and reception seem to react in the same general direction to a decrease in temperature. Physiological systems (except biological clocks) are expected to function differently according to temperature.

It seems likely that the coordination of the control systems of IPI production, temperature dependence of IPI and IPI reception could be selectively advantageous. A female would then be able to "choose" the correct IPI range specific to males of her own species, regardless of temperature. At 25°C, *D. simulans* males produce a song with a mean IPI of 48ms-55ms (Ewing & Bennet-Clark, 1968; Cowling & Burnet, 1981). Thus, by choosing to mate when stimulated by a song with a 33ms mean IPI (rather than a 48ms IPI), *D. melanogaster* females avoid mating with *D. simulans* males. If, at 18°C, *D. simulans* males continue to produce a mean IPI in the range of 48ms-55ms, there would be no benefit to the *D. melanogaster* female in discriminating in favour of a 48ms IPI, as she would be as likely to mate with a *D. simulans* male as with a conspecific *D. melanogaster* male. Equally, she would not benefit by continuing to "choose" a 33ms mean IPI, as few conspecific males would be producing a song with this IPI. To investigate this possibility, the songs of *D.*

simulans males were recorded at 18°C and at 25°C. It appears that the mean IPI of the *D. simulans* courtship song does become longer at lower temperatures. By "choosing" a song with a mean IPI of 48ms, *D. melanogaster* females should be able to discriminate against *D. simulans* males at low temperatures.

Why then, do *D. melanogaster* females fail to discriminate between songs with a mean IPI of 33ms and of 48ms? From Table 9.7, it is clear that the IPI of *D. simulans* males is more variable at 18°C than at 25°C. It is possible that the same may be true of *D. melanogaster* males. In this case it may be that the IPI range is a less reliable indicator of a conspecific male at low temperatures. Thus there may be no selective advantage for females to evolve preferences for different IPIs at low temperatures, especially given their ability to discriminate conspecific males using the IPI cycle of the courtship song.

Chapter 10

General Discussion

Most organisms living on the surface of this planet demonstrate a variety of behavioural and physiological rhythms which are "tuned-in" to the normal cycles of the Earth. Biological clocks have been well characterised at the physiological and anatomical levels in a variety of species, particularly insects (eg Saunders, 1982). However, little is known about how such clocks may function at the cellular and molecular levels. Such knowledge may have tremendous potential for the alleviation of certain medical and social problems. For example, it may be possible to alleviate jet-lag by phase shifting the clock of sufferers so that it is tuned-in to local time. If the cellular nature of the clock was understood, it may be possible to design drugs which could achieve this.

To what degree has current research contributed to such knowledge? The study of the *per* gene has revealed that it influences several biological rhythms, suggesting that it may control a fundamental mechanism common to all clock functions. It is thought that *per* may code for a proteoglycan (Jackson *et al*, 1986; Reddy *et al*, 1986; Bargiello *et al*, 1987). This putative proteoglycan may be involved in intercellular communication via gap junctions (Bargiello *et al*, 1987). However, the proteoglycan hypothesis must remain tentative, as it is largely based on the supposition that the Threonine-Glycine repeats in the *per* protein have similar biochemical properties to the Serine-Glycine repeats found in the rat chondroitin sulphate proteoglycan. However, glycosylation can be disrupted in mammals when Ser-Gly repeats are altered to Thr-Gly repeats (Bourdon *et al*, 1987; also see Hall & Kyriacou, 1990). In addition, the Thr-Gly region can be deleted without affecting the 24 hour period of the locomotor activity rhythm and thus is clearly not vital for circadian rhythmicity (Yu *et al*, 1987b).

Thus it is not yet clear exactly what kind of protein *per* produces nor what it does. Immunoreactive staining of an anti-per antibody has revealed a rather widespread pattern of staining (Saez & Young, 1988; Siwicki *et al*, 1988), making it difficult to focus on a particular site of *per* action. However, the anti-per antibody of Siwicki *et al* (1988) may provide a way of directly examining the function of *per*. Siwicki *et al* (1989) have shown that this antibody will stain the pacemaker cells in the eyes of the marine molluscs, *Bulla* and *Aplysia*. Thus it appears that a *per*-like gene may exist in these organisms. The large size of the pacemaker neurons in *Aplysia* makes them relatively easy to manipulate neurophysiologically (Jacklet, 1985). It should be possible to clone the *per* gene relative in *Aplysia* by screening expression libraries of *Aplysia* DNA with the anti-per antibody (Hall & Kyriacou,

1990). This done, the *Aplysia* gene could be subjected to *in vitro* mutagenesis and the mutated material injected into the pacemaker neurons. Any changes in the neurophysiology of these neurons could be detected and may provide an insight into the actual function of *per* (Hall & Kyriacou, 1990). The majority of rhythm mutations are semi-dominant (Hall & Rosbash, 1988; Ralph & Menaker, 1988), so the effect of such a mutation should be detected within a wild type background.

The pacemaker function of Earth dwelling organisms seems likely to be complicated, involving the action of many genes. Therefore, the study of more clock genes is probably necessary for a more complete understanding of the structure and functioning of pacemaker systems. *Drosophila* may again lead the field in this respect, given its easily manipulated genetic system. It will be useful to characterise more "clock" phenotypes in this organism, so that the effects of any one gene can be studied with respect to a variety of phenotypes. This process has already begun, with the recent discovery that clock genes also affect development time in *Drosophila* (Kyriacou et al, 1990a & this thesis).

The study of the *Clk6* and *andante* genes in Chapters 3 & 5 provides a small step in the analysis of further clock mutants. The *andante* mutation is similar to *per* in that it seems to affect all the clock phenotypes examined. The *Clk6* mutation is perhaps more interesting, as it produces short rhythms in circadian behaviour (locomotor activity and eclosion) and yet does not seem to affect song rhythms nor development time. However, the *Clk6* story is complicated by the close proximity of this gene to *per*, as it is possible that *Clk6* could be part of the *per* locus. There appear to be at least 3 possibilities regarding the *Clk6* mutation:

1. it is part of the *per* locus, perhaps defining a regulatory site
2. it is a separate locus, with a modifying effect on the *per* gene
3. it is a separate locus, encoding a structural clock protein

If *Clk6* is a separate locus from *per*, then it is located very close to *per* (Dushay et al, 1990) and is likely to encode either the 0.9kb or the 2.7kb RNA transcript shown in Figure 1.2. This could be confirmed by cloning DNA from this region in *Clk6* mutants and separately transforming the 0.9kb and 2.7kb encoding regions back into wild type flies. As *Clk6* is semi-dominant, such flies would be expected to demonstrate locomotor activity rhythms with periodicities intermediate to those of *Clk6* and wild type (Dushay et al, 1990). However, this procedure would not distinguish between options 2 & 3 described above.

It is clearly desirable to create more clock mutants. This

could be achieved either by standard EMS mutagenesis, which tends to generate point mutations, or by P-factor mutagenesis (eg Roberts, 1986). An advantage with the latter method is that it allows easy retrieval of the DNA into which the P-element insertion has occurred, facilitating molecular analysis of the region.

Another method of investigating clock function is to use neurological mutants. This approach has been successfully adopted by Helfrich (1986) who showed that severe optic lobe mutants have quasi-normal locomotor activity rhythms. This suggests that the locomotor activity clock of *Drosophila* is not located in the optic lobes, as is believed to be the case in Orthopteran insects (Saunders, 1982). The problem with many neurological mutations is that they are rather unspecific and may have pleiotropic effects (Kyriacou, 1990). This is illustrated by the study of the effects of *disco* on biological rhythms (Dushay *et al*, 1989 & this thesis). The evidence may suggest that *disco* mediates its effects on rhythmicity by blocking communication between the extraocular photoreceptors and the pacemaker structures (Dushay *et al*, 1989). However, it is not possible to rule out a direct effect of *disco* on the pacemaker itself. Thus neurological mutations may have a rather limited value in the study of clock or other behavioural functions.

As well as being of value to the study of biological rhythmicity, the courtship song rhythm of *Drosophila* is also of interest to evolutionary biologists because it acts as a species recognition signal (Kyriacou & Hall, 1982). In fact, the *per* gene provides a perfect example of the potential of molecular and genetic techniques to solve evolutionary and behavioural problems. The *per* gene has been cloned and sequenced in several *Drosophila* species, revealing that some regions are highly conserved between species whilst others are highly diverged (Bargiello & Young, 1984; Reddy et al, 1984; Colot *et al*, 1988; Thackeray, 1989; Wheeler *et al*, ms submitted).

The Thr-Gly region of *per* is particularly important in the control of song rhythms (Yu *et al*, 1987b; Wheeler *et al*, op cit). Results from Chapter 4 and Wheeler *et al* (op cit) suggest that a small number of amino acid substitutions may be responsible for species specific differences in song rhythm. When *D. simulans* DNA from this region is transduced into *D. melanogaster* per^{01} flies, a *D. simulans*-like song rhythm is produced. It may be possible to further clarify which amino acids are important using in vitro mutagenesis. For example, there are four species specific amino acid differences between *D. melanogaster* and *D. simulans* (Wheeler *et al*, op cit). These amino acids could be

mutagenised in *D. simulans* DNA to the *D. melanogaster* motif and the resulting hybrid gene transduced into per^{01} *D. melanogaster*. If the resulting transformants produce a *D. melanogaster*-like song rhythm, then this would indicate that the altered amino acid was responsible for the change in species specific behaviour. Any one or a combination of these amino acids could be mutagenised to the *D. melanogaster* form. In this way the effects of individual and combinations of amino acids on species specific song rhythms could be studied.

There is clearly little point in male *Drosophila* producing species specific courtship signals if females are not "tuned-in" to the signal of conspecifics. Both the rhythm and the IPI range of the song seem to act as species recognition signals (Kyriacou & Hall, 1982). Evidence from Chapter 7 confirms that wild type *D. melanogaster* females mate faster when stimulated by a song with a *D. melanogaster*-like rhythm of 55s than with a *D. simulans*-like rhythm of 40s. A novel result was obtained, showing that wild type *D. melanogaster* females do not discriminate against a song with an 80s rhythm. The results from Chapter 7 also suggest that female song rhythm preferences and male song rhythm production are genetically coupled, as per^{01} females do not discriminate between different song rhythms.

It would have been interesting to have been able to further investigate the role of the *per* gene in the production of species specific song rhythms and their reception by females. For instance, the preferences of *D. melanogaster* females transformed with a *D. simulans per* gene could have been examined. Would such females prefer a *D. simulans*-like rhythm of 40s? The Thr-Gly region of the *per* gene is important in the production of species specific song rhythms (Wheeler *et al* op cit; Chapter 4, this thesis). The role of this region in establishing species specific song preferences could have been tested by transforming *D. melanogaster* flies with chimaeric *per* genes, containing, for example, the Thr-Gly region from *D. simulans* and the remainder of the *per* gene from *D. melanogaster*.

Unfortunately, per^{01} females transformed with per^{+} DNA (the 13.2;2a strain discussed in Chapter 7) continue to show per^{01}-like behaviour; the song rhythm preferences seen in wild type females are not restored. Thus, no suitable control is available for the experiments described above. Other *per*-affected behaviours such as locomotor activity, eclosion and song rhythms <u>are</u> restored in these transformants, so it is puzzling that the preference phenotype is not. A possible explanation is that the *per* DNA is required to be inserted into its

correct position on the X-chromosome before song rhythm preferences are expressed. This may indicate the presence of a modifier gene, perhaps suggesting that *per* is part of a supergene complex. It is noteworthy that *Clk6* is very closely linked to *per* and may itself play a role in *per* regulation. However, it must be remembered that *Clk6* could actually be part of the *per* locus itself. Clearly, the idea of modifier genes being involved in the regulation of *per* is highly speculative. If they exist, then any modifier genes affecting rhythms of song, locomotor activity and eclosion do not require to be physically adjacent to transformed *per* DNA in order to function correctly.

Using brain mutants, Chapter 8 has attempted to characterise and locate a possible mechanism for the control of female song rhythm preferences. The results illustrate the problems associated with the use of brain mutants discussed earlier. Both optic lobe mutants tested, *omb* and the *sol;so* double mutant, demonstrate no song rhythm preferences. This may suggest an optic lobe location for the song rhythm preference mechanism, but equally the lack of preferences shown by these mutants may be due to other effects of the mutant genes. However, some support for the highly tentative optic lobe hypothesis is provided by the observation that transformed flies, which show no song rhythm preferences, also show no *per* immunoreactive staining in the optic lobes (Zerr *et al*, ms submitted). Normal per^+ flies do show staining in these ganglia (Siwicki *et al*, 1988). Speculatively, it is possible that a modifier gene is required to be in close proximity to the *per* DNA in order to promote expression specifically in the optic lobes. This is again somewhat reminiscent of the *Clk6* situation, where the *Clk6* gene affects the locomotor activity clock in the head, but not the song clock in the thorax. If *Clk6* is a modifier of *per*, then it appears to have tissue specific properties.

The results from Chapter 9 show that wild type females continue to demonstrate a preference for a 55s song rhythm at low temperatures. The song rhythms of wild type males are temperature compensated ie do not alter according to the ambient temperature (Kyriacou & Hall, 1980; Chapter 9, this thesis). Thus, the song rhythm appears to be a reliable species recognition signal, regardless of temperature. The same does not appear to be true of the IPI range of the song. IPIs tend to become longer as the temperature decreases in both *D. melanogaster* and *D. simulans* (Shorey, 1962; Chapter 9, this thesis). *D. melanogaster* males produce a song with a mean IPI of about 33ms at 25°C and of about 48ms at 18°C. It might therefore be expected that *D. melanogaster* females will "prefer" a song

with a 33ms mean IPI at 25°C and a song with a 48ms mean IPI at 18°C. The results show that the former is true, but at 18°C, females do not discriminate between the two songs. This is a puzzling result, but may suggest that the IPI range is a less reliable courtship signal at low temperatures than the song rhythm.

The *per* gene also appears to be involved in temperature compensation of the locomotor activity rhythm, as per^{s} and per^{L1} mutants both demonstrate a degree of temperature sensitivity (Konopka *et al*, 1989). This temperature sensitivity of clock mutants is also seen in the *frq-9* mutant of *Neurospora*, whose clock speeds up considerably when the temperature is raised (Loros & Feldman, 1986). However, the results described in Chapter 9 show that the courtship song rhythms of per^{s} and per^{L1} males are not altered at low temperature. Thus, per^{s} and per^{L1} mutants do not demonstrate temperature sensitivity with respect to their song rhythms.

Kyriacou & Hall (1982) demonstrated that both the song rhythm and the IPI range are important characteristics in allowing *Drosophila* females to select a conspecific mate. It is interesting to speculate about how these song characters and female song preferences may have evolved. At first glance, the function of the song rhythm is puzzling; given the possible short length of courtships in the wild (Ewing & Ewing, 1983), it is unlikely that *Drosophila* females are able to "count" whole IPI cycles. It is possible that individual females may be most stimulated by individual IPI values. In this case, males which sing with an unvarying IPI will stimulate only a limited number of females, whilst males which vary their IPI will stimulate a greater number. The song rhythm could be a method by which males systematically vary their IPI production.

Chapter 6 provides some evidence that there may be inherited variation in female IPI preferences and that females show preferences for individual IPIs. This evidence arose from a selection experiment in which it was attempted to select 2 separate lines of females which preferred either a song with a constant 30ms IPI or a song with a constant 40ms IPI. In the early generations of selection, IPI preferences were observed in the predicted directions, but these disappeared in later generations. This was probably due to the preferences being swamped by the simultaneous selection of fast mating genes, as discussed in Chapter 6. It should not be allowed to obscure the evidence of the early generations of selection that females did show predictable preferences for individual IPI values. Thus it seems possible that a male could stimulate more females by varying

his IPI production and hence increase his chance of mating.

The song rhythm of *Drosophila* clearly has a central role to play in courtship communication and recognition of conspecific mates. Thus any genes affecting this character have the potential to participate in evolutionarily important events such as speciation. The experiments described in this thesis have contributed in a small way to the understanding of how such genes may exert their effects on both the production and reception of courtship song in *Drosophila*.

Appendices

APPENDIX 1

Differences in the distribution of song periods from the CLEAN and van den Berg analyses observed in the andante courtship songs. (see p52)

It is notable that different distributions of the *andante* song periods are obtained with the CLEAN and van den Berg analyses. There are 7 songs whose periods differ by more than 10s with the two methods of analysis. Theoretically, the greatest difference between the van den Berg and CLEAN analyses should lie in their treatment of empty 10s bins (ie missing data points. See Kyriacou et al, 1990b). The van den Berg analysis ignores missing data points, whilst the CLEAN analysis estimates them by a procedure approximating to a least squares interpolation. Table A.1.1 shows the number of empty bins in each *andante* song. The table is divided into those songs in which the CLEAN and van den Berg periods were similar and those in which they differed by more than 10s. The percentage of empty bins in each song was calculated and transformed to degrees. A one way ANOVA was then performed on the two song types ie those in which the CLEAN and van den Berg periods were similar and those in which they were different. No significant difference was observed ($F=0.71$; $df=1,13$).

Thus the absolute number of empty bins does not appear to contribute towards the differences observed in the CLEAN and van den Berg periodicities. It is possible that the number of consecutive (ie 2 or more) empty bins may contribute towards the differences observed. Table A.1.1 also shows the number of consecutive empty bins (2 or more) in the andante songs. A one way ANOVA was again performed on the two song types, this time with respect to whether there were 2 or more consecutive empty bins. Again, no significant differences were observed ($F=0.01$; $df=1,13$). Only *andante* no20 and *andante* no30 had 3 consecutive empty bins and only *andante* no24 had 4 consecutive empty bins. Thus it appears that differences in song periods obtained from the CLEAN and van den Berg analyses must be due the CLEAN algorithm itself which "cleans" the spectral analysis of the frequencies of sampling. The van den Berg analysis, however, produces a "dirty" spectrum and contains the periodic components that are inherent in the way the data are sampled. See Kyriacou et al (1990b) and Roberts et al (1987).

Table A.1.1 Empty bins (missing data points) in *andante* songs

FLY	NO. OF EMPTY BINS (missing data points)	NO. OF CONSECUTIVE EMPTY BINS (2 or more)	TOTAL NO. OF BINS (total no of data points, including empty bins)
A			
2	8	0	37
3	2	0	33
4	5	0	35
5	7	1	27
25	9	0	32
30	11	1	35
36	9	3	32
B			
7	2	0	33
9	2	0	25
20	8	1	32
21	9	1	33
24	6	1	33
28	5	0	35
29	10	2	32
40	7	1	46

A Songs from which different periods were obtained with the van den Berg & CLEAN analyses

B Songs from which similar periods were obtained with the van den Berg & CLEAN analyses

APPENDIX 2

Population cages

A.2.2 Introduction

The relative fitness of the various *per* alleles has never been investigated. The results from Chapter 7 of this thesis show that per^{+}, per^{s} and possibly per^{L1} females mate faster with a courtship song with a 55s or an 80s rhythm. Thus it may be expected that per^{s} and per^{01} males, who produce 40s song cycles and arrhythmic songs respectively, may be at a disadvantage compared to per^{+} or per^{L1} males, who produce 55s and 80s song cycles, with respect to mate competition. However, Chapter 5 has shown that per^{s} and per^{01} flies have shorter developmental times than per^{+} or per^{L1}, perhaps conferring a developmental advantage to the former genotypes. To investigate the relative fitness of per^{+}, per^{s}, per^{L1} and per^{01}, a population cage experiment was initiated.

A.2.2 Method and Materials

It was not possible to directly compare the relative fitnesses of the *per* genotypes by mixing them together in a population cage. The *per* genotypes are not distinguishable by eye, but must be tested in the locomotor activity measurement apparatus (see Section 2.3, p31). In a population cage experiment, the genotype of a large number of flies must be recorded at each generation to obtain a statistically acceptable sample size. It would not be feasible to test a sufficiently large number of flies in the activity monitoring apparatus currently available.

To overcome this problem, 8 population cages (2 replicates of each *per* genotype) were established, in which each *per* genotype was separately mixed with FM7a flies. The *per* strains used were as described in Section 5.2.1. The FM7a balancer derivative of FM7 (Lindsley & Grell, 1968) prevents recombination on the X chromosome. It is marked with the visible mutations *yellow*, *white* and *Bar*. Female *per*/FM7a heterozygotes can be distinguished by their characteristic kidney shaped eyes. It was expected that the fittest *per* genotype would take over from the FM7a flies in the shortest time and that the least fit *per* genotype would take the longest time to swamp the FM7a flies.

The cages consisted of a perspex box (24cm height x 26cm width

x 10cm depth), with 12 holes into which food vials (described in Section 2.2) could be inserted. A wire mesh top provided ventilation and a sliding tray at the bottom allowed the cages to be cleaned.

The population cages were established with an initial 90% of FM7a chromosomes and 10% *per* chromosomes. 200 flies formed the starting population - 90 FM7a males, 90 FM7a females, 10 per males and 10 per females. The flies were all 4 days old and were virgins. 6 food vials were inserted into the cage and the remaining 6 food vial holes were plugged with foam stoppers. The food vials were used both as a source of food and as an egg laying medium. The cages were maintained at 25°C, in a 12:12 LD cycle.

After 10 days, the first flies of the next generation began to emerge. By this stage, most of the flies in the initial population had died, as the food supply had been exhausted. Consequently, the generations were relatively discrete and did not overlap. When the new flies began to eclose, 3 new food vials were inserted into the empty positions to provide a source of food for the newly emerged adults. 6 days later (16 days after the start of the experiment) these 3 vials were removed and replaced with 6 new vials into the empty positions. These vials were left in place for 1.5 hours, in order for the new generation of flies to lay eggs. The progeny from these vials formed the sample of the first generation. After 1.5 hours, the vials were removed and replaced with 6 new vials. The old vials (put in place at the beginning of the experiment) were left in place until the next generation began to emerge, when they were removed and replaced with the next set of new vials. This procedure was repeated at each generation for a total of 9 generations.

After sampling, the sample vials were maintained at 25°C, in a 12:12 LD cycle. The sample progeny were counted and classified as they emerged. All progeny from each vial were scored and scoring took place until no more flies emerged. From these samples, the proportion of FM7a and *per* chromosomes were calculated at each generation.

A.2.3 Results and Discussion

The percentage of *per* chromosomes from each cage, in each generation are shown in Table A.2.1 and Figures A.2.1 and A.2.2. No samples were taken in Generation 7.

Unfortunately, the experiment was not a success, as the FM7a frequency was so rapidly reduced by the competing *per* chromosome that

Table A.2.1 Percentages of per chromosomes at each generation

Generation		per^{+}	per^{s}	per^{L1}	per^{01}
Initial		10.0	10.0	10.0	10.0
1	Replicate 1	36.4	74.9	51.5	30.4
	Replicate 2	39.2	69.1	54.4	63.8
2	Replicate 1	54.4	56.9	60.3	57.0
	Replicate 2	66.7	72.4	73.5	79.2
3	Replicate 1	68.7	78.3	72.2	76.4
	Replicate 2	83.9	81.7	81.8	87.1
4	Replicate 1	83.9	81.0	86.7	93.1
	Replicate 2	86.1	81.5	86.4	89.3
5	Replicate 1	94.7	86.5	86.5	76.5
	Replicate 2	91.3	94.4	no result	94.8
6	Replicate 1	98.4	88.4	93.0	91.9
	Replicate 2	93.6	97.3	95.6	96.2
8	Replicate 1	no result	97.2	no result	99.1
	Replicate 2	98.3	98.2	no result	99.7
9	Replicate 1	97.2	98.9	97.5	97.7
	Replicate 2	98.6	98.6	98.2	98.5

Fig A.2.1 Replicate 1

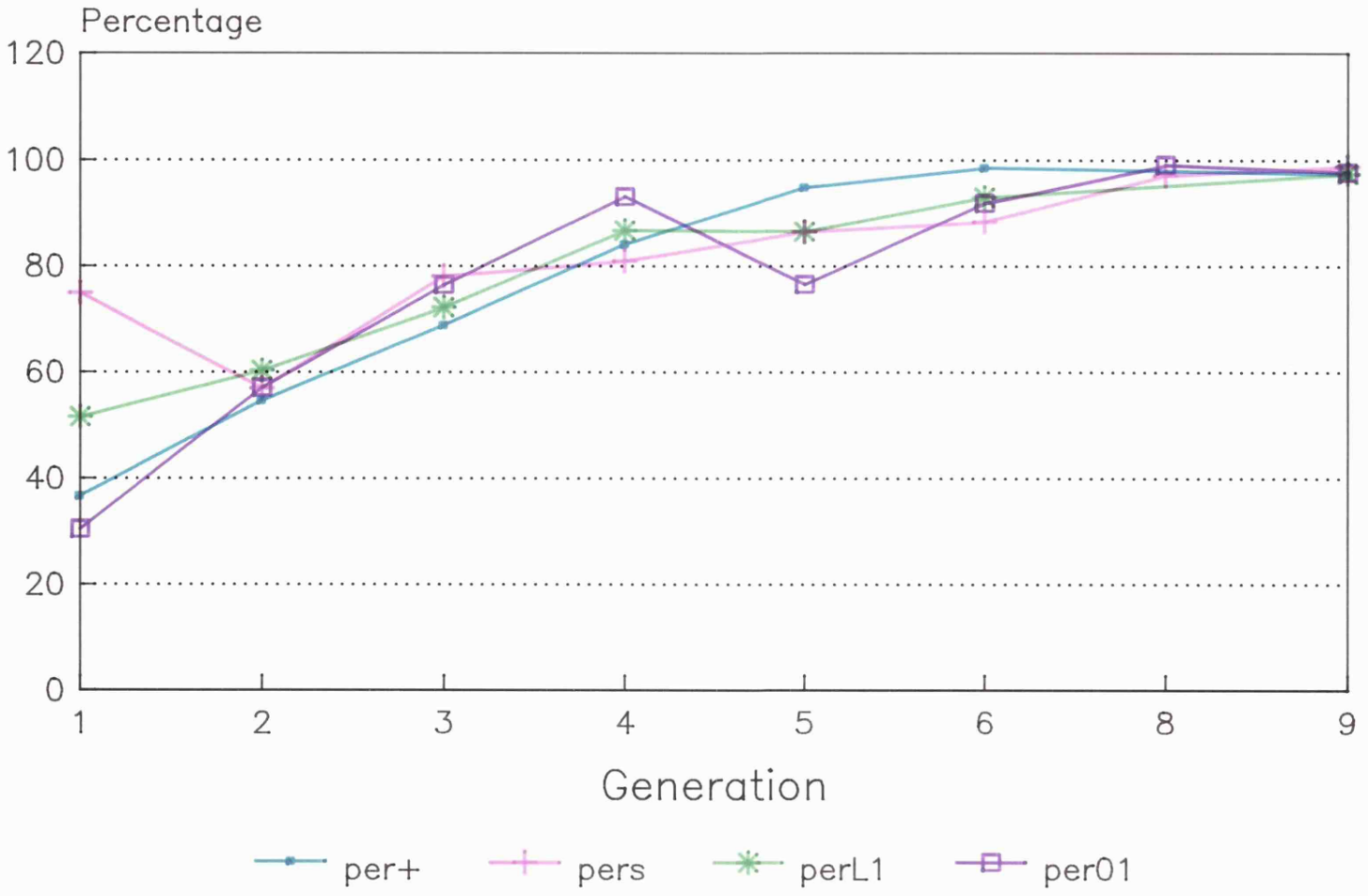
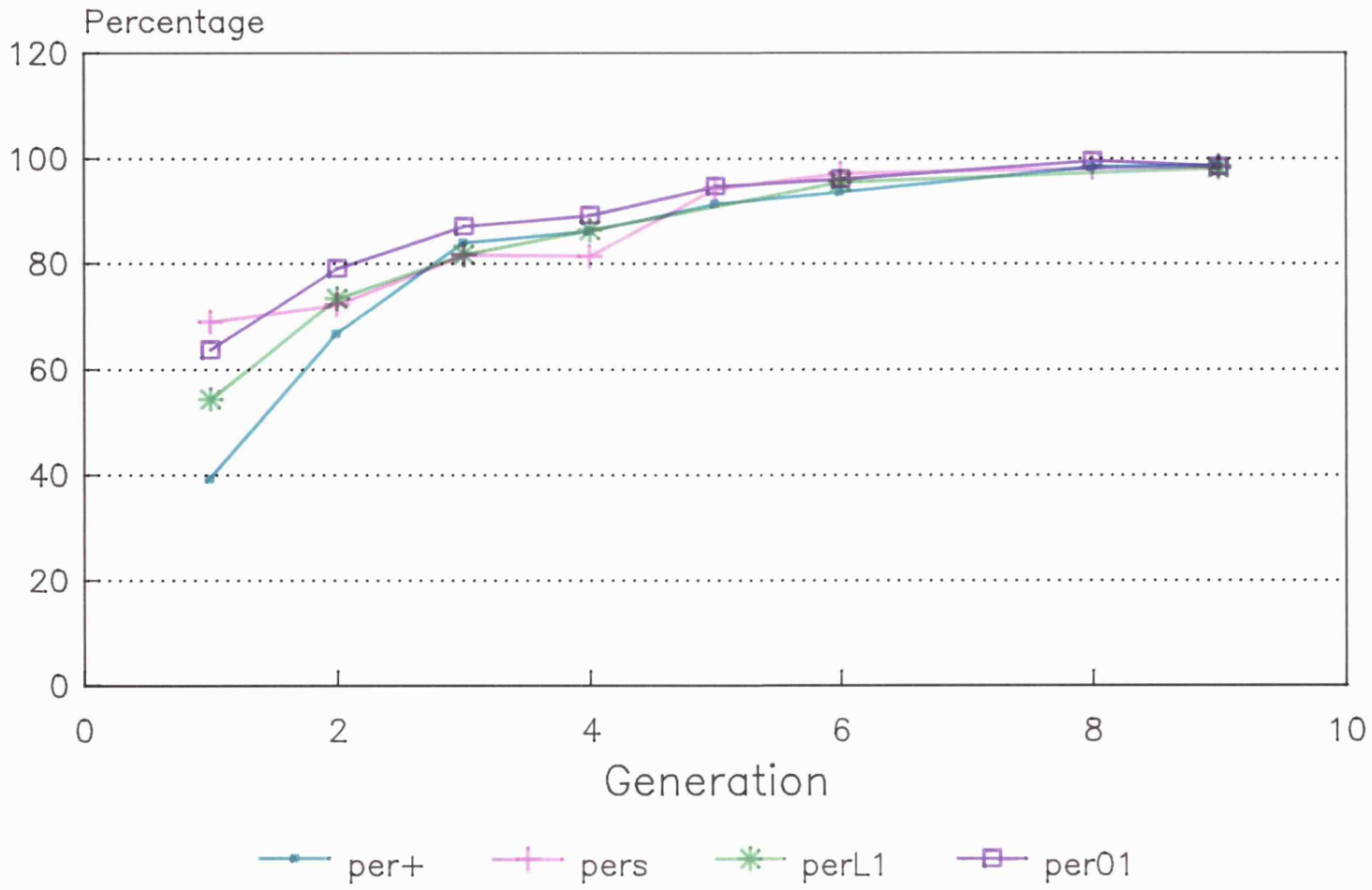


Fig A.2.2 Replicate 2



nothing could be deduced about the relative fitness of per^{+}, per^{s}, per^{L1} and per^{01}. A similar experiment is currently being planned in this laboratory (R Costa & C P Kyriacou, pers comm). The Thr-Gly region of the *per* gene shows a length polymorphism in natural populations of *D. melanogaster* (Yu et al, 1987b; R Costa, pers comm). There are at least 3 variants, with either 17, 20 or 23 Thr-Gly pairs. Northern populations from Austria and northern Italy show a high degree of polymorphism, whereas southern populations from Greece are monomorphic (R Costa, pers comm). Population cages will be set up in order to study how the 17-, 20- and 23-Thr-Gly flies will compete with one another under various conditions. The changing frequencies of the Thr-Gly variants will be monitored using the polymerase chain reaction (PCR). Flies from each cage can be individually examined for their Thr-Gly length using PCR, and thus the variants can be easily distinguished. If there is no selective advantage to any of these variants, the Thr-Gly region could be used to mark the per^{+} and competing *per* variants in population cages. For example, a 17-Thr-Gly per^{+} variant could be monitored against the 20-Thr-Gly per^{s} mutant.

APPENDIX 3 (see Chapter 5, pp68-70)

Table A.3.1 Viability of eggs, larvae, and pupae of *andante* and *andante*$^+$

Developmental stage	*andante*$^+$		andante	
	No completing development	Total no tested	No completing development	Total no tested
Embryonic 11am	92	104	72	104
Embryonic 1.30pm	94	100	72	98
Embryonic 4.30pm	96	100	59	105
Embryonic + Larval	160	201	62	200
Embryonic + Larval + Pupal	108	200	59	200

Table A.3.2 Viability of eggs, larvae and pupae of *Clk6*$^+$ and *Clk6*

Developmental stage	*Clk6*$^+$		*Clk6*	
	No completing development	Total no tested	No completing development	Total no tested
Embryonic 11am	46	52	49	52
Embryonic 1.30pm	97	99	97	100
Embryonic 4.30pm	81	100	94	104
Embryonic + Larval	163	250	132	200
Embryonic + Larval + Pupal	163	250	115	200

Table A.3.3 Viability of eggs, larvae and pupae of per^{+} and per^{s}

Developmental stage		per^{+}		per^{s}	
		No completing development	Total no tested	No completing development	Total no tested
Embryonic	11am	73	100	-	-
	1.30pm	67	100	41	44
	4.30pm	50	100	94	100
Embryonic + Larval		63	200	107	200
Embryonic + Larval + Pupal		59	200	85	200

Table A.3.4 Viability of eggs, larvae and pupae of per^{L1} and per^{01}

Developmental stage		per^{L1}		per^{01}	
		No completing development	Total no tested	No completing development	Total no tested
Embryonic	11am	72	85	-	-
	1.30pm	90	100	52	56
	4.30pm	93	100	88	100
Embryonic + Larval		105	200	115	200
Embryonic + Larval + Pupal		97	200	110	200

References

Alexander, R D (1962) Evolutionary change in cricket acoustical communication. *Evolution* **16**: 443-67

Andersson, M (1982) Female choice selects for extreme tail length in widowbirds. *Nature* **299**: 818-20

Bargiello, T A & Young, M W (1984) Molecular genetics of a biological clock in Drosophila. Proc Nat Acad Sci *USA* **81**: 2142-6

Bargiello, T A, Jackson, F R & Young, F W (1984) Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*. *Nature* **312**: 752-54

Bargiello, T A, Saez, T A, Baylies, M K, Gasic, G, Young, M W & Spray, D C (1987) The *Drosophila* gene, *per*, affects intercellular junctional communication. *Nature* **328**: 686-91

Bateman, A J (1948) Intrasexual selection in *Drosophila*. *Heredity* **2**: 349-68

Bauer, M & von Helversen, O (1987) Separate localisation of sound recognising and sound producing neural mechanisms in a grasshopper *J Comp Physiol* **161**: 95-101

Baylies, M, Bargiello, T A, Jackson, F R & Young, M W (1987) Changes in abundance or structure of the *per* gene product can alter periodicity of the *Drosophila* clock. *Nature* **326**: 390-92

Block, G D & Wallace, S F (1982) Localisation of a circadian pacemaker in the eye of a mollusc, *Bulla*. *Science* **217**: 155-57

Bourdon, M A, Shiga, M & Ruoslahti, E (1987) Gene expression of the chondroitin sulfate proteoglycan core protein PG19. *Molec Cell Biol* **7**: 33-40

Breden, F (1988) Sexual selection and predation risk in guppies. *Nature* **332**: 594

Breden, F & Stoner, G (1987) Male predation risk determines female preference in the Trinidadian guppie. *Nature* **329**: 831-33

Burley, N. (1988) The differential allocation hypothesis: an experimental test. *Am Nat* 132: 611-628

Butlin, R K & Ritchie, M G (1989) Genetic coupling in mate recognition systems: What is the evidence? *Biol J Linn Soc 37: 237-46*

Cain, A J & Sheppard, P M (1954) Natural selection in *Cepaea*. *Genetics* 39: 89-116

Card, J P & Moore, R Y (1984) The suprachiasmatic nucleus of the golden hamster: immunohistochemical analysis of cell and fiber distribution. *Neuroscience* 13: 415-31

Chatfield, C (1984) The analysis of Time Series: An Introduction. Third edition, Chapman & Hall, London

Citri, Y, Colot, H V, Jacquier, A C, Yu, Q, Hall, J C Baltimore, D & Rosbash, M (1987) A family of unusually spliced and biologically active transcripts is encoded by a *Drosophila* clock gene. *Nature* 326: 42-47

Colot, H V, Hall, J C & Rosbash, M (1988) Interspecific comparisons of the *period* gene in *Drosophila*. *EMBO J* 7: 3929-37

Coté, G G & Brody, S (1986) Circadian rhythms in *Drosophila melanogaster*: analysis of period as a function of gene dosage at the *per (period)* locus. *J Theoret Biol* 121: 487-503

Cowling, D E & Burnet, B (1981) Courtship songs and genetic control of their acoustic characteristics in sibling species of the *Drosophila melanogaster* subgroup. *Anim Behav* 29: 924-35

Cox, F E G (1989) Parasites and sexual selection. *Nature* 341: 289

Crossley, S A (1988) Failure to confirm rhythms in *Drosophila* courtship song. *Anim Behav* 36: 1098-1109

Dixon, W J (1985) BMDP Statistical Software Manual. University of California Press, Berkeley

Doherty, J A (1985) Temperature coupling and "trade-off" phenomena in the acoustic communication system of the cricket, *Gryllus bimaculatus* De Geer (Gryllidae). *J Exp Biol* **114**: 17-35

Doherty, J A & Gehardt, H (1984) Acoustic communication in hybrid tree frogs: Sound production by males and selective phonotaxis by females. *J Comp Physiol* **154**: 319-30

Doherty, J A & Hoy, R R (1985) Communication in insects III. The autitory behaviour of crickets: some views of genetic coupling, song recognition and predator detection. *Quarterly Review of Biology* **60**: 457-72

Dover, G A (1989) Slips, strings and species. *Trends in Genetics* **5**: 100-102

Dowse, H B, Hall, J C, & Ringo, J M (1987) Circadian and ultradian rhythms in *period* mutants of *Drosophila melanogaster*. *Behav Gen* **17:1**: 19-35

Dowse, H B & Ringo, J M (1989) Rearing *Drosophila* in constant darkness produces phenocopies of *period* clock mutants. *Physiol Entomol* **62**: 785-803

Dowse, H B, Dushay, M S, Hall, J C & Ringo, J M (in press) High resolution analysis of locomotor activity rhythms in *disconnected*, a visual system mutant of *Drosophila melanogaster*. *Behav Genet*

Dushay, M S, Rosbash, M & Hall, J C (1989) The *disconnected* visual system mutations in *Drosophila melanogaster* drastically disrupt circadian rhythms. *J of Biological Rhythms* **4**: 1-27

Dushay, M S, Konopka, R J, Orr, R, Greenacre, M L, Kyriacou, C P, Rosbash, M & Hall, J C (in press) Phenotypic and genetic analysis of *Clock*, a new circadian rhythm mutant in *Drosophila melanogaster*. *Genetics*

Elsner, N & Popov, A V (1978) Neuroethology of acoustic communication. *Advances in Insect Physiology* **13**: 229-335

Endler, J A (1980) Natural selection of color patterns in Poecilia *reticulata*. *Ecology* **34**: 76-91

Endler, J A (1988) Sexual selection and predation risk in guppies. *Nature* **332**: 593-594

Engelmann, W & Mack, J (1978) Different oscillators control the circadian rhythm of eclosion and activity in *Drosophila*. *J Comp Physiol* **127**: 229-237

Eskin, A J (1977) Neurophysiological mechanisms involved in photoentrainment of circadian rhythm in *Aplysia* eye. *J Neurobiol* **8**: 273-99

Ewer, J, Rosbash, M & Hall, J C (1988) An inducible promotor fused to the *period* gene of *Drosophila* conditionally rescues adult *per* mutant arrhythmicity. *Nature* **333**: 82-84

Ewing, A W (1969) The genetic basis of sound production in *Drosophila pseudoobscura* and *D. persimilis*. *Animal Behaviour* **17**: 555-60

Ewing, A W & Bennet-Clarke, H C (1968) The courtship songs of *Drosophila*. *Behaviour* **31**: 288-301

Ewing, L S & Ewing, A W (1987) Courtship in *Drosophila melanogaster* in large observation chambers: the influence of reproductive state. *Behaviour* **101**: 243-52

Ewing, A W (1988) Cycles in the courtship song of male *Drosophila melanogaster* have not been detected. *Anim Behav* **36**: 1091-97

Feldman, J F (1971) New mutations affecting circadian rhythmicity in *Neurospora*. In Biochronometry, ed M Menaker. National Academy of Sciences

Fischbach, K F & Heisenberg, M (1984) Neurogenetics and behaviour in insects. *J Exp Biol* **112**: 65-93

Fischbach, K F & Technau, G (1984) Cell degeneration in the developing optic lobes of the *sine oculis* and *small-optic-lobes* mutants of *Drosophila melanogaster*. *Developmental Biology* **104**: 219-39

Fisher, R A (1958) The Genetical Theory of Natural Selection. Dover Books

Garcia-Bellido, A & Merriam, J R (1969) Cell lineage of the imaginal discs in *Drosophila* gynandromorphs. *J Exp Zool* **170**: 61-76

Garcia-Bellido, A, Ripoll, P & Morata, G (1976) Developmental compartmentalisation in the dorsal mesothoracic disc of *Drosophila*. *Dev Biol* **48**: 132-47

Georges, H, Cochaux, P, Lequarre, A-S, Young, M W & Vassart, H (1987) DNA fingerprinting in man using a mouse probe related to part of the *Drosophila* "per" gene. *Nucleic Acid Research* **15**: 7193

Gerhardt, H C (1978) Temperature coupling in the vocal communication system of the gray tree frog, *Hyla versicolor*. *Science* **199**: 992-94

Hall, J C (1979) Control of male reproductive behaviour by the central nervous system of *Drosophila*: dissection of a courtship pathway by genetic mosaics. *Genetics* **92**: 437-457

Hall, J C (1984) Complex brain and behavioral functions disrupted by mutations in *Drosophila*. Develop Genet **4**: 355-378

Hall, J C & Rosbash, M (1988) Mutations and molecules affecting biological rhythms. *Ann Rev Neurosci* **11**: 373-93

Hall, J C & Kyriacou, C P (1990) Genetics of Biological Rhythms in *Drosophila*. *Advances in Insect Physiology* (in press)

Halliday, T R (1978) Sexual selection & mate choice. In Behavioural Ecology - an evolutionary approach ed J R Krebs & N B Davies. Blackwell Scientific Publications

Hamblen, M, Zehring, W A, Kyriacou, C P, Reddy, P, Yu, Q, Wheeler, D A, Zwiebel, L J, Konopka, R J, Rosbash, M & Hall, J C (1986) Germline transformation involving DNA from the *period* locus of *Drosophila melanogaster*. Overlapping genomic fragments that restore circadian and ultradian rhythmicity to per^{01} and per^{-} mutants. *J Neurogenet* **3**: 249-91

Hamblen-Coyle, M, Konopka, R J, Zwiebel, L J, Colot, H V, Dowse, H B, Rosbash, M & Hall, J C (1989) A new mutation at the *period* locus of *Drosophila melanogaster* with some novel effects on circadian rhythms. *J Neurogenet* 5: 229-56

Hamilton, W D & Zuk, M (1982) Heritable true fitness and bright birds: a role for parasites ? *Science* 218: 384-386

Hamilton, W D & Zuk, M (1989) Parasites and sexual selection. *Nature* 341: 289-90

Handler, A M & Konopka, R (1979) Transplantation of a circadian pacemaker in *Drosophila*. *Nature* 279: 236-38

Hara, K, Tydeman, P & Kirschner, M (1980) A cytoplasmic clock with the same period as the division cycle in *Xenopus* eggs. *Proc Natl Acad Sci USA* 77: 462-66

Hardin, P E, Hall, J C & Rosbash, M (1990) Feedback of the Drosophila *period* gene on circadian cycling of its messenger RNA levels. *Nature* 343: 536-40

Hays, W L (1973) Statistics for the Social Sciences.

Heisenberg, M (1980) Mutants of brain structure and function: What is the significance of the mushroom bodies for behaviour? In Development and Neurobiology of *Drosophila*. Ed O Siddiqi, P Babu, L M Hall & J C Hall, Plenum Press

Heisenberg, M, Borst, A, Wagner, S & Byers, D (1985) *Drosophila* mushroom body mutants are deficient in olfactory learning. *J Neurogen* 2: 1-30

Helfrich, C (1986) Role of the optic lobes in the regulation of the locomotor activity rhythm in *Drosophila melanogaster*: Behavioural analysis of neural mutants. *J Neurogen* 3: 321-43

von Helversen, D & von Helversen, O (1975) Verhaltensgenetische untersuchungen am akustischen kommunikationssystem der feldheuschrecken (Ortherptera: Acridae). I. Der gesang von artbastarden zwischen *Chorthippus biggutulus* und *Ch. mollis*. *J Comp Physiol* 104: 273-99

von Helversen, O (1979) Angeborenes Erkennen akustischer Schluesselreize. *Verh Dtsch Zool Ges* 42-59

Hoikkala, A (1988) The importance of different courtship stimuli in the mating behaviour of different European species of the Drosophila *virilis* group. *Ann Zool Fennici* **25**: 257-63

Hoy, R R, Hahn, J & Paul, R C (1977) Hybrid cricket auditory behaviour: Evidence for genetic coupling in animal communication. *Science* **180**: 82-83

Jacklet J W (1989) Cellular neuronal oscillators. In "Neuronal and Cellular Oscillators" (ed J W Jacklet) New York, Marcel Dekker pp 482-527

Jacklet, J W & Geronimo, J (1971) Circadian rhythm: Population of interacting neurons. *Science* **174**: 299-302

Jackson, F R, Gailey, D A & Siegal, R W (1983) Biological rhythm mutations affect an experience dependent modification of male courtship behaviour in *Drosophila melanogaster*. *J Comp Physiol* **151**: 545-52

Jackson, F R, Wilson, S D, Strichartz, G R & Hall, L M (1984) Two types of mutants affecting voltage sensitive sodium channels in *Drosophila melanogaster*. *Nature* **308**: 189-91

Jackson, F R, Bargiello, T A, Yun, S H & Young, M W (1986) Product of *per* locus of *Drosophila* shares homology with proteoglycans. *Nature* **320**: 185-88

James, A, Ewer, J, Reddy, P, Hall, J & Rosbash, M (1986) Embryonic expression of the *period* clock gene in the central nervous system of *Drosophila melanogaster*. *EMBO* **5**: 2313-20

Jones, J S (1987) The heritabilty of fitness: bad news for good genes? *TREE* **2**: 35-38

Kankel D R & Hall, J (1976) Fate mapping of nervous system & other internal tissues in genetic mosaics of *Drosophila melanogaster*. *Dev Biol* **48**: 1-24

Konopka, R J (1987a) Genetics of biological rhythms in Drosophila. *Ann Rev Genet* **21**: 227-36

Konopka, R J (1987b) Neurogenetics of *Drosophila* circadian rhythms. In "Evolutionary Genetics of Invertebrate Behaviour" (ed M D Huettel) pp 215-21 Plenum Press, New York

Konopka, R J & Benzer, S (1971) Clock mutants of *Drosophila melanogaster* *Proc Nat Acad Sci* **68**: 2112-5

Konopka, R J & Orr, D (1979) Effects of a clock mutation on the subjective day - Implications for the membrane model of the *Drosophila* circadian clock. In: Development and neurobiology of *Drosophila*. Plenum Books

Konopka, R J, Wells, S & Lee, T (1983) Mosaic analysis of a *Drosophila* clock mutant. *Mol Gen Genet* **190**: 284-88

Konopka, R J, Pittendrigh, C & Orr, D (1989) Reciprocal behaviour associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants. *J Neurogenet* **6**:1-10

Kripke, D F, Risch, S C & Janowsky, D (1983) Bright white light alleviates depression. *Psychiatry Research* **10**: 105-112

Kyriacou, C P (1990) Genetic and molecular analysis of eukaryotic behaviour. *Seminars in the Neurosciences* (in press)

Kyriacou, C P & Hall, J C (1980) Circadian rhythm mutations in *D. melanogaster* affect short term fluctuations in the male's courtship. *Proc Nat Acad Sci* **77**: 6729-33

Kyriacou, C P & Hall J C (1982) The function of courtship song rhythms in *Drosophila*. *Anim Behav* **30**: 794-801

Kyriacou, C P & Hall, J C (1984) Learning and memory mutations impair acoustic priming of mating behaviour in *Drosophila*. *Nature* **308**: 62-65

Kyriacou, C P & Hall, J C (1985) Action potential mutations stop a biological clock in *Drosophila*. *Nature* **314**: 171-73

Kyriacou, C P & Hall, J C (1986) Interspecific genetic control of courtship song production and reception in *Drosophila*. *Science* **232**: 494-97

Kyriacou, C P & Hall, J C (1989) Spectral analysis of *Drosophila* courtship song rhythms. *Animal Behav* **37**: 851-59

Kyriacou, C P, Oldroyd, M, Wood, J, Sharp, M & Hill, M (1990a) Clock mutations alter developmental timing in *Drosophila*. *Heredity* (in press)

Kyriacou, C P, van den Berg, M & Hall, J C (1990b) *Drosophila* courtship song cycles in normal and *period* mutant males revisited. *Behav Genet* (in press)

Lachaise, D, Cariou, M-L, David, J R, Lemeunier, F, Tsacas, L & Ashburner, M (1988) Historical biogeography of the *Drosophila melanogaster* species subgroup. *Evol Biol* **22**: 157-225

Lande, R (1982) Rapid origin of sexual isolation and character divergence in a cline. *Evolution* **36**: 213-23

Lindsley, D L & Grell, E H (1968) Genetic variations of *Drosophila melanogaster*. Carnegie Institution of Washington Publication No 627

Lloyd, D & Edwards, S W (1984) Epigenetic oscillation during the cell cycles of lower eukaryotes are coupled to a clock. In "Cell Cycle Clocks" ed L N Edmunds. pp 27-46, N Y Marcel Dekker

Lorenz, L J, Hall, J C & Rosbash, M (1989) Expression of a *Drosophila* mRNA is under circadian control during pupation. *Development* **107**: 869-80

Loros, J J, Denome, S A & Dunlap, J C (1986) Molecular cloning of genes under control of the circadian clock in *Neurospora*. *Science* **243**: 385-88

Mackay, T F C (1986) A quantitative genetic analysis of fitness and its components in *Drosophila melanogaster*. *Genet Res* **47**: 59-70

Majerus, M E N, O'Donald, P & Weir, J (1982) Evidence for preferential mating in *Adalia bipunctata*. *Heredity* **49**: 37-49

Majerus, M E N, O'Donald, P & Weir, J (1982) Female mating preference is genetic. *Nature* **300**: 521-23

Majerus, M E N, O'Donald, P, Kearns, P W E & Ireland, H (1976) Genetics and evolution of female choice. *Nature* **321**: 164-66

Manning, A (1961) The effects of artificial selection for mating speed in *Drosophila melanogaster*. *Anim Behav* **9**: 82-92

McClung, C R, Fox, B A & Dunlap, J C (1989) The *Neurospora* clock gene, *frequency*, shares a sequence element with the *Drosophila* clock gene, *period*. *Nature* **339**: 558-62

Moodie, G E E (1972) Predation, natural selection and adaptation in an unusual three spine stickleback. *Heredity* **28**: 155-167

Moore-Ede, M C & Burr, R G (1973) Circadian rhythm of therapeutic effectiveness of oxymetholone in paraplegic patients. *Clin Pharmacol Ther* **14**: 448-54

Moore-Ede, M C, Sulzman, F M & Fuller, C A (1982) The Clocks That Time Us. Harvard Univ Press

Mrosovsky, N (1989) Mutant hamster in a hurry. *Nature* **337**: 213-14

Mukai, T & Nagano, S (1983) The genetic structure of natural populations of Drosophila melanogaster XVI Excess of additive variance of variability. *Genetics* **105**: 115-34

Nishitsutsuji-Uwo, J & Pittendrigh, C S (1968) Central nervous system control of circadian rhythmicity in the cockroach III The optic lobes - locus of the driving oscillation? *Z vergl Physiol* **58**: 14-46 Berlin-Heidelberg-N York Springer

Njus, D, Sulzman, F M & Hastings, J W (1974) Membrane model for the circadian clock. *Nature* **248**: 116-120

O'Donald, P (1980) Genetic Models of Sexual Selection. Camb Univ Press

O'Donald, P & Majerus, M E N (1985) Sexual selection and the evolution of preferential mating in ladybirds I Selection for high and low lines of female preference. *Heredity* **55**: 401-12

Pape-Moller, A (1988) Female choice selects for male sexual tail ornaments in the monogamous swallow. *Nature* **332**: 640-42

Pape-Moller, A (1989) Viability costs of male tail ornaments in a swallow. *Nature* **339**: 132-35

Pavlidis, T (1969) Populations of interacting oscillators and circadian rhythms. *J Theor Biol* **22**: 418-36

Petersen, G, Hall, J C & Rosbash, M (1988) The *period* gene of *Drosophila* carries species specific behavioural instructions. *EMBO J* **7**: 3939-47

Pirrotta, V, Hadfield, C & Pretorius, G H J (1983) Microdissection and cloning of the *white* locus and the 3B1-3C2 region of the *Drosophila* X chromosome. *EMBO J* **2**: 927-34

Pittendrigh, C S (1954) On temperature independence in the clock controlling emergence time in *Drosophila*. *Proc Natl Acad Sci USA* **40**: 1018-1029

Pittendrigh, C S (1960) Circadian rhythms and the circadian organisation of living systems. *Cold Spring Harbor Symp Quant Biol* **25**: 159-84

Pittendrigh, C S (1965) On the mechanism of entrainment of a circadian rhythm by light cycles. In Circadian Clocks ed J Aschoff pp277-97. North Holland, Amsterdam

Pittendrigh, C S & Daan, S (1976) A functional analysis of circadian pacemakers in nocturnal rodents. IV Entrainment: pacemaker as clock. *J Comp Physiol* **106**: 291-331

van den Pol, A N & Tsujimoto, K L (1985) Neurotransmittors of the hypothalamic suprachiasmatic nucleus: immunocytochemical analysis of 25 neuronal antigens. *Neuroscience* **15**: 1049-86

Pomiankowski, A (1989) Choosing parasite-free mates. *Nature* **338**: 115-16

Poulton, E D (1890) The colours of animals: Their meaning and use, especially considered in the case of insects. London: Keegan Paul

Ralph, M R & Menaker, M (1988) A mutation of the circadian system in golden hamsters. *Science* **241**: 1225-27

Read, A F (1987) Comparative evidence supports the Hamilton & Zuk hypothesis on parasites and sexual selection. *Nature* **328**: 68-70

Read, A F & Harvey, P H (1989) Reassessment of comparative evidence for Hamilton and Zuk theory on the evidence of secondary sexual characters. *Nature* **339**: 618-20

Read, A F & Harvey, P H (1989) Validity of sexual selection in birds. *Nature* **340**: 105

Reddy, P, Zehring, W, Wheeler, D, Pirotta, P, Hadfield, C, Hall, J & Rosbash, M (1984) Molecular analysis of the *period* locus in *Drosophila melanogaster* and the identification of a transcript involved in biological rhythms. Cell **38**: 701-10

Reddy, P, Jacquier, A, Abovich, N, Petersen, G & Rosbash, M (1986) The *period* clock locus of *D melanogaster* codes for a proteoglycan. *Cell* **46**: 53-61

Redman, J, Armstrong, S & Ng, K T (1983) Free-running activity rhythms in the rat: Entrainment by melatonin. *Science* **219**: 1089-92

Roberts, D B (1986) *Drosophila*: a practical approach. IRL Press, Oxford

Roberts, D H, Lehar, J & Dreher,J W (1987) Time series with CLEAN. I Derivation of a spectrum. *Astron J* **93**: 968-89

Roelofs, W, Glover, T, Tanf, X, Sreng, I, Robbins, P, Eckenrode, C, Lofstedt, C, Hansson, B S & Bengtsson, B O (1987) Sex pheromone production and reception in European corn borer moths is determined by both autosomal and sex-linked genes. *Proc Natl Acad Sci USA* **84**: 7585-89

Saez, L & Young, M W (1988) *In situ* localisation of the *per* clock protein during development of *Drosophila melanogaster*. *Mol Cell Biol* **8**: 5378-85

Sargent, M L, Briggs, W R & Woodward, D O (1966) The circadian nature of a rhythm expressed by an invertaseless strain of *Neurospora crassa*. *Plant Physiol* **41**: 1343-49

Saunders, D S (1982) Insect Clocks. Pergamon Press Ltd, Oxford

von Schantz, T, Goransson, G, Andersson, G, Froberg, I, Grahn, M, Helgee, A & Wittzell, H (1989) Female choice selects for a viability-based male trait in pheasants. *Nature* **337**: 166-169

von Schilcher, F (1976) The function of pulse song and sine song in the courtship of *Drosophila melanogaster*. *Anim Behav* **24**: 622-25

von Schilcher, F & Hall, J C (1979) Neural topography of courtship song in sex mosaics of *D melanogaster*. *J Comp Physiol* **129**: 85-95

von Schilcher, F (1989) Have cycles in the courtship song of *Drosophila* been detected? *Trends in Neurosciences* **12**: 311-13

Schildberger, K (1981) Some physiological features of mushroom body linked fibres in the house cricket brain. *Naturwissenschaften* **67**: 623

Semler, D E (1971) Some aspects of adaptation in a polymorphism for breeding colours in the three-spine stickleback. *J Zool* **165**: 291-302

Serras, F & van den Bigelaar (1987) Is a mosaic embryo also a mosaic of communication compartments? *Dev Biol* **120**: 132-38

Shin, H S, Bargiello, T A, Clarke, B T, Jackson, F R & Young, M W (1985) An unusual coding sequence from a *Drosophila* clock gene is conserved in vertebrates. *Nature* **317**: 445-48

Shorey, H H (1962) Nature of the sound produced by *Drosophila melanogaster* during courtship. *Science* **137**: 677-78

Siwicki, K K, Eastman, C, Petersen, G, Rosbash, M & Hall, J C (1988) Antibodies to the *period* gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* **1**: 141-150

Siwicki, K K, Strack, S, Rosbash, M, Hall, J C & Jacklet, J W (1989) An antibody to the *Drosophila period* protein recognises circadian pacemaker proteins in *Aplysia* and Bulla. *Neuron* 3: *51-58*

Smith, R & McClaren, A (1977) Factors affecting the time of formation of the mouse blastocoele. *J Embryol Exp Morph* **41**: 79-92

Smith, R F (1982) Genetic analysis of the circadian clock system of *Drosophila melanogaster*. Dissertation. California Institute of Techhnology, Pasadena

Smith, R F & Konopka, R (1981) Circadian clock phenotypes of chromosome abberations with a breakpoint at the per locus. Mol Gen Genet **183**: 243-51

Smith, R F & Konopka, R (1982) Effects of dosage alterations of the *per* locus on the period of the circadian clock of *Drosophila*. *Mol Gen Genet* **185**: 30-36

Snow, M H L & Tam, P L (1980) Timing in embryological development. Nature **286**: p107

Spieth, H T (1952) Mating behaviour within the genus *Drosophila*. Bull *Am Mus Nat Hist* **99**: 395-474

Spray, D C, Fujita, M, Saez, J C, Choi, H, Watanabe, T, Heitzberg, L, Rosenberg, C & Reid, L M (1987) Proteoglycans and glycosaminoglycans induce gap-junction synthesis and function in in primary liver cell cultures *J Cell Biol* **105**: 541-51

Steller, H, Fischbach, K F & Rubin, G (1987) *disconnected*: A locus required for neuronal pathway formation in the visual system of *Drosophila*. Cell **50**: 1139-53

Suzuki, D T, Grigliatti, T & Williamson, R (1971) Temperature sensitive mutations in *Drosophila melanogaster* - mutation ($para^{ts}$) causing reversible adult paralysis. *Proc Natl Acad Sci USA* **68**: 890-93

Thackeray, J R (1989) Molecular analysis of behavioural rhythms in *Drosophila*. PhD thesis, University of Leicester

Truman, J W (1974) Physiology of insect rhythms:IV. Role of the brain in the regulation of the flight rhythm of the giant silkmoths. *J Comp Physiol* **95**: 281-96

Truman, J W (1985) Rhythmic regulation of eclosion hormone release and action in insects. In Current Trends in Comparative Endocrinology pp 667-669 Hong Kong University Press

Turek, F W (1985) Circadian neural rhythms in mammals. *Ann Rev Physiol* **47**: 49-64

Turek, F W & Losee-Olsen, S (1986) A benzodiazepine used in the treatment of insomnia phase-shifts the mammalian circadian clock. *Nature* **321**: 167-8

van Vactor, D Jr, Krantz, D E , Reinke, R & Zipursky, S L (1988) Analysis of mutants in *chaoptin*, a photoreceptor cell-specific glycoprotein in *Drosophila*, reveals its role in cellular morphogenesis. *Cell* **52**: 281-90

Warner, A (1985) The role of gap junctions in amphibian development. *J Embryol Exp Morph* supplement **89**: 365-80

Wadepuhl, M & Huber, F (1979) Elicitation of singing and courtship movements by electrical stimulation of the brain of the grasshopper. *Naturwissenschaften* **66**: 320

Walker, T J (1957) Specificity in the response of female tree crickets (Orthoptera, Gryllidae, Oecanthinae) to calling songs of males. *Ann Entomol Soc Am* 50: 626-36

Watt, W B, Carter, P A, & Donahue, K (1986) Females' choice of "good genotypes" as mates is promoted by an insect mating system. *Science* 233: 1187-90

Wehr, T A & Wirz-Justice, A (1982) Circadian rhythm mechanisms in affective illnes and in antidepressant drug action. *Pharmacopsychiatry* 15: 31-39

Weitzel, G & Rensing, L (1981) Evidence for cellular circadian rhythms in isolated fluorescent dye labelled salivary glands of wild type and an arrhythmic mutant of *Drosophila melanogaster*. *J Comp Physiol* 143: 229-35

Wheeler, D A, Kyriacou, C P, Greenacre, M L, Yu, Q, Rutila, J, Rosbash, M & Hall, J C Molecular transfer of a species-specific courtship behaviour from *D. simulans* to *D. melanogaster*. (ms submitted)

Winfree, A T (1987) The Timing of Biological Clocks. Scientific American Library, Inc

Wu, C F & Ganetsky, B (1980) Genetic alteration of nerve membrane excitability in temperature sensitive paralytic mutants of *Drosophila melanogaster*. *Nature 286*: 814-16

Yu, Q, Jacquier, A, Citri, Y, Hamblen, M, Hall, J & Rosbash, M (1987a) Molecular mapping of point mutations in the *period* gene that stop or speed up the biological clock in *D melanogaster*. *Proc Nat Acad Sci* 84: 784-88

Yu, Q, Colot, H, Kyriacou, C P, Hall, J & Rosbash, M (1987b) Behaviour modification by *in vitro* mutagenesis of a variable region within the *period* gene of *Drosophila*. *Nature* 326: 765-69

Zehring, W, Wheeler, D, Reddy, P, Konopka, R, Kyriacou, C P, Rosbash, M & Hall, J (1984) P-element transformation with *period* locus DNA restores rhymicity to mutant, arrhythmic *Drosophila melanogaster*. *Cell* **39**: 369-76

Zerr, D N, Rosbash, M, Hall, J C & Siwicki, K K Circadian rhythms of *period* protein immunoreactivity in the CNS and the visual system of *Drosophila*. (submitted to *Neuron*)

Zouros, E (1981) The chromosomal basis of sexual isolation in two sibling species of Drosophila: D. *arizonensis* and D. *mojavensis*. *Genetics* **97**: 703-18

Zuk, M (1989) Validity of sexual selection in birds. *Nature* **340**: 104-05