The Evolution of Life Histories in *Drosophila melanogaster.* Costs of Reproduction and the Responses to Artificial Selection on Age at Reproduction

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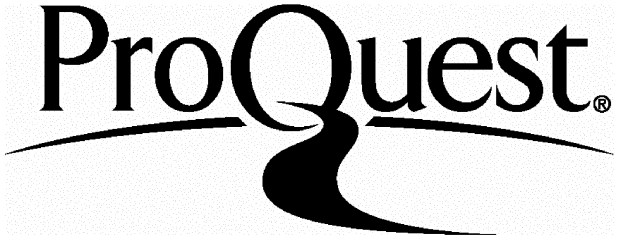

Abstract

The demographic and physiological effects of reproduction on male survivorship and fertility in *Drosophila melanogaster* were examined by manipulating reproductive status part way through life. Reproductive costs can take the form of either an instantaneous elevation of risk or an irreversible decline in fitness, and the relative importance of these was considered. The impact of variation in individual fitness was also investigated. Early reproduction permanently damaged both survival and fecundity, with the cost to fertility being the greater of the two. Sterility appeared attributable to a reduced sperm count. Risk played an apparently minor role. Analyses of survival rates using the Gompertz parameters gave a misleading measure of ageing, which should also include the impact on fertility. Potential indicators of individual frailty were used to assess the effect of cohort heterogeneity on the interpretation of reversal experiments. The impact of such variation on survival and fertility was negligible.

Two established selection regimes, selected for early and late life fitness under controlled density conditions in the absence of inadvertant selection, were examined for correlated responses to selection. Two hypotheses were tested. The first, that selection on age at reproduction would cause a divergence in adult body size and dry weight, larval viability and pre-adult development time, was disproved. The second hypothesis was that selection would result in reduced reproductive investment in favour of survival, and a trade off between survival and early fecundity was found in both sexes to support this. Apart from early life fecundity, no evidence was found for the evolution of female reproductive biology under selection. Comparisons were made with reference to the original base stock. The significance of the observed selection responses in the context of the evolution of senescence was discussed.

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Chapter 1. Introduction

1.1. Life Histories and their Evolution

A life history is the product of the age-specific survival probabilities and fecundities of an organism (Law, 1979a; Charlesworth, 1994), and can be described in terms of age and size at first reproduction, patterns of lifetime reproductive investment and mortality rates (Stearns, 1992; Charlesworth, 1993). The study of life histories is the analysis of how natural selection acts to maximise the contribution of progeny to the next generation (Law, 1979a; Smith, 1991). Natural selection can also be said to maximise fitness, a relative measure of the success of an organism in a given environment (Smith, 1991). The most commonly used measure of fitness in a population is known as the intrinsic rate of increase or Malthusian parameter, *r*, derived from the Euler-Lokta equation (Smith, 1991), and is calculated from the probability of survival to age *x*, l_x, and fecundity at that age, m_x (Smith, 1991; Stearns, 1992).

$$1 = \sum e^{-rx} l_x m_x$$

Calculation of the intrinsic rate of increase requires one to assume that the environment is stable and that population growth is independent of density (Stearns, 1992). It can be used to predict the fitness of a given genotype in a population totally made up of that genotype (Charlesworth, 1993), the one with the highest *r* value being favoured (Roff, 1992). The Malthusian parameter remains the most widely used predictor of fitness (Smith, 1991; Stearns, 1992). There are more complex measures, but these are more likely to be used for specific problems such as non-equilibrium populations and density-dependent growth. The stochastic growth rate, *a*, allows the estimation of fitness in non-equilibrium populations and produces more predictions than the Malthusian parameter (Stearns, 1992). But it also leads to more complex mathematics and increasing difficulty for biologists trying to collect the data needed for the calculations (Stearns, 1992). The use of population carrying capacity, *K*, as a measure of fitness may be appropriate in circumstances where density

dependence is important (Stearns, 1992), but runs into problems in certain situations (see Roff, 1992).

Natural selection will favour a genotype that increases an organism's fitness, but it is impossible for life history characters such as growth, survival probability and fecundity to be simultaneously maximised. The immortal, ever growing, constantly reproducing "Darwinian demon" (Law, 1979a) cannot exist because of constraint. The optimality approach to life history evolution considers how fitness is maximised when natural selection acts upon constrained characters (Parker and Maynard Smith, 1990). Constraints may be ecological or physiological in nature (Smith, 1991). Ecological constraints are functions of the environment (Roff, 1992) and could for example involve the patterns of predation on an organism. This type of constraint is known to affect the guppy, *Poecilia reticulata*, which in certain areas has a size constraint placed upon it by a predator species that favours larger fish (Reznick and Bryga, 1987). Ecological constraints may also affect the availability of resources. Once acquired by the organism, resources have to be allocated to different functions (Stearns, 1992). Physiological constraints, or trade offs, exist between life history characters because these resources are finite. Fitness may be maximised by a particular pattern of resource allocation between activities such as growth and reproduction (Stearns, 1992), the result being a life history that reflects the balance between the different activities in which the resources have been invested (Law, 1979a). Patterns of trade offs are not always obvious, and it is possible to find a positive correlation between life history characters that would otherwise be expected to be negatively correlated. This may depend upon the variability of available resources, and also on the degree of flexibility with which they can be allocated to different life history traits (van Noordwijk and de Jong, 1986). Physiological trade offs result in a number of costs; the price paid in terms of one character for investment in another. Some common examples, for which there is much empirical evidence, include types of reproductive cost whereby current reproduction can be increased at the expense of future reproduction (e.g. red deer; Clutton-Brock *et al.*, 1982), survival probability (e.g. *Drosophila melanogaster*; Partridge and Farquhar, 1981; Fowler and Partridge, 1989), or growth (e.g. *Poa annua*; Law, 1979b).

The environment is extremely important in determining the success of a particular life history. If the chance of dying before the next bout of reproduction

is high, selection will favour a genotype that invests heavily in current reproduction (Charlesworth, 1994). Providing that such a genotype is fitter than one that also invests in future reproduction and growth, semelparity will evolve (Bell, 1980; Charlesworth, 1994). Iteroparity will evolve under lower environmental mortality and will result in more than one bout of reproduction and a certain amount of investment in growth and tissue maintenance (Bell, 1980; Smith, 1991; Charlesworth, 1994). A high degree of mortality amongst progeny would also favour iteroparity so that the chance of not having any surviving offspring is reduced (Smith, 1991; Charlesworth, 1994). Thus, environmental mortality influences the relative importance of reproduction, growth and somatic maintenance (Law, 1979a) and determines the fitness of a particular genotype. Costs of reproduction have a part to play here. For example, if reproduction is very costly in terms of survival, then it may pay to sacrifice future survival in order to gain improved fertility (Bell, 1980). This situation could result in the life history tending towards semelparity (Law, 1979a; Bell, 1980). Whereas extrinsic mortality and reproductive costs affect the organism's pattern of reproduction, these factors also influence decisions about when to first reproduce. Selection generally favours early over late reproduction, so the observation that many species appear to delay maturity needs to be explained (Charlesworth, 1994). Theoretical models support the idea that first reproduction will be delayed if this increases fitness. In a species where fecundity increases with age and current reproduction reduces future fecundity, it pays to delay maturity so that it is late enough to sufficiently increase fecundity (Bell, 1980) and early enough for that benefit still to exceed the cost of extended generation time and increased juvenile mortality (Stearns, 1992). Mortality rate will also affect the extent to which maturity is delayed (Bell, 1980). Very similar results were obtained by Charlesworth (1994). Comparative evidence seems to support these conclusions: poikilotherms such as teleosts grow throughout adult life and show a more pronounced delay in maturity than many homeothermic species, which do not generally increase in size with advancing age (Bell, 1980).

It can be seen that the allocation of limited resources to life history characters results in a process of constrained optimisation (Stearns, 1992) in which natural selection acts to maximise fitness in a given ecological niche within the boundaries of constraint (Smith, 1991). Life history patterns, such as age at maturity and the evolution of semelparity or iteroparity, are shaped by the

conflict between reproductive costs and the selective advantage of early reproduction that are caused by patterns of survival and growth (Charlesworth, 1994). This process is governed by the extrinsic mortality rate (Charlesworth, 1994) which affects the fitness of each genotype. The pattern of allocation that maximises fitness in a particular niche results in the optimal life history (Law, 1979a). This requires that there is sufficient genetic variation present for the traits under selection to allow the optimal genotype to evolve (Law, 1979a; Stearns, 1992). Life history theory predicts that traits connected to fitness will have low additive genetic variation because selection on them will remove genotypes of low fitness, thus reducing the amount of genetic variation for the trait (Roff, 1992). This suggests that life history characters, which have a strong effect on fitness, will have generally low heritabilities (Mousseau and Roff, 1987; Roff, 1992). This is indeed true; heritability for life history characters is lower than for morphological traits (Roff and Mousseau, 1987; Mousseau and Roff, 1987). However, another measure of variability, the additive coefficient of variation, which is based upon trait means rather than the phenotypic variance, V_P, suggests that life history characters have higher variability than morphological traits (Houle, 1992). This is probably because they are affected by more environmental and genetic factors than other types of character (Houle, 1992). Genetic variation is maintained by, amongst other things, mutation, immigration, negative genetic correlations and pleiotropy (Stearns, 1992). It is therefore unlikely to be a limiting factor in evolutionary adaptation to environmental change (Roff, 1992).

1.2. The Evolution of Senescence as part of the Life History

1.2.1. An Unsolved Problem of Biology

Senescence, or ageing, is the tendency for fecundity to decline and the likelihood of death to increase with age (Charlesworth, 1994). It occurs in almost all groups of vertebrates and many invertebrates, although there is some doubt about the Coelenterata, Annelida and Platyhelminthes (Rose, 1991). It has been suggested that most wild animals die before senescing (Medawar, 1952), and that ageing is a product of domestication. Senescence is certainly difficult to

measure in the field, not least because of the problem of estimating the age of a wild animal. For this reason the existence of ageing in many species has been established under captive conditions in zoos or laboratories. A decline in vitality with age is more easily observed in this way, because sources of mortality other than senescence can be removed. However, comprehensive studies have shown that senescence occurs in the wild in many taxa, including the mammals (Promislow, 1991; Gaillard *et al.*, 1994) and birds (Holmes and Austad, 1995). From an evolutionary viewpoint, senescence is apparently paradoxical because it is disadvantageous to the organism. Theories to elucidate its occurrence must therefore explain this paradox while accounting for the patterns that have been observed in wild and laboratory populations.

There are forms of damage that occur during an organism's lifetime that may represent proximate causes of ageing (Kirkwood, 1992; Partridge and Barton, 1993). By far the simplest and most obvious of these is the wear and tear of non-replaceable structures such as teeth, which could indeed lead to death (Comfort, 1979). Far more subtle events have also been identified as causing an age related decline in vitality. One such process is the accumulation of macromolecules that have been damaged by oxidation, the significance of which is that it can occur in any organism. Free radicals, or reactive oxygen metabolites, are the by-products of the partial reduction of oxygen which can cause damage to DNA, proteins and membrane fatty acids in living tissue (Sohal and Weindruch, 1996). They are produced by aerobic metabolism, especially in the mitochondria, and by other physiological processes such as the phagocytic destruction of bacteria and viruses (Ames *et al.*, 1993). The level of oxidants accumulates with age (Ames *et al.*, 1993; Agarwal and Sohal, 1994; Forster *et al.*, 1996), as does the quantity of damaged macromolecules that needs degrading or repairing. The phenomenon has been associated with, amongst other events, declining cognitive function in mice (Forster *et al.*, 1996) and activity rates in houseflies (Agarwal and Sohal, 1994). Oxidative damage is seen as a very important cause of ageing in many species (Ames *et al.*, 1993; Kowald and Kirkwood, 1994, and 1996; Sohal and Weindruch, 1996; Martin *et al.*, 1996). Mechanisms are known that combat oxidative damage on all levels, from the removal of potentially harmful radical species (Orr and Sohal, 1994) to the repair of damage once it has been inflicted (Ames *et al.*, 1993) and the replacement of irreparably damaged cells (Martin *et al.*, 1996). The existence of this type of

defence is proof that free radicals pose a threat to viability (Kowald and Kirkwood, 1994).

In addition, molecules such as DNA, RNA or polypeptides are constantly under threat of chemical modification or cleavage, and both DNA and RNA can be damaged by ultraviolet radiation (Holliday, 1995). Abnormal molecules can accumulate with age if they are not removed efficiently, and the effects of damage to important molecules and the problems associated with inefficient proteolysis and repair are implicated in senescence (Kowald and Kirkwood, 1996). Mitochondrial proteins and DNA are particularly prone to the accumulation of damage, as mitochondria are the primary source of free radicals in the cell and mitochondrial DNA has no capacity for repair (Ames *et al.*, 1993). Errors in proteins themselves involved in protein or DNA synthesis could lead to the proliferation of those errors, resulting in error catastrophe (Orgel, 1963) and the eventual loss of cell function. In the same way that oxidative damage has prompted the evolution of anti-oxidant defence, the existence of proof-reading enzymes in DNA and protein synthesis suggests that the correction of errors in synthesis is important in the maintenance of vitality and the avoidance of premature senescence.

However, whereas a decline in fitness may be bought about by these mechanisms, senescence cannot be explained simply in terms of bodily damage. This is because mechanistic explanations do not account for two things. First, genetic variation for senescence rate exists (Partridge and Barton, 1993; Hughes and Charlesworth, 1994; Promislow *et al.*, 1996) and indeed, life span has been successfully altered by artificial selection in species such as *Drosophila melanogaster* (for example Luckinbill *et al.*, 1984; Rose, 1984; Partridge and Fowler, 1992), *D. subobscura* (Wattiaux, 1968), the flour beetle, *Tribolium castaneum* (Sokal, 1970; Mertz, 1975), the melon fly, *Bactrocera cucurbitae* (Miyatake, 1997), the bean weevil, *Acanthoscelides obtectus* (Tucic *et al.*, 1996) and by other means in *Caenorhabditus elegans* (Johnson and Wood, 1982). Second, maximum life span and rates of ageing are species-specific, even when environmental factors are controlled (Partridge and Barton, 1993). This shows that ageing has a genetic component, rather than simply being determined by events during an individual's life time. Therefore, an explanation must be sought that takes account of these facts and that is compatible with the existence of accumulated damage as outlined above.

1.2.2. Theories on the Evolution of Senescence

The first theory of how senescence evolved was formulated by Weismann in the last century (see Kirkwood and Cremer, 1982). Weismann's theory states that selection would act against an organism that is past its reproductive peak and no longer of any use to the species (Kirkwood and Cremer, 1982). This idea has been criticised (Medawar, 1952; Williams, 1957; Kirkland, 1989), not least because it is circular (Medawar, 1952; Kirkwood and Cremer, 1982) and supposes that non senescing animals become decrepit. In addition, positive selection for genes late in life is not likely to occur because the force of natural selection declines with age (Williams, 1957) and the reduction of life span to relieve pressure on subsequent generations invokes group selection. Group selection is a weak evolutionary force that will not lead to change if it opposes the direction of individual selection (Ridley, 1993). Indeed, on the individual level, there is a natural tendency to lengthen life not shorten it (Edney and Gill, 1968), because genes that extend life span will increase fitness. Later theories that have also failed to explain the existence of ageing include the developmental theory of ageing, which describes senescence as "the ultimate step in a chain of developmental sequences" (Lints, 1988), and the longevity assurance hypothesis (Sacher, 1978) which outlines a mechanism for the evolution of a predetermined life span. The developmental theory is based upon the premise that life span and development are correlated, which has been refuted using environmental (Zwaan *et al.*, 1991, and 1992) and genetical (Zwaan *et al.*, 1995a) manipulation. The longevity assurance argument proposes that genes which actively postpone death will be selected late in life. It is therefore flawed for the same reason as Weismann's theory: natural selection will not positively favour genes that are expressed late in life.

A solution to explaining senescence in an evolutionary context comes with viewing it as a maladaptive trait that has come about as a by-product of evolution (Martin *et al.*, 1996). It was realised by Medawar (1952) and Williams (1957) that the force of natural selection gets progressively weaker with age. Without ageing, individuals risk death and accumulate insults to fertility through predation, disease and accident (Partridge and Barton, 1993). A gene that is expressed early in life when most of these individuals are still alive and fertile will be subject to the full force of natural selection, because the fitness of the

gene will influence its carrier's chance of survival. If the gene is expressed later on in life, however, it will have already been passed on to the next generation without affecting the fitness of its carrier. For this reason, the late expressed gene will be under less selection pressure than the one expressed in a young individual (Kirkwood, 1981) because of the number that are either dead or sterile at the later time. Two theories have been proposed on this basis. The first is that such genes are deleterious and will be able to accumulate due to the weakness of natural selection at late ages (Medawar, 1952). The second proposes that pleiotropic genes with age-specific action can be selected early in life, despite having deleterious side effects that are expressed later on when they cannot be removed (Williams, 1957).

The mutation accumulation hypothesis (Rose and Charlesworth, 1980) conjectures that age-specific mutant alleles that are expressed late in life will cause senescence (Medawar, 1952). They would be selected out of the population if expressed in a young individual, but later on they will not be selected for or against (Hoekstra, 1993) and will spread through the population by mutation pressure and drift (Martin *et al.*, 1996). Consequently they will be relatively high in the mutation-selection balance (Hoekstra, 1993; Partridge and Barton, 1994). Ageing could be caused in this way if enough age-specific mutations arise (Charlesworth, 1994). Medawar also suggested that genetic modifiers would influence diseases, such as Huntingdon's chorea (Medawar, 1952) and arteriosclerosis (Medawar, 1957), that have a known genetic component and would, in his view, be selected against if expressed when the animal's reproductive potential was still high. This would lead to these genes being expressed gradually later and later in the life span. However, the intensity of selection for the complete removal of a rare recessive allele by a modifier gene is similar to the mutation rate (Charlesworth, 1994). Thus a slight advantage, such as a delay in the age of expression of that rare allele, is under very weak selection indeed. Whereas mutation accumulation has been widely accepted, Medawar's case for modifiers is not strong.

The antagonistic pleiotropy theory of ageing (Rose and Charlesworth, 1980) states that alleles with dual effects can be expressed in different ways when in different somatic environments (Williams, 1957). This could mean the expression of a gene at two stages of life within the same cell line. The theory proposes that one effect may be deleterious and the other beneficial. If the

deleterious effect was expressed late in life then natural selection would not be able to act against it. Therefore the earlier expressed, beneficial effect could be favoured, allowing the gene to spread through the population. Thus, senescence could evolve as the consequence of selection for increased early life fitness. An extension of the antagonistic pleiotropy hypothesis is the disposable soma theory of ageing (Kirkwood and Holliday, 1979), which puts senescence in the context of physiological ecology (Kirkwood and Rose, 1991). It suggests that there is a trade off between somatic maintenance and reproductive effort and that senescence evolves as a consequence of this (Kirkwood and Rose, 1991). It is a special case of the antagonistic pleiotropy theory (Kirkwood and Holliday, 1979), which is a general description of the genetic principles involved, whereas the disposable soma theory identifies a physiological mechanism that may be responsible for the evolution of senescence (Kirkwood and Rose, 1991).

Mutation accumulation and antagonistic pleiotropy propose that senescence is caused in different ways. The mutation accumulation hypothesis postulates that the optimal life history is suppressed by the expression of deleterious alleles late in life. Once established, positive feedback will accelerate the decline in fitness because as ageing evolves, selection against it will get weaker (Partridge and Barton, 1993). Antagonistic pleiotropy can be described in terms of the evolution of an optimal life history, with increased fertility or survival probability early in life being traded off against a later reduction, resulting in ageing (Partridge and Barton, 1993). This could indeed happen if investment in current reproduction elevates fitness at the expense of later survival. An optimal life history does not necessarily require pleiotropic gene action, however, and could be reached by genes with a single effect (Partridge and Barton, 1993).

It is probable that once these mechanisms evolve, they increase susceptibility to extrinsic and intrinsic mortality risks. For example, a senescent decline in general activity rate could make an animal more prone to attack from predators. In the same way, an age-specific reduction in the efficiency of the immune system will increase the risk of death by disease. It is likely that death in a senescent organism will be due to the combined effect of a number of factors, because death by a single cause at a given age will reduce the selection pressure for other systems of repair and defence to be maintained beyond that age. Various causes of death should therefore converge in terms of their time of

onset (Williams, 1957). Under the disposable soma hypothesis repair mechanisms should deal with many types of damage, both intracellular and extracellular, in equal measure (Kirkwood and Rose, 1991), and so this model also predicts that senescent death should be due to a combination of factors acting at a similar time (Kirkwood and Franceschi, 1992).

Criticism has been levelled at the antagonistic pleiotropy and mutation accumulation theories about how the concept of "lateness" could have existed before senescence evolved (Kirkwood and Holliday, 1979). Cells in the ageless organism are in a steady state, so how can deleterious mutations or side effects be expressed at a particular time? The disposable soma hypothesis gets around this apparent circularity because the decisions on how to allocate energy to somatic and reproductive causes are taken at a point early in life, so the problem of timing does not arise. However, if the deleterious side effect of a hypothetical pleiotropic gene is the gradual accumulation of a toxic waste product that is triggered at the same time as the beneficial effect, then the side effect will cause death some time after the gene has been expressed, when some lethal threshold for toxicity is reached. This still comes under the definition of pleiotropy because it is the timing of the gene's effect that is important, not the time of its expression (Partridge and Barton, 1993). The human genetic disorder, idiopathic hemochromatosis, causes increased iron absorption until it eventually reaches toxic levels in some organs. The allele causing it is thought to be maintained in the population by some selective advantage (Albin, 1993), and as such is a potential example of antagonistic pleiotropy by the mechanism just described. In this way, a pleiotropic gene could abolish the steady state and define "lateness", thus allowing the acceleration of the evolution of ageing by either mutation accumulation or antagonistic pleiotropy. Therefore, for the purposes of the following review of the literature, I shall treat the disposable soma theory as a special case of the antagonistic pleiotropy model, in which the function of the genes have been defined.

The optimality framework accommodates the concept of the species-specific life span. Life span evolves in response to the hazard factor, the extrinsic mortality risk in a given niche (Edney and Gill, 1968). Individuals in a species will invest in the necessary somatic growth and maintenance to allow them to optimise survival and reproduction. Part of this investment is in mechanisms for the repair of cell and tissue damage that can threaten survival

and fecundity, but these are costly (Kirkwood, 1981; Kirkwood and Franceschi, 1992). Intracellular homeostasis uses a significant proportion of a cell's energy budget in tasks such as the repair of oxidative damage to DNA, proteins and membrane lipids, accurate DNA replication and the removal of abnormal polypeptides (Kirkwood, 1992). Therefore, the hazard factor will have a bearing on the level of somatic repair throughout life (Kirkwood and Rose, 1991). For a given hazard factor, there will be an age that members of a species will have no chance of living past (Kirkwood and Rose, 1991). Natural selection should act to produce a balance between somatic maintenance and reproduction that will optimise energy investment over this expected life span, and this includes investment in repair and homeostasis. The consequence of repair being shaped by extrinsic mortality is that only limited protection from damaging agents will be offered (Kirkwood and Franceschi, 1992), and a decline in the efficiency of somatic maintenance towards the end of the average life span will occur, since these mechanisms will not be maintained indefinitely. A decline in fitness towards the end of life will not affect the probability of an organism having previously passed on its genes. Optimality theory therefore predicts that senescence will evolve (Kirkwood and Rose, 1991).

The rate of ageing for a species is expected to be positively correlated with the rate of extrinsic, age independent mortality (Williams, 1957). The intensity of selection against genes causing senescence will increase with decreasing mortality rate, due to the higher numbers of individuals surviving to old age. There is some evidence from natural history studies to suggest that ageing rates may be related to levels of risk. In wild mammals the rate of increase of age-specific mortality is greater in short lived species (Promislow, 1991), although comparative data on age-specific mortality rates must be treated with caution because assumptions made about the age structure of wild populations may not hold true, and sampling can be biased against certain age classes (Gaillard *et al.*, 1994).

The biology of an organism will also have an effect upon the hazard factor that members of a species experience. If an organism can avoid death by predation or accident, then it will live longer. Thick shelled bivalve molluscs are longer lived than other molluscs, and turtles and tortoises live longer than other reptiles (Partridge and Barton, 1993). Birds are able to fly and escape predators on the ground and they have a longer potential life span than mammals of the

same size (Holmes and Austad, 1995). Flightless species such as emus and ostriches are very short lived for their size (Partridge and Barton, 1993) whereas swifts, which are fast, agile fliers, have very low mortality rates (Holmes and Austad, 1995). The evolution of flight may have shaped the evolution of avian life histories (Holmes and Austad, 1995). Comparatively low mortality risk has relaxed selection to reproduce rapidly and extended life span has evolved due to selection for postponed senescence. This pattern is pronounced in large sea birds (Holmes and Austad, 1995). An extensive study of mammalian data has shown that bats are a minimum of three times longer lived than flightless eutherian (non marsupial) mammals (Austad and Fischer, 1991). There was no suggestion that bats lived longer because they had a lower basal metabolic rate (Austad and Fischer, 1991), it is more likely due to reduced mortality risk brought about by bats' ability to fly.

The association between external hazard and the evolution of the rate of mortality may be more complicated than first thought. If the dependence of population growth on density is constant throughout life (or even if it is density independent), any change in extrinsic mortality will affect all age classes equally and this will not alter the schedule of senescence (Abrams, 1993). This could apply if a predator threatened all age classes equally, in which case a decline in survivorship would most likely occur across ages. The rate of ageing would therefore not change because the decrease in survivorship would be age independent, rather than being biased towards older individuals.

1.2.3. The Ubiquity of Senescence: the Germ/Soma Distinction

Ageing has been suggested to occur in all organisms that have a clear distinction between germ line and soma, and if there is no distinction between cells of each type, then natural selection cannot act differentially upon them (Williams, 1957). Whereas senescence has evolved in many species (Rose, 1991), experimental evidence and logic suggest that Williams did not get his conditions for its occurrence quite right. Species such as *Stenostomum incaudatum* and *Paranais litoralis* have little distinction between germ and soma but do actually age (Martinez and Levinton, 1992). Bell (1984) has shown that two species of oligochaete that reproduce by paratomical fission, where the

products are a similar size, do not show an age-specific decline in fitness, whereas other ovigerous (egg producing) species do. It appears that in order for senescence to evolve there must at least be a difference between parent and offspring (Partridge and Barton, 1993), but some animals that were previously thought not to senesce do indeed have enough distinction between germ and soma to do so (Martinez and Levinton, 1992). An organism that has an equal chance of any of its cells turning into gametes has no germ/soma distinction, yet it would still experience a non-zero probability of death over time (Partridge and Barton, 1993). Species such as *Stenostomum* have a defined anterior and posterior and the latter buds off to become the new animal (Martinez and Levinton, 1992). Thus, despite not having a distinct germ or soma, the roles of parent and juvenile apply and so senescence can occur since each parent will be subject to an age-specific decline in fitness and an increased likelihood of death (Partridge and Barton, 1993). The argument should also apply to a unicell that buds off distinct daughters because the probability of death increases with time, although in this case the parent and daughter share a nucleus until the point of budding (Hoekstra, 1993). Does this mean that age-specific mutations will affect both parent and offspring? Hoekstra (1993) suggests that the cytoplasm differentiates the parental cell from the newly formed daughter, acting as a trigger for age-specific gene expression in the former only. Williams' original conjecture should be modified. Germ and soma do not need to be distinct, but there must be a recognisable part that functions as a soma and produces offspring for the age-specific probability of death to increase and ageing to evolve.

1.3. Testing Evolutionary Theories of Senescence

Theories on the evolution of senescence provide a number of readily testable predictions. Bell (1984) addressed one of these in a very simple way. As discussed above, senescence should not occur in organisms that do not have a recognisable difference between germ and soma. Bell (1984) looked at the rates of age-specific mortality in two species that reproduce by paratomical fission and four ovigerous species. By finding a decline in survivorship with age in the ovigerous but not the paratomical species, he showed that mechanistic

theories of ageing, which do not require a germ-soma distinction to operate, do not satisfactorily explain senescence, and that evolutionary theories of ageing are required (Bell, 1984). Edney and Gill (1968) predicted that it would be possible to genetically alter life span by varying the hazard factor for an organism. By selecting for shortened life span, senescence would start earlier because selection on the later part of the natural life span would be removed, thus allowing the accumulation of mutations and the incidence of late expressed effects of pleiotropic alleles. This was tried by Wattiaux (1968) on *Drosophila subobscura*, and by Sokal (1970) and Mertz (1975) on *Tribolium castaneum*, who all succeeded in selecting for shortened life span. These studies showed that further work on the predictions specific to each theory of ageing was required. The mutation accumulation and antagonistic pleiotropy hypotheses are not mutually exclusive. The two mechanisms by which they work, the rise in frequency of deleterious mutations late in life and the existence of constraints between life history traits, can and do co-occur (Partridge and Barton, 1993). Thus, the relative importance of each these theories needs to be assessed.

1.3.1. Testing Mutation Accumulation: Measuring Genetic Variance

Under mutation accumulation, the amount of additive genetic variance, V_A, for survival or fecundity will increase with age (Partridge and Barton, 1993; Charlesworth, 1994) because of the increasing frequency of mutant alleles at late ages (Hughes and Charlesworth, 1994; Charlesworth, 1994). However, more recently, theoretical models have been used to show that this could also occur with antagonistic pleiotropy (Charlesworth and Hughes, 1996). This means that studies that have provided supposedly firm conclusions before this model was formulated have in fact resolved nothing.

Additive variance for egg laying rate in *Drosophila* did not increase significantly for twenty five days after eclosion (Rose and Charlesworth, 1980), which did not support the mutation accumulation hypothesis, or indeed either hypothesis, as later shown by Charlesworth and Hughes (1996). Hughes and Charlesworth (1994) then measured genetic (additive and dominance, V_D) and environmental (V_E) components of variance of mortality in males that were standard third chromosome heterozygotes (created by crossing different

extracted lines). They sampled for longer and used bigger sample sizes than Rose and Charlesworth, and found that the mean value of all variance components of male mortality greatly increased with age. Large sample size is crucial to avoid a decrease in V_A due to mortality, which could mask the effect of increasing variance (Clark, 1987). At the time, this provided strong evidence for the mutation accumulation hypothesis and suggests that the sampling period was too short in the, 1980 study, as genetic and environmental variance for male mortality did not significantly increase until week nine (Hughes and Charlesworth, 1994). However, following the recent change in understanding, the pattern of increasing additive variance could also fit the antagonistic pleiotropy model (Charlesworth and Hughes, 1996).

Charlesworth and Hughes (1996) did resolve the problem somewhat by showing that under mutation accumulation, inbreeding depression for a trait should also increase with age, whereas under pleiotropy, inbreeding is independent of age. Inbreeding and V_A for male mortality has been shown to significantly increase with age (Charlesworth and Hughes, 1996), giving good support to the mutation accumulation hypothesis in a way that previous studies have not. However, there is doubt as to where this leaves antagonistic pleiotropy, which does not necessarily require genetic variation to contribute to senescence, and so may in fact not affect patterns of inbreeding and genetic variances in the population (Charlesworth and Hughes, 1996). This study has been of benefit in highlighting the failings of assumptions made about mutation accumulation and antagonistic pleiotropy, but the picture is still no clearer; the question of the relative contribution of each mechanism to the evolution of ageing has yet to be answered. Indeed, more questions are raised each time an experiment is performed. For example, Promislow *et al.* (1996) obtained a result that does not support either theory. Additive variance for mortality in *Drosophila* increased with age in both sexes for the first half of life, but then declined again. In addition, no dominance variance was found, a necessity for antagonistic pleiotropy. The study raises questions about the pattern of genetic variance throughout life, the influence of the laboratory environment upon them and the problem of diminishing sample size. If this is a true biological phenomenon, then it may reflect a change in gene regulation at late ages, such that V_A decreases with a decline in gene regulation across genotypes (Promislow *et al.*, 1996). It is clear that to date this type of investigation has not resolved the issue of the

importance of different theories for the evolution of ageing. Artificial selection for extended life span has been used extensively to test other predictions arising from the theories.

1.3.2. Trade Offs between Early and Late Fitness Components

Artificial selection upon reproduction in *Drosophila* has been used repeatedly to examine the correlated responses to selection for late life fitness. This approach has two advantages. It deals with the genetic correlations that exist between phenotypic characters, because a response to selection has to have a genetic basis. It also directly tests for antagonistic pleiotropy, since the existence of trade offs between characters at different ages is predicted by the theory. However, it does not provide a measure of mutation accumulation, except to suggest that in cases where no pleiotropy is found, it may have a role. Results from this type of study must be used in conjunction with a positive test for mutation accumulation, as detailed in the last section, to assess the importance of each.

Artificial selection on age at reproduction involves taking eggs from successively older females in each generation, so that extended life span is selected. Evidence for the antagonistic pleiotropy theory of senescence was given when a number of these experiments independently showed that when life span was selected, life span and late fecundity increased while early life fecundity decreased (Rose and Charlesworth, 1981; Luckinbill *et al.*, 1984; Rose, 1984), precisely as Williams (1957) predicted. The method has also been used to show support for the theory in species other than *Drosophila*, such as *A. obtectus* (Tucic *et al.*, 1996) and *B. cucurbitae* (Miyatake, 1997).

In contrast, when Lints and Hoste (1974) tried to extend life span by selection on age at breeding, they obtained wildly fluctuating longevity from generation to generation and virtually no overall response to selection. They concluded that ageing is controlled by non-genetic maternal effects, or the Lansing effect (Lints and Hoste, 1974). Low density rearing is the one consistent difference between work by Lints and Hoste (1974) and the subsequent successful selection studies. Luckinbill and Clare (1985), Arking (1987) and Service *et al.* (1988) have showed that selection on age at reproduction at low,

controlled densities produced no consistent response to selection. There may be suppression of genetic variation for life span in conditions of low environmental stress, leading to the suppression of additivity of genes for life span and the expression of a single, short lived phenotype that will not respond easily to selection (Luckinbill and Clare, 1985). A clear gene by environment interaction was demonstrated when F_1 hybrids of short and long lived stocks showed intermediate longevity when reared at high, uncontrolled density, but only a short lived phenotype when reared at low density (Clare and Luckinbill, 1985). A similar interaction between strain and temperature has been shown in *D. simulans* (Parsons, 1977). Selection studies are limited because the results apply only in a single environment. Gene by environment interactions can confound any indication that genetic correlations exist (Clark, 1987), and the sign of genetic correlations may change with environment (Service and Rose, 1985). Therefore, exact replication of previous environmental conditions is essential for any worthwhile comparisons to be made, as is making measurements in the environment in which selection was carried out (Leroi *et al.*, 1994). This has been highlighted by the demonstration in lines selected for long and short life span that a relative difference in early fecundity between regimes can be reversed by changing the assay environment (Leroi *et al.*, 1994). In addition, the application of selection for many generations can result in changes to correlated responses. This may be due to either variation between regimes other than the intended demographic differences, or the adaptation of each population to its own environment (Leroi *et al.*, 1994).

Artificial selection on life span has been used to provide evidence for mutation accumulation. In the *r*-populations, *Drosophila* adults were only allowed to reproduce and lay eggs until day six, after which they were discarded. These were small and grew rapidly. The *K*-populations were kept at high density and allowed to reproduce until they died. After 120 generations, the *r*-populations showed significantly lower fertility in the fourth week after eclosion compared to the *K*-population flies, and also a higher rate of adult mortality (Mueller, 1987). This indicates that the *r*-populations had evolved an accelerated senescent decline in both fertility and survival. There was no decline in early life fertility in the *K*-populations, suggesting that the accelerated senescence in the *r*-populations was due to the accumulation of deleterious mutations under weakened selection pressure. This study must be treated with some caution,

because rearing density differed between the *r*- and *K*- selected populations, which may have lead to responses not solely due to selection for late or early life fitness. Mueller (1987) did, however, provide further evidence for mutation accumulation, because hybridisation between replicates within each selection regime showed that the accelerated senescence in the *r*-lines had been all but removed by hybridisation. Under mutation accumulation, deleterious alleles would fix at different loci in each replicate. The fact that hybridisation had any effect on fecundity means that ageing cannot have been caused by a small number of fixed, dominant alleles, a pattern which would be consistent with pleiotropy (Mueller, 1987).

Two further studies found no evidence for a correlated decline in early fecundity when late life fitness was selected (Partridge and Fowler, 1992; Roper *et al.*, 1993). In one case, this was demonstrated in two strains of *D. melanogaster* with different origins (Partridge and Fowler, 1992), confirming that the result was not peculiar to any one strain and that it was not due to differences in the additive genetic variation for the characters under selection (Partridge and Fowler, 1992). It is possible, therefore, that either mutation accumulation alone was responsible for rapid ageing observed in the 'young' lines, that there was a correlated response to selection elsewhere in the life history, or that some other aspect of the selection environment confounded the result. This does not explain findings by other authors; it may be that the selection environment applied to the Dahomey and Brighton stocks lead to different correlated responses compared to those seen in the previous experiments (Partridge and Fowler, 1992).

Partridge and Fowler (1992) and Roper *et al.* (1993) looked at characters in the pre-adult life history for correlated responses to selection. They found that the 'old' line larvae took longer to develop than 'young' line larvae and that, in the case of the Brighton strain, the 'old' adults were larger and heavier than 'young' ones. In addition, larval competitive ability was greater in the 'young' lines, suggesting a trade off between one or both of life span and late life fecundity versus larval viability (Partridge and Fowler, 1992; Roper *et al.*, 1993). Extended larval development time leads to larger adults, and positive correlations between body size and both longevity and fecundity have been found (Robertson, 1957; Tantawy and Rakha, 1964; Partridge and Farquhar, 1983; Santos *et al.*, 1992). The longer development time may therefore have been the result of pressure to

get bigger and live longer, and the larger body size of the 'old' lines the reason for their higher life time fecundity compared to the 'young' lines.

This confusion highlights a recurring problem with the studies that have involved selection on age at reproduction. It is that despite, in some cases, efforts to avoid it, there has been selection upon larval characters that has confounded the results of selection on longevity and fecundity. This has largely been the result of inadvertent selection on larvae to develop faster (Partridge and Fowler, 1992; Chippindale *et al.*, 1994; Roper *et al.*, 1993; Tucic *et al.*, 1996; Miyatake, 1997), but also due to lack of controlled rearing density (Luckinbill *et al.*, 1984; Partridge and Fowler, 1992; Roper *et al.*, 1993). This has lead to differences in larval development time (Luckinbill *et al.*, 1984; Partridge and Fowler, 1992; Chippindale *et al.*, 1994; Roper *et al.*, 1993), adult body size (Partridge and Fowler, 1992; Roper *et al.*, 1993; Tucic *et al.*, 1996), adult body weight (Rose *et al.*, 1984; Partridge and Fowler, 1992; Tucic *et al.*, 1996) and pre-adult viability (Partridge and Fowler, 1992; Chippindale *et al.*, 1994; Roper *et al.*, 1993) between selection regimes. This is why it is necessary to examine these issues under rigorously controlled conditions: with standardised larval density, relaxed selection upon eclosion time and in relation to the base stock (Roper *et al.*, 1993). It is quite possible that these difficulties are the root of the discrepancy between the studies that showed a trade off between early and late life fecundity and those that did not. Inadvertent selection on early fecundity in long lived lines may also be an important factor (Partridge and Fowler, 1992), since all studies selected for increased early fecundity by requiring females to reproduce soon after eclosion. Interpretation is difficult because some selection regimes (e.g. Luckinbill *et al.*, 1984; Rose, 1984) did not have a proper control with which to compare responses to selection for both early and late life fitness. This is important, because application of selection for early life fitness is not necessarily the same as keeping a base stock for reference.

One further question that arises from studies of artificial selection on life span and especially the results of Mueller (1987) and Partridge and Fowler (1992) is whether life span and late life fertility are inseparable. The crux of the problem has been that selection on age at reproduction selects both. The results of one study (Zwaan *et al.*, 1995b), which involved selection on life span alone, have shown that lifetime fecundity declines when life span is extended. This suggests that early fecundity is correlated with longevity (Zwaan *et al.*, 1995b).

Lipid content is implicated in the function of both survival and fertility, because fat fraction increased in the 'old' lines, and starvation resistance increased while fecundity decreased (Zwaan *et al.*, 1995b). Indeed, fecundity and starvation resistance were found to be negatively correlated elsewhere (Service *et al.*, 1988). Lipids are utilised by both egg and sperm production as well as tissue maintenance (Geer *et al.*, 1970). Measurement of fat fraction by Zwaan *et al.* (1995b) has shown a possible physiological basis for the trade off that is the principle of the disposable soma mechanism of ageing.

1.4. Alternative Approaches to Studying Evolutionary Aspects of Ageing

1.4.1. Evidence from Physiological Studies

Work on physiological and morphological characters in lines selected for postponed ageing has helped increase understanding of the mechanisms that alter life span and fertility. It seems that life span extension requires the enhancement of physiological adaptation to several different kinds of stress, since stress resistance characters have changed in response to selection on age at reproduction. The ability to withstand ethanol vapours, desiccation and starvation (Service *et al.*, 1985) and increased flight duration (Graves *et al.*, 1988) were all greater in 'old' line flies compared to controls. In addition, flies selected for high desiccation resistance lived longer than controls (Hoffmann and Parsons, 1993), suggesting a direct relationship between enhanced resistance to stress and survival probability. There is evidence that there are at least two distinct physiological mechanisms involved, since desiccation resistance depends on the level of glycogen (Graves *et al.*, 1992), and starvation resistance depends on the level of lipids (Service, 1987; Zwaan *et al.*, 1995b). The partial independence of these mechanisms, as shown by the response of starvation resistance but not desiccation or ethanol resistance to reverse selection (Service *et al.*, 1988), suggests that life span can be enhanced in a number of ways. There are a number of physiological processes that decline in rate and efficiency with progressing senescence, so it is logical that an improvement in just a few of them may prolong life (Tower, 1996).

If life span is extended then a cost may be paid in terms of fertility, because somatic functions and reproduction both draw their resources from the same source within the same organism. This has been suggested by artificial selection studies (Rose and Charlesworth, 1981; Luckinbill *et al.*, 1984; Rose, 1984; Zwaan *et al.*, 1995b), and also by morphological studies showing that in young 'O' line females, selected for late life fitness, ovary weight was a half to a third of that of the control, or 'B', line females of the same age (Rose *et al.*, 1984). 'O' line females also had high lipid content early in life, which is positively correlated with starvation resistance (Service, 1987; Zwaan *et al.*, 1995b), so resources such as lipids may be diverted away from the ovaries and into somatic functions in these flies (Service *et al.*, 1985). This is also supported by females selected for high desiccation resistance, which have low early life fecundity, and a lower metabolic rate than controls (Hoffmann and Parsons, 1989). Young 'O' females also showed reduced metabolic rate compared to 'B' females of the same age (Service, 1987). These patterns could be due to linkage disequilibrium, in which case stress resistance characters and genes for extended life span have different genetic backgrounds, but are likely to occur together. However, this is unlikely because the data are from several independent sources. Alternatively, the positive correlation of stress resistance characters with life span, and their negative correlation with early life fertility provide support for the antagonistic pleiotropy mechanism of senescence. The data also fit the disposable soma model, which involves a decision to divert resources, including lipids, away from reproduction and towards survival.

In addition to effects on fertility and lipid metabolism, anti-oxidant defence has also been shown to have a role in life span extension in one set of lines selected for age at reproduction (Dudas and Arking, 1995). It is clear that the extension of life span in response to selection is caused by a number of different mechanisms that combine to produce the long lived phenotype in *Drosophila*. This fits the pattern of there being many loci involved with extended longevity in certain selected populations (Luckinbill *et al.*, 1988a; Buck *et al.*, 1993), be they concerned with anti-oxidant defence, reproductive behaviour, lipid and glycogen metabolism or any other mechanism that selection acts upon to prolong life.

1.4.2. Candidate Genes for Senescence

There are a number of physiological and biochemical events that affect fitness and have been linked with senescence because they show an age-specific trend. Examples include the shortening of telomeres (Harley *et al.*, 1990), the accumulation of mutations in mitochondrial DNA (Curtsinger *et al.*, 1995) and the increase of protein half-life with age (Young and Munro, 1978; Sharma *et al.*, 1979; Richardson and Cheung, 1982). However, mechanisms that are sometimes cited as causing ageing may in fact only contribute to an age-specific decline in state after the process has started. Mechanistic explanations for ageing must be scrutinised to see if they require an intrinsic decline in state for them to occur. The occurrence of these phenomena does not preclude the existence of a smaller number of underlying mechanisms that do actually cause senescence (Tower, 1996). For example, the reduction in the rate of protein turnover with age in many species is likely to be the result of the progressive accumulation of oxidative damage to enzymes involved in synthesis and proteolysis, and of errors in the synthesis of proteins that are themselves involved in protein synthesis. The disposable soma theory predicts that investment in cellular defence processes that perform functions such as ensuring the accuracy of DNA replication and protein synthesis, repairing damaged DNA and removing free radicals is limited (Kirkwood and Rose, 1991; Kirkwood and Franceschi, 1992), because infinite survival is not possible, even with infinite investment in repair. The network theory of ageing models the interactions of the various forms of damage that are known to occur and the way in which the cellular defence network responds (Kowald and Kirkwood, 1996). It predicts that the level of protection, in terms of anti-oxidant defence and proteolysis, given to an organism in which senescence has evolved is not adequate to avoid the accumulation of damage to mitochondrial DNA or aberrant proteins (Kowald and Kirkwood, 1996). Mechanisms such as protein turnover therefore become progressively less efficient with age and contribute to the phenomenon of ageing, but are not a primary cause.

Explanations of how ageing is caused must be in accordance with the theories on how it evolved. In terms of mutation accumulation, any disease gene expressed late in life is a possible candidate (Martin *et al.*, 1996). One possible example is the apolipoprotein E type 4 allele, strongly suggested to be

connected with late-onset Alzheimer's disease (Corder *et al.*, 1993). The accumulation of mutations late in life is determined by mutation pressure and drift, so causes of senescence by this mechanism are likely to vary widely across lineages (Martin *et al.*, 1996). Pleiotropic genes, on the other hand, have a beneficial effect that is selected and so pleiotropy may be the mechanism by which common causes of ageing arise (Martin *et al.*, 1996). There are genes that are known to fit the pattern of having an early benefit and late cost, as would be required for agreement with the pleiotropy model. In humans, Huntingdon's disease is a possible candidate because there is evidence that carriers have above-average fecundity prior to the onset of the disease (Albin, 1993). In addition, steroid hormones in humans appear to have pleiotropic effects. Despite having a beneficial role during development and early life, later on, testosterone exacerbates the development of coronary arteriosclerosis in cynomolgus monkeys (Adams *et al.*, 1995) and prostate growths in men (Finch and Rose, 1995), whereas estrogens stimulate uterine growths in women (Finch and Rose, 1995). In *Drosophila mercatorum*, the abnormal abdomen mutation causes early development and enhanced fecundity at the expense of shortened survival, mediated by juvenile hormone (Finch and Rose, 1995). Indeed, hormones potentially play a very great role in the evolution of the life history and mechanisms of antagonistic pleiotropy, because one hormone plays many different roles throughout the life history of the organism, and such hormones therefore exhibit pleiotropy (Finch and Rose, 1995). This suggests that many candidate genes for senescence may be connected with endocrine function.

Oxidative damage to macromolecules is an important factor in senescence, and so genes with a role in anti-oxidant defence are likely candidates in the determination life span. Much has been revealed about the action of free radicals through the manipulation of some of the enzymes that degrade them, catalase and copper, zinc-superoxide dismutase (SOD) (Sohal and Weindruch, 1996). The genes coding for catalase and Cu, Zn-SOD were inserted, by way of a p-element construct, into the genome of *D. melanogaster*, which were then shown to have longer life, a lower mortality rate doubling time, and higher rates of activity later in life than controls (Orr and Sohal, 1994). In addition, the level of protein carbonyls, the by-products of certain oxidising reactions, was shown to be lower in the transgenic flies across ages (Orr and Sohal, 1994). This result on its own must be treated cautiously, because recent

work on another potential gerontogene, EF-1α, has shown that the effects of insert position and size, and their interaction with the genetic background, can mimic the supposed effects of the inserted gene (Kaiser *et al.*, 1997). However, in the case of free radicals, other experimental evidence supports the argument. The pattern of accumulating oxidative damage to DNA in houseflies is well correlated with that of protein carbonyl accumulation in the same flies (Agarwal and Sohal, 1994). In addition, flies restricted to different levels of activity showed similar levels of damage when at the same physiological age (Agarwal and Sohal, 1994). Oxidative damage has also been associated with the age related loss of motor and cognitive function in mice (Forster *et al.*, 1996), evidence that this mechanism is applicable to mammalian senescence.

How does the evidence for genes with a role in combating ageing fit in with predictions from life history theory? It has important evolutionary implications because it suggests that certain genes can have a major influence on life span and the incidence of senescence. Population genetics usually deals with the combined impact of many minor genes, but the notion of genes with large effects does not contradict theories of ageing (Stearns *et al.*, 1993). In addition, despite the fact that life history theory postulates a number of different causes of senescence (Williams, 1957; Kirkwood and Rose, 1991), it is possible to reconcile this notion with the potentially large effect that certain genes involved with for example, anti-oxidant defence, may have on life span. This is because such genes do not operate in isolation, but in an integrated system of defence, as postulated by the network theory of ageing (Kowald and Kirkwood, 1996), which models the combined effects of a number of different systems of cellular repair and defence. In this context, evidence for the action of genes that combat particular aspects of senescence complements the predictions of life history theory.

1.4.3. Studies on Ageing in Other Species

The majority of studies reviewed here concern *Drosophila,* which is invaluable in life history studies. We can compare data from phenotypic manipulations and quantitative genetic studies to build up a wealth of valuable information on *Drosophila* life histories (Partridge and Barton, 1993). However,

there is always the problem that once we have evidence for a phenomenon in *Drosophila* we can only speculate on its significance in other species. The evolution and mechanisms of ageing have been examined in *Drosophila*, but there is little need to study a wide range of species for this purpose. It would be valuable, however, to gain information regarding species of importance, especially humans (Rose, 1988).

Selection for long life in rodents would be useful for examining ageing in mammals and therefore humans, but would be expensive. Artificial selection, either directly upon life span or indirectly upon late life fertility, may not be the best solution. Studies in *Drosophila* have been started with prior knowledge of the species' life history, for example of genetic correlation patterns (Rose, 1988). Such information is not held for rodents and more is needed before embarking on such a project. Minor changes in the environment can have large consequences for the outcome of selection, which has been shown in *Drosophila* (Luckinbill and Clare, 1985; Johnson, 1988). Rigorous standardisation procedures are needed to make results reliable. However, there might not be such a problem in mammals as in *Drosophila* because their life histories are less flexible (Rose, 1988). Artificial mutagenesis and construction of cross bred lines could be used to create mice with increased longevity (Johnson, 1988), but induced mutations invariably come with large deleterious effects which would confound the study of longevity (Rose, 1988). In the case of hybridisation, longer lived hybrids would only exist in the F_1 generation, after which the hybrid genotype would break down (Rose, 1988). Selection for longer lived rodents would be very useful for studying the genetic and physiological mechanisms of ageing in mammals, with special reference to humans. However, the problems will be far from easy to solve.

Life span manipulations have been carried out upon species other than *Drosophila*, including *Tribolium* (Mertz, 1975), the bean weevil, *Acanthoscelides* (Tucic *et al.*, 1996) and the nematode, *Caenorhabditis elegans* (Johnson and Wood, 1982; Johnson, 1987; Friedman and Johnson, 1988; Johnson, 1990; Kenyon *et al.*, 1993). This highly inbred hermaphrodite has a very high proportion of homozygous loci in both laboratory and natural environments (Johnson and Wood, 1982), removing the problem of heterosis effects occurring when different strains are crossed, and allowing the study of single gene effects on life span (Johnson and Wood, 1982). Recombinant inbred lines used by

Johnson and Wood (1982) showed a range of life spans between 10 and 31 days. The lack of genetic variation in such populations means that *Caenorhabditis* is not suitable for artificial selection studies such as those carried out on *Drosophila*, but other types of genetic manipulation have been possible. For example, mutants of the *age-1* gene showed a 40% extension of life span at 20°C, whereas *age-1* hermaphrodites had a 75% reduction in fertility (Freidman and Johnson, 1988). Senescence in this species does not seem to affect the length of the reproductive period; short lived recombinant inbred lines had a rapid mortality rate but did not have a significantly shorter reproductive period (Johnson, 1987). Despite this, the results of Freidman and Johnson (1988) suggest that the level of reproduction does affect life span, as would be predicted by life history theory, even if the picture is not as clear as in *Drosophila*. In a later study, Kenyon *et al.* (1993) found that *daf-2* mutants of *C. elegans* live twice as long as wild type individuals. The *daf-2* gene governs the developmental step taken to form the long lived dauer hermaphrodite, used to endure environmental hardship. The mutants produced as many progeny as wild type adults, but in twice the time (Kenyon *et al.*, 1993). This also fits the idea of a trade off between life span and reproductive effort. The ability of *daf-2* to double life span when in mutant form is the result of its regulatory effect on many genes further along the developmental pathway, and can be regarded as a polygenic effect (Partridge and Harvey, 1993). It has since been found that *daf-2* and *age-1* appear to extend life span along similar pathways, because both are reliant on the function of the same two genes downstream (Dorman *et al.*, 1995). This suggests that the mutant *age-1* also acts as a regulator for many other genes to extend life span.

Mating costs remain unclear in *C. elegans*; one study has shown that spermatogenesis is the sole cost of reproduction, and that only males experience this (Van Voorhies, 1992), whereas another study has evidence that hermaphrodites alone experience a cost of copulation, and that gamete production is costly to neither males nor hermaphrodites (Gems and Riddle, 1996). Kenyon *et al.* (1993) ablated the germ cells of wild type hermaphrodites, and found no life span extension compared to control animals which supports the findings of Gems and Riddle (1996) that spermatogenesis is not costly. If copulation is indeed costly to hermaphrodites, then the extension of life span at the expense of fertility, as seen in the *daf-2* and *age-1* mutants, does support

the hypothesis that survival and fertility are traded off against one another. Therefore, this provides tentative support for the antagonistic pleiotropy theory of ageing in a species other than *Drosophila*.

1.5. Senescence and the Cost of Reproduction

Natural selection acts upon a combination of age-specific fecundity and survival to shape the life history. Senescence affects both of these traits (Partridge and Barton, 1994) and must always be considered in light of this fact. Much work has been carried out on the effect of reproductive schedules upon patterns of mortality, and the role of reproductive costs in the evolution of senescence. However, questions still need to be answered concerning the way in which the reproductive biology of *Drosophila melanogaster* is shaped by selection on life span, and the way in which fitness components related to reproduction, such as the ability of males to maintain sperm supplies, are affected by changes in mating and survival probability.

1.5.1. Types of Reproductive Cost

When an organism reproduces, it must face the dilemma of having to allocate resources to that task, while maintaining all the other physiological functions of the body. The result is a cost of reproduction that may take two forms. There may be a trade off between current and future reproduction, the fecundity cost (Bell and Koufopanou, 1986), and there could also be a trade off between current reproduction and survival probability, or survival cost (Bell and Koufopanou, 1986). Certain factors will influence the extent of such costs. In general, situations that either limit resource availability further or make a greater demand on those resources are going to make the costs more severe. Therefore, environmental stress and increased reproductive effort will both accentuate costs. For example, in *Artemia*, the brine shrimp, a cost of mating was only seen when food was limiting (Browne, 1982) and nutritional stress also increased the risk of mortality in the aphid, *Megoura* (Ward *et al.*, 1983). A cost of reproduction in terms of survival has been demonstrated in other vertebrate

and invertebrate species, such as the killifish, *Oryzias* (Hirshfield, 1980), the bruchid beetle, *Callosobruchus* (Tatar *et al.*, 1993), *Notophilus*, a carabid beetle (Ernsting and Issaks, 1991), and *Drosophila melanogaster* (Malick and Kidwell, 1966; Partridge and Farquhar, 1981; Fowler and Partridge, 1989) as well as in plants such as the perennial alpine flower *Polemonium* (Galen, 1993). A fertility cost has been seen in wild populations of the collared flycatcher (Gustaffson and Part, 1990). Evidence for costs to both survival and fertility as a result of reproduction has been found in red deer (Clutton-Brock *et al.*, 1982), the water skink (Schwarzkopf, 1993), the dioecious shrub, *Lindera* (Cipollini and Whigham, 1994) and the meadow grass, *Poa* (Law, 1979b).

In *Drosophila*, the exact causes of reproductive costs have been investigated. Both male and female *Drosophila* (Partridge and Farquhar, 1981; Fowler and Partridge, 1989 respectively) experience shortened life span as a result of reproductive activity. In females the matter is complicated, however, because they lay eggs before they have mated. Egg laying is costly in terms of life span (Partridge *et al.*, 1987b). Independently of this, exposure to males affects female survival when no mating occurs (Partridge and Fowler, 1990), possibly as a result of harassment by courting males. The act of mating itself also reduces life span (Fowler and Partridge, 1989), as does the receipt of male seminal fluid (Chapman *et al.*, 1995). Mating costs in males are removed when females are absent, although they do have pre-mating stresses in the form of a cost of courtship (Cordts and Partridge, 1996).

A cost of reproduction can affect survival in two ways. The instantaneous risk of death may be elevated, but once mating stopped subsequent survival probability would not be affected. Alternatively, the rate of senescence could be accelerated, meaning that mating would cause an irreversible decline in survival. In male *Drosophila* the effects of mating were found to be reversible, and males that stopped mating had similar survival prospects to life-long virgins (Partridge and Andrews, 1985). The same may be true in females, although the reversal was not as quick, and a complete recovery was never made (Partridge *et al.*, 1986). Under the conditions used in these studies, mating did not accelerate ageing in males, and possibly did not in females. In female *Callosobruchus maculatus* beetles, this was not the case. Different aspects of reproduction were partitioned, and Tatar *et al.* (1993) showed that while raising the initial mortality rate, egg production had no effect on the change in the rate of ageing, but

mating itself did accelerate the ageing rate. This raises the question of how discrete reproductive costs and senescence actually are. If reproductive costs implicate ageing, then they may have had a role in its evolution in a particular species. There are other ways in which reproductive costs could influence the pattern of life history evolution. For example, in a certain low risk environment, reducing current reproductive output might be beneficial in the long term for increasing future mating prospects (Partridge, 1987). This kind of adaptation to the level of extrinsic mortality risk can lead to the evolution of extended life span. Costs of mating may provide a mechanism for the evolution of ageing by antagonistic pleiotropy (Partridge, 1987) because the enhancement of current reproduction is at the cost of future reproduction and survival. In the evolution of an optimal life history this trade off would be favoured because increased reproduction early on in life will have a relatively greater impact on fitness (Partridge and Barton, 1994).

1.5.2. Costs of Reproduction and Selection on Age at Breeding

The mechanism by which antagonistic pleiotropy is said to work has bought the relationship between reproductive costs and the responses to selection for postponed senescence into consideration. Indeed, it is possible to conclude on first inspection of the data that the longevity difference between lines selected for early and late life fitness (Rose and Charlesworth, 1981; Luckinbill *et al.*, 1984; Rose, 1984) may be due to elevated levels of risk associated with higher reproductive rates in the short lived lines (Partridge, 1987). This is true of more recent studies too (e.g. Partridge and Fowler, 1992). The fact that one could draw such conclusions from these studies illustrates the need for data on levels of sexual activity and relative reproductive costs in these lines, as well as a base stock control, so that the true nature of the responses with respect to the original stock can be seen.

Do the responses to selection on age at reproduction in terms of life span and early or late life fecundity reflect altered schedules of senescence or just changes in the level of risk between selection regimes? Longevity differences between regimes do persist in virgins (Luckinbill *et al.*, 1988b; Service, 1989; Partridge and Fowler, 1992) with one exception (Zwaan *et al.*, 1995b), who have

been the only group to select on virgin flies, and their longevity differences were seen solely in virgins. Both Service (1989) and Partridge and Fowler (1992) found that mating costs occurred equally across selection regimes. Luckinbill *et al.* (1988b) found that the survival effects of mating were not as great in females selected for late life fitness as in those selected for early life fitness, suggesting that the response to selection included a better tolerance of mating in the late reproduced lines. Differences between studies may be due to experimental conditions (Service, 1989), but may also reflect remating rate differences in Luckinbill *et al's* lines (Partridge and Barton, 1994). Elevated female remating rates can decrease life span (Fowler and Partridge, 1989). In one instance, males of short lived populations have been shown to induce faster remating in females than males from long lived populations (Service and Vossbrink, 1996), which could have elevated female mating costs in short lived lines. If it did, then there was another factor involved, since the same lines showed no overall differences in the extent of reproductive costs (Service, 1989). Although elevated risk has been demonstrated through phenotypic manipulation, the persistence of life span (Partridge and Fowler, 1992) and early fecundity differences (Service, 1989) in virgins from selected populations means that in the selection regimes discussed so far the role of mating costs in these patterns is minor compared to the effects of pleiotropic genes controlling trade offs across ages.

1.5.3. The Conflict Between the Sexes

When copulation occurs between male and female *Drosophila melanogaster*, sperm is transferred, along with fluid from the male's paired accessory glands. The receipt of these ejaculate components triggers a behavioural change in the female. She will begin to lay eggs at a faster rate, and will become unreceptive to courting males (Kalb *et al.*, 1993). These responses are mediated in the short term by components of the accessory gland fluid (Manning, 1962; Chen *et al.*, 1988) and in the longer term by the presence of sperm (Manning, 1962; Letsinger and Gromko, 1985; Kalb *et al.*, 1993). After a period of intense egg production, the female will remate, and there is good evidence that this is triggered by the depletion of sperm within the female

storage organs (Letsinger and Gromko, 1985; Service and Vossbrink, 1996), although there has been some debate over the timing of this event (Newport and Gromko, 1984). When males mate several times in succession, the number of sperm transferred drops off very quickly. By the fourth or fifth mating without a break, they are very nearly infertile (Lefevre and Jonsson, 1962), although this does not reduce sex drive; males that are almost certainly sterile still attempt to mate frequently (Partridge and Farquhar, 1981). Lefevre and Jonsson (1962) showed that accessory gland fluid is required for fertility, because when the level of fluid declined, so did fertility. If sperm is transferred without seminal fluid, only on the injection of fluid after mating will eggs be fertilised (Hihara, 1981). These studies both observed very fast depletion of the accessory glands during serial mating. It would be informative to examine the incidence of male sterility using rates of mating that are closer to those that exist in natural populations. Manufacture of accessory gland fluid is triggered by mating (DiBenedetto *et al.*, 1990), more specifically by the act of copulation itself (Herdon *et al.*, personal communication). This rapid response means that the apparent loss of fertility can be recovered from in two hours (Lefevre and Jonsson, 1962). How fast is the recovery when sperm supplies are depleted as well?

Males have evolved mechanisms to induce females to i) remate at a rate above the optimum for their long term reproductive interests (Chapman *et al.*, 1995) and ii) increase their egg laying rate after mating (Chen *et al.*, 1988), which also reduces life span (Partridge *et al.*, 1987b). The evolutionary interests of the male and female differ because male fitness is maximised by producing as many progeny as possible per mating whereas females maximise their fitness by spreading progeny output across their life span (Chapman *et al.*, 1995). Evidence for antagonistic evolutionary interests between the sexes was found by Rice (1996) using an elaborate experimental design that allowed males to adapt to a static female phenotype. Remating frequency increased, as did the efficiency of male seminal fluid at maintaining low female receptivity after mating. This produced an elevated cost of mating in the females. A number of characters relating to male reproductive success have been examined in populations selected for accelerated or postponed ageing. Selection on age at reproduction resulted in behavioural and physiological enhancement of reproductive success early in life for short lived populations, and late in life for long lived populations. Males from postponed ageing lines had better sperm

"defence" capability; they ensured that greater numbers of their sperm were used to fertilise eggs prior to the female remating (Service and Fales, 1993). Males from the short lived populations, however, induced faster female egg laying rates immediately after copulation (Service and Vossbrink, 1996). Although the physiological aspects of reproductive success in males did not show trade offs between early and late ages (Service and Fales, 1993), behaviour did. Long lived males had elevated late life competitive mating ability at its expense early on (Service, 1993). Apart from fecundity, females in populations selected for divergent life span have rarely been examined for differences in other reproductive characteristics. However, a divergence in female mating rate has been suggested in one set of selected populations (Pletcher *et al.*, 1997). The adaptations of females in such populations needs to be investigated further. It would be interesting to examine remating rate in females, and the ability of males to induce the same, in the same population of flies selected for accelerated ageing. There is an evolutionary conflict between females and males (Chapman *et al.*, 1995), and if such adaptations were found in the same population of flies selected for early life fitness it would be an example of both sexes sharing an evolutionary goal, to reproduce as fast as possible in early life.

1.6. The Measurement of Ageing and Cohort Heterogeneity

A number of studies have used demographic survival data and the Gompertz equation to measure ageing rate in insect populations (Carey *et al.*, 1992; Curtsinger *et al.*, 1992; Tatar *et al.*, 1993; Hughes and Charlesworth, 1994). This model assumes that the mortality rate increases exponentially with age, and estimates the initial mortality rate and the rate at which it increases (Tatar *et al.*, 1993). Exponentially increasing mortality rates are theoretically possible, but are not thought to be the norm (Abrams and Ludwig, 1995). Empirically, diverse patterns may be found; mortality rate has even been found to slow down or decrease in some cases (Carey *et al.*, 1992; Curtsinger *et al.*, 1992; Brooks *et al.*, 1994). These patterns might be due to declining density in population cages, such that environmental mortality risk declines with age. However, individually held flies have yielded similar results (Carey *et al.*, 1992).

Genetically uniform flies have also produced the same pattern of levelling rates at late ages (Curtsinger *et al.*, 1992). One possible explanation is that variation in individual frailty produces these patterns. As a cohort ages the average fitness of the remaining individuals increases, eventually leaving only the hardiest ones alive, so that cohort mortality rate declines. This has been proven experimentally (Brooks *et al.*, 1994). A population of *C. elegans* that consisted of genotypes with characterised life spans showed a flattening of mortality late in life, but when subdivided on the basis of life span, death rate showed a near exponential increase within each group. This does not explain the patterns seen in genetically uniform flies, but there is a suggestion that the basis of the heterogeneity in such populations may be environmental (Curtsinger *et al.*, 1992). A similar levelling pattern has been looked for in human populations, but has not been found (Kannisto, 1988), although death rates in the elderly of many countries are improving (Kannisto *et al.*, 1994). This is unlikely to be the result of a change in the variation in individual frailty, rather an improvement in medical care and living standards. Constant environmental conditions are needed to examine the situation properly in humans, which would require exceptional circumstances.

At first it seems that the results from insect populations imply a reduction in the rate of ageing at late ages. However, they may simply reflect genetic or environmental variation for mortality rate. Recently, researchers have increasingly emphasised the importance of fertility data in the assessment of ageing. Survival and fertility are not independent, since the intensity of selection on fertility at a certain age is governed by survival at the same age (Partridge and Barton, 1996). Therefore, a better measure of ageing would be the product of age-specific survival and fertility (Partridge and Barton, 1994). The implication of this is that mortality patterns alone cannot tell us about ageing, and thus a decline in mortality rate does not necessarily mean that the rate of ageing is also declining.

Partridge and Barton (1996) provide an explicit measure of ageing that takes into account survival probability and fertility schedules. Their "residual reproductive value" gives an indication of the reproductive potential of an organism from a particular age. A decline in state in terms of either survival or reproduction will affect this value, indicating ageing. If a population is at equilibrium, then the "reproductive value" will stay at one, its initial value,

because reproductive potential is effectively constant. This method also allows different populations to be compared, because it gives a relative rather than absolute measure of fitness. When applied to data from the studies that have recently shown mortality rate to decline at late ages, this method indicated that reproductive potential would also have had to decline for the rate of ageing to slow down (Partridge and Barton, 1996). In fact, reproductive potential would already have been zero, and therefore constant, at the time when a decline mortality rate was observed, which illustrates that patterns of mortality, if viewed independently of fertility, do not give a reliable measure of ageing rate.

Survival curves showing the progress of a genetically and environmentally variable population will reflect the heterogeneity between individuals, if indeed they vary with respect to a character other than mortality rate (Partridge and Barton, 1994). This uncontrolled variable could possibly confound interpretations based on differences in mortality. This is an important consideration when interpreting experiments on reproductive costs to survival that involve the examination of mortality schedules of two or more treatments. Given that individual frailty varies within each treatment, the one experiencing the greatest mortality during the experiment will, on later comparison, have the lowest mean frailty because more individuals will have died in that treatment, starting with the weakest ones, and so only the hardiest individuals will be left. This effect will be reflected in the survival data and will therefore bias mortality rates and estimates of ageing. This confounding effect is especially relevant to experiments involving reversals of sexual activity (e.g. Browne, 1982; Partridge and Andrews, 1985; Partridge *et al.*, 1986), because the effect of differing reproductive histories is unknown. Another problem with interpreting studies of this type is comparing the relative influences of senescence and short term risk on mortality. How is heterogeneity affected by these factors? By generating heterogeneity in characters that vary with frailty, and by controlling for their effects, it may be possible to gain information on the effect of cohort variability in experimental populations. Fertility might be important in this kind of study as a more reliable indicator of heterogeneity patterns than survival (Partridge and Barton, 1994).

1.7. The Aims of the Work in this Thesis

The work presented in this thesis is concerned with the relationship between senescence and the cost of reproduction in *Drosophila melanogaster*, and associated issues. The specific aims were as follows:

- I have investigated reproductive costs to fertility in male *Drosophila melanogaster.* Survival costs have been demonstrated, but a fertility cost has not. If mating does affect future fertility, what are the mechanisms underlying this cost?
- In relation to male fertility and survival costs, I looked at the question of whether mating accelerates ageing or elevates current mortality risk. In conjunction with this, I considered the role of cohort heterogeneity in the interpretation of such experiments. Does individual variation in frailty affect the pattern of survival in males that have had their reproductive status manipulated? Using the Gompertz equation, I analysed mortality rates in these cohorts and used fertility schedules in the same males to provide a measure of ageing that incorporated survival and reproduction. The merits of using measures of ageing based solely upon survival data were considered against a method that combines survival and fertility data.
- I examined some important life history characters in recently established lines selected for either early or late life fitness. The selection regimes had been designed with previous difficulties in mind, and incorporated controlled larval and adult densities and a complete absence of covert selection for accelerated development. All experiments were carried out with reference to a base stock control.
- Potential correlated responses to selection in pre-adult development time, larval competitive viability, adult thorax length and adult dry weight were monitored to assess the effect of selection on life span on these characters. In light of the results, an assessment of previous studies and the difficulties arising from uncontrolled differences between regimes was made.
- The evolution of female reproductive characters in response to selection on life span was examined. Behavioural, physiological and morphological aspects of female reproduction were considered. The potential

role of female survival costs of non-virginity in the extension of longevity was also investigated.

Chapter 2. General Methods And Materials

2.1. Fly Stocks

All flies used in these experiments were *Drosophila melanogaster* taken from the random bred Dahomey strain (Partridge and Farquhar, 1981) that has been kept in population cages with a 12 hour:12 hour light/dark cycle at 25°C for over 20 years. A population cage is a clear plastic box, approximately 22 cm by 22 cm by 31 cm, that can be used for the large scale culture of fruit flies. Dahomey population cages are fed weekly on sugar-yeast medium contained in four 190ml culture bottles that are rotated and removed after four weeks. Wild type individuals were the focus of all the experiments described here. Eggs were collected from the population cages by allowing females to lay on yeasted Lewis medium in 190 ml culture bottles.

In addition, two marked stocks were used, both of which had single-locus eye colour mutations; one stock was *sparkling poliert*, with dark red sparkling eyes, and the other was *scarlet*. These mutations had been backcrossed into the Dahomey strain so that marked flies had comparable fitness to wild type Dahomey flies, which is an important consideration in life history experiments.

2.2. Food

2.2.1. Lewis Medium

Lewis medium was used to rear flies in culture bottles and shell vials. The bottles are of 190 ml capacity, contain 70 ml of medium and were used to rear large numbers of flies whenever the exact rearing density of larvae was not important. Shell vials are 75 mm tall and 25 mm in diameter, hold 7 ml of food and can be used to culture an exact number of larvae in controlled density experiments. Lewis medium in vials, with a sprinkling of dried yeast granules, was also used for housing small numbers of adult flies. Unless

otherwise stated, all culture bottles and vials mentioned throughout this thesis contained Lewis medium. The Lewis recipe is as follows.

Water	1000 ml
Agar	5.6 g
Sugar	85.2 g
Maize meal	94.2 g
Yeast	17 g
Niapigin	27.2 ml

2.2.2. Sugar-Yeast Medium

Sugar-yeast medium is a highly nutritious food medium that is suitable for keeping adult flies on because of its firm texture. It is used in the same quantities as Lewis medium; 70 ml in culture bottles, 7 ml in shell vials. It is made using the recipe below.

Water	1000 ml
Agar	27 g
Sugar	100 g
Yeast	100 g
Niapigin	30 ml
Propionic Acid	3 ml

2.2.3. Grape Juice Medium

Red grape juice medium facilitates the easy collection of eggs because of its colour and firm texture. It was used to collect eggs from flies that had been placed into upturned laying pots with grape medium in their lids. A small amount of yeast paste, made by mixing dried yeast granules with water, was placed on the medium in each lid to encourage oviposition. This medium was also used to feed flies in population cages for experiments involving artificial selection on age at reproduction (see chapter 4 for

details). In these cases, 30 ml grape juice medium was placed into a petri dish with a large piece of yeast paste. The yeast provided nutrition and the exposed grape medium allowed prompt egg collection whenever necessary. The recipe is as follows.

Water	1000 ml
Agar	50 g
Red grape juice	600 ml

2.3. Standard Procedures

2.3.1. Virgin Collection

Whenever flies needed to be sorted, counted or sexed, CO_2 anaesthesia was used. The exception to this was when flies had to be collected or sorted when they were less than three hours old, because CO_2 can kill or injure newly eclosed adults by rupturing the gut (Ashburner, 1989). In these cases, ice was used as an alternative. If virgin flies were needed for any reason, bottle or vial cultures containing eclosing adults would be cleared so that any fly emerging within the following eight hours could be guaranteed as virgin. Males do not mate for at least seven to eight hours after eclosion (Fowler, 1973), and females will not mate before they are twelve hours old (Ashburner, 1989). These adults would be collected over ice, sexed and placed into food vials in single sex groups of less than 20 with a few granules of dried yeast. Females could be monitored for virgin status a few days later by checking for larvae in the food vials.

2.3.2. Standard Density Cultures

Larval density plays a very important role in experiments in which a standardised rearing environment is required. When appropriate, two steps were taken in order to minimise environmental variation between individuals reared for experiments. First, the laying time allowed for egg collections was

kept as short as possible, usually about three hours, so that the age range of larvae was no larger than it had to be in order to collect the desired number of eggs. The second precaution was to make sure that all the shell vials used to rear flies for an experiment were laid up with the same number of first instar larvae. After eggs had been collected, they would be incubated for 24 hours at 25°C to allow the larvae to hatch. First instar larvae were then transferred to shell vials containing Lewis medium using a mounted needle. In this way, the number of larvae per culture could be accurately controlled.

2.4. General Statistical Methods

2.4.1. Software and Common Procedures

All the statistics described in this thesis were performed on the JMP statistics package (versions 3.1.5 and 3.1.6, SAS Institute Inc.) except for the Mantel-Cox Log-Rank test, which was carried out upon BMDP software (BMDP Life Tables and Survival Functions, BMDP Software Ltd. (Dixon, 1988)).

Where appropriate, data sets were tested to see if they conformed to the assumptions of parametric analysis. Deviation from the normal distribution was tested using the Shapiro-Wilk W test, and homogeneity of sample variances was verified using the O'Briens test. Any data violating these assumptions were either transformed using an appropriate procedure, so that parametric tests could be used, or analysed with a suitable non-parametric method. Unless otherwise mentioned, it may be assumed that data subjected to parametric analysis had homogeneous variances and were normally distributed. The main parametric method used was analysis of variance (ANOVA), but linear regression and Product-moment correlations were also employed.

In the majority of cases, data that could not be analysed parametrically were tested using the Kruskal Wallis one-way analysis of variance by ranks. If such a test yielded a significant result, pairs of groups could then be tested using the Kruskal Wallis multiple comparisons test

(Siegel and Castellan, 1988). All Kruskal Wallis analyses were tested at the 5% significance level.

To avoid the possibility of obtaining spurious positive results, p values in multiple comparisons tests were corrected using the sequential Bonferroni method (Rice, 1989). This method involves reducing the significance level in a test by an amount that depends upon the number of seperate comparisons that are being made. Certain multiple comparisons procedures already account for the number of separate comparisons made; these include the Kruskal Wallis multiple comparisons test and the Tukey Kramer Honest Significant Difference (HSD). Therefore, Bonferroni correction was not used in these cases.

2.4.2. The Analysis of Survival and Mortality

A number of different analyses were carried out on the survival data collected during the experiments described in the following chapters.

- To assess lifetime survivorship, survival curves were analysed using the Mantel-Cox Log-Rank test. This compares cumulative mortality in each sampling interval with the expected mortality based on the number of live individuals in each group that enter the sampling interval. Accidental losses are taken into account. Cumulative survival probabilities for pairs of groups can be compared using an observed versus expected chi-squared statistic.
- The rate of increase in age-specific motality with age was estimating using the Gompertz survival model. The Gompertz model has been widely used to measure changes in rates of age-specific mortality (Curtsinger *et al.,* 1992; Fukui *et al.,* 1993; Tatar *et al.,* 1993; Brooks *et al.,* 1994; Hughes and Charlesworth 1994). The Gompertz parameters were estimated using the following model fitted to survival data.

$S_t = ((A/G)*(1-exp(G-day))$

(Finch, 1990).

S_t is the proportion of the population surviving to age t, A is the age-independent mortality rate, and G is the age-dependent rate of increase in mortality (Finch, 1990). G can be used as a measure of the rate of senescence in a cohort, and was of particular interest. The model was fitted to the survival data for each experiment by least squares. The first step in testing G for variation between treatment groups was to estimate A for each treatment group and make a single estimate of G for all groups. Secondly, G was estimated individually for each group. The residual sums of squares error (SSE) for the two fits were then compared with an F test; an improvement in fit was indicated by a reduction in the SSE. A significant difference between the two fits would indicate that one or more of the individual estimates of G differed from the original single value. If this was shown, then pairs of treatments could be compared in the same manner to establish exactly which ones differed.

For analysis of the age-dependent Gompertz parameter estimated for different treatment groups in experiments on populations selected for age at reproduction, described in chapters 4 and 6, an estimate of G was made for each replicate line within each selection regime. Comparisons between regimes were then made with either Kruskal Wallis or ANOVA, using the estimate of G for each replicate line within a regime to calculate a mean value of G for that regime.

- Age-specific mortality rate was also monitored in different way. In situations where it was desirable to measure mortality rates during a specific part, or interval, of the life span, the following formula was used.

$$\text{Age-Specific Mortality Rate} = \frac{\text{Number dying during interval}}{\text{Number alive at the start of that interval}}$$

The Kruskal Wallis test or analysis of variance was then used to compare mean mortality rates of different cohorts within a particular interval.

- The point of 10% mortality in a cohort, when 10% of individuals have died, has been described previously as the start of a cohort's senescent period (Arking, 1987), with this period continuing until 90% mortality. The point of 10% mortality was used as an additional way of monitoring the schedule of mortality in certain populations to assess the timing of the onset of the senescent period. Sample means were compared using the Kruskal Wallis test or ANOVA, depending on whether the data conformed to the assumptions of parametric analysis.

Chapter 3. The Effects of Reproduction on Longevity and Fertility in Male *Drosophila melanogaster*

(a version of this chapter has been accepted for publication in the *Journal of Insect Physiology*; Prowse, N. and Partridge, L., 1997)

3.1. Summary

The effect of reproduction on subsequent survival and fertility of male *Drosophila melanogaster* was examined by reversing the reproductive status of individuals part-way through life. Reproduction had a much more marked effect on fertility than survival: males with a history of reproduction showed complete sterility at a time when upwards of 80% of their cohort were still alive. Analyses of survival rates alone gave a misleading measure of the impact of ageing. Sterility appeared to be attributable mainly to a reduction in sperm count. Early reproduction caused permanent, irreversible damage to both survival and fecundity, with risk playing an apparently minor role. Individual differences in frailty appeared to be of little consequence for the interpretation of these reversal experiments, although its possible occurrence made definite detection of risk difficult.

3.2. Introduction

A cost of reproduction for survival can take two forms that can be detected in experiments where the reproductive status of individuals is switched part way through life (Partridge and Andrews, 1985; Partridge and Barton, 1994). Reproductive activity may simply increase the current risk of death or reduce current fecundity; if the risk is survived, future survival probability and fecundity are unaffected. These risks can be endogenous, for example there may be an increased risk of heart attack or metabolic failure while reproductive activity is occurring; or ecological, whereby an individual may be open to an elevated chance of predation when searching for or courting a potential mate. Alternatively, reproduction may cause irreversible

damage that affects long term survival probability or future fecundity. This could occur if resources are diverted from repair to reproduction (Kirkwood and Holliday, 1979; Abrams and Ludwig, 1995), or if reproduction causes damage that cannot be repaired. Both patterns of reproductive cost have been reported (Partridge and Andrews, 1985; Partridge *et al.*, 1986; Tatar *et al.*, 1993). Irreversible damage as a consequence of reproduction increases the rate of ageing because it causes a deterioration of state with age.

The physiological mechanisms determining the links between present reproductive rate, survival probability and subsequent fecundity in *Drosophila melanogaster* are not well understood, particularly in males. Reproduction in male *D. melanogaster* has been shown to be costly for survival (Partridge and Farquhar, 1981; Partridge and Andrews, 1985; Luckinbill *et al.*, 1988b; Service, 1989), apparently as a result of short term risk (Partridge and Andrews, 1985) and mainly because of a cost of courtship (Cordts and Partridge, 1996). It is not known if reproduction in males can affect subsequent fertility, or under what circumstances reproductive costs can cause accelerated ageing. It is important that survival and fecundity are examined together because ageing is a reduction in the potential for future progeny production and, as such, is affected by both traits. Therefore, the appropriate measure of the extent to which ageing has occurred is the residual reproductive value, or RRV (Partridge and Barton, 1996). This is the index of an organism's potential to produce offspring after a certain point in its lifetime. Using this index, it would be possible to assess the effect of reproduction on the schedule of senescence, as described by future progeny production. In this study the reproductive rate of male *D. melanogaster* was manipulated and tested for effects on both fecundity and survival. Conclusions derived from death rates alone were compared with those gained from the measurement of survival and fecundity. The physiological basis of what turned out to be a substantial cost of reproduction for subsequent fecundity was also investigated.

Individuals may differ in their susceptibility to ageing. In a heterogeneous population it will be the weakest individuals that die first, so those individuals that reach later ages will be a non-random, less frail subset of their original cohort (Vaupel and Yashin, 1983; Carey *et al.*, 1992; Curtsinger *et al.*, 1992; Kowald and Kirkwood, 1993; Vaupel and Carey, 1993; Brooks *et al.*, 1994). Similarly, those individuals that are more fecund at later ages may

be a non-random subset of their cohort. Interpretation of experiments involving a reversal of sexual activity that are used to discriminate between risk and long term damage could, therefore, be confounded by individual variation in frailty. Where early reproductive rate is experimentally increased, death rates are generally higher. The survivors of such treatment are therefore likely to be, on average, less frail than the survivors of lower rates of reproduction where death rates have also been lower. If the subsequent performance of these groups of survivors is compared under standard conditions, the role of risk could be over-estimated. A history of higher rates of reproduction would elevate subsequent death rate if permanent damage resulted, but the effect could be masked if the survivors were also a less frail subset of their cohort with better prospects for survival or fecundity. In this study potential indices of individual frailty such as body size, development time and culture of origin were used to examine whether individual variation in frailty is likely to confound the results of this type of experiment.

3.3. Methods and Materials

3.3.1. General Methods

Parents of the experimental flies were obtained from yeasted bottles of Lewis medium placed in Dahomey population cages for a few hours. To rear males under standard conditions, adults emerging from these bottles were placed approximately 50 mating pairs to a laying pot and allowed to acclimatise for 24 hours. Eggs were then collected for three hours on the grape juice medium in the lid. Twenty four hours later first instar larvae were collected and placed at a density of 200 larvae into shell vials. High density rearing conditions were used to increase the variation in body size within cultures, based upon the idea that scramble competition between larvae accentuates the importance of individual variation in feeding rate (Bakker, 1961), so that whereas some larvae eat quickly and become large, slower larvae do not eat as much because of the limited food supply. Emerging males were collected as virgins. Bottle cultures similar to those used to rear the parents of the

experimental males were used to generate virgin females. All experimental flies were collected over ice.

3.3.2. The Effects of the Removal of Females on Male Survival

The aim was to investigate the effect of early reproduction on the survival of subsequently celibate males, and the role of individual variation in shaping the results. Virgin males were allocated randomly to one of four experimental groups. 'Never' exposed males (n = 60) were kept in single sex groups of six throughout the experiment. Dead males were replaced with mutant *sparkling poliert* males to maintain constant density. 'Always' exposed males (n = 50) were kept singly in vials and supplied with five virgin females every two days when males were transferred into fresh food vials. All transfers were carried out using carbon dioxide anaesthesia. '7 day' males (n = 40) were exposed to females until day 7, and '29 day' males (n = 50) until day 29, after which these groups were kept as 'never' males. Day 7 was chosen for the initial removal because survival rates did not differ by then and variation in frailty should have had little effect, whereas by day 29 a statistically significant divergence in survival between the 'never' and 'always' groups had appeared, allowing variation in frailty to have a potential role. Each vial was checked once a day for deaths. The 'vial of origin' and the thorax length of each male were used as possible indices of individual frailty. Longevity and lifetime mating success of adult males have been shown to be correlated with body size (Ewing, 1961; Partridge and Farquhar, 1983; Santos *et al.*, 1992) which is in turn affected by, amongst other things, culture of origin. Six vials of origin were chosen to supply an equal number of males to each treatment group. Thorax length was measured to the nearest 0.02 mm using an eyepiece graticule on a binocular microscope.

3.3.3. The Effects of Addition of Females on Male Survival and Fecundity (High Reproductive Rate)

The aims were to investigate the effects of the addition of females on subsequent male survival and fecundity, with increased variation in frailty between individual males. Average body size declines and the variation between individuals increases as the emergence of a cohort from a particular vial or bottle culture progresses (Partridge and Farquhar, 1983). To exploit this, first instar larvae were put into vials on two consecutive days, and the latest emerging flies from the first day's vials and the earliest emerging flies from vials set up on the second day were collected, so that flies from extremes of the emergence range were collected during the same 36 hour period. This was done to further increase variation in frailty between individual males without introducing a three or four day age-range. Males from six culture vials, three in each of the two 'emergence groups' ('early' and 'late'), were allocated equally to each of three treatments (all n = 78). Two treatments, 'never' and 'always', were identical to those in the first experiment. Males in the third treatment, the 'addition' treatment, were kept in single sex groups until day 19, the first day that the 'never' and 'always' groups differed significantly in survival, and were then given females in the same way as the 'always' males.

The methods were otherwise as for the first experiment except that from day 19 fecundity of the 'always' and 'addition' males was examined. Five males from each vial of origin were chosen at random and the females that they had mated with were put individually into vials of yeasted Lewis medium containing charcoal to facilitate egg counts. These females were put on fresh food each day for four days, and the eggs that they laid in each vial were counted. The vials were then incubated until progeny emerged, which were counted. This procedure was repeated six times at two day intervals after day 19, when the 'addition' males were first given females. The measurement was not continued because the 'always' males proved to be sterile.

3.3.4. The Effects of Addition of Females on Male Survival and Fecundity (Low Reproductive Rate)

In view of the large cost to subsequent fecundity of males found in the previous experiment, the extent of costs when males reproduced at a lower rate was investigated. All treatments were of 86 male flies, reared in the same way as in the previous experiment. Each mated male was kept with just two virgin females every two days. Males without females were therefore kept three to a vial. Females were supplied to the 'addition' males for the first time on day 34, again a choice based on survival differences, and fecundity samples were taken every two days following day 34. After the fourth sample, fecundity assays were discontinued due to 'always' male sterility.

3.3.5. The Effects of Mating on Sperm Number and Accessory Gland Volume

The effects of mating on sperm number and accessory gland volume in males were investigated in order to examine the physiological basis of the fecundity costs observed in the previous experiments. Males and virgin females were reared as in the first experiment. There were four treatments, corresponding to those used in previous experiments: 'never', 'always', 'removal' and 'addition'. The removal and addition of females was on day 28, the day that a significant difference in survival between the 'always' and 'never' groups first appeared. Males were kept with four virgin females when mated, with four other males when celibate. Females were replaced with virgins once every two days. Measurements of sperm number and accessory gland dimensions were taken on the days between replacement by destructive sampling of virgin and continuously mated flies throughout the experiment, and of 'removal' and 'addition' groups starting shortly before and then for 11 days after day 28. Each treatment had between 140 and 180 males at the beginning of the experiment, and six or eight males per group were taken for each sample.

Prior to dissection, males were put on ice for a short time, decapitated and their thorax length measured. The internal sex organs were dissected out

in a drop of phosphate buffered saline. The seminal vesicles containing mature sperm were cut free and transferred to a fresh drop of saline. Without severing the duct between it and the ejaculatory duct, each gland was teased out and laid straight, but not stretched, using a fine needle. The length and the width at one, two and three quarters of the way along each gland were measured using an eyepiece graticule. The seminal vesicles were then ruptured and the sperm inside allowed to dissociate. The preparations were left for ten minutes and then allowed to dry. The time allowed for dissociation and drying ensured that the sperm were well spread out. The slides were then fixed with 20 µl 3:1 methanol:acetic acid solution and stained with 3.2 µl/ml 4,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) in Vectashield mounting medium (Vector Laboratories Inc.), which protects DAPI from degradation by light. Each slide was then covered with a coverslip. DAPI is a DNA-specific stain that is visible in fluorescent light. It was therefore possible to count sperm heads with a compound microscope connected to a fluorimeter. A video camera and monitor were also attached to the microscope in order to make counting easier. On slides where the sperm were not well spread out, and where the density of sperm was high, estimates were taken by counting half of the screen and then doubling the number. Prior to the experiment, six males were dissected as described and their sperm counted several times to ensure repeatable results. The correlations between the first and second and the first and third counts were significant in both cases ($r = 0.985$, $p = 0.003$ and $r = 0.972$, $p = 0.029$ respectively).

Fecundity was sampled in the 'always' and 'addition' treatments by taking the females that had mated with experimental males and keeping them for two days in one vial before they were transferred to a fresh vial for a further two days. Both sets of vials were kept. This was first done on day 30 and females mating with males on days 32, 34 and 36 were treated in the same way. Progeny were counted when they emerged from the incubated vials. Fecundity was also sampled once in the 'removal' males ten days after they had been isolated from females.

3.3.6. Statistical Analysis

Survivorship data and estimates of Gompertz parameters were analysed as described in section 2.4.2. The following statistical tests were also carried out on the data, with multiple comparisons tested against Bonferroni corrected significance levels where necessary. Thorax length and survival were examined for variation between different vials of origin, emergence group and reproductive treatment using either two-way nested ANOVA or the Kruskal Wallis test, depending on whether the data conformed to the assumptions of parametric analysis. On the same basis, for tests of correlations between variables Product-moment correlation coefficients or Spearmans Rho coefficients were calculated. All tests on age-specific fecundity were carried out using the Kruskal Wallis test, tested at the 5% significance level.

The total subsequent progeny production of a male after a particular age was used as an index of residual lifetime reproductive success, the "residual reproductive value" or RRV. Distributions of this index for males from the 'always' and 'addition' treatments were compared using the Wilcoxon rank sum test. For these comparisons, RRV was calculated from the day when males in the 'addition' treatment of that particular experiment started to mate.

The length (l) and width (w) data from the accessory gland measurements were used to calculate their volume using the equation $\pi(0.5w)^2l$, which assumes that the accessory gland is cylindrical (Pitnick and Markow, 1994). Average accessory gland volume was obtained from the two values obtained from each male, and sperm number was treated in the same way. This meant that in cases where there was just one value per fly, for whatever reason, the single measurement could be substituted for the mean. The data were log transformed so that they were normally distributed. Linear regression and ANOVA were used to analyse differences in sperm number and accessory gland volume between groups over time.

3.4. Results

3.4.1. The Effects of the Removal of Females on Male Survival

The survival curves for treatment groups in this experiment are shown in Figure 3.1. Five separate comparisons were made between survival curves, and the 5% significance level was corrected for each comparison using the sequential Bonferroni method. Virgin males were significantly longer lived than continuously mated males ($X^2 = 37.75$, df = 1, $p < 0.0001$), and '7 day' males did not differ significantly in subsequent death rate from the virgins ($X^2 = 0.48$, $p = 0.49$). After day 29, '29 day' males were not shorter lived than the '7 day' males ($X^2 = 5.50$, $p = 0.019$; this result was only marginally non significant because it was tested at a Bonferroni corrected significance level of 0.017). Following the removal of females on day 7, '7 day' males were significantly longer lived than 'always' males ($X^2 = 37.75$, $p < 0.0001$), but '29 day' males were not longer lived than the continuously mated group after day 29 ($X^2 = 0.64$, $p = 0.42$).

The age-dependent rate of decline in survival, G, was compared between treatments (see Table 3.1a for estimates of G and A). An F test was first used to show a significant difference in the estimates of the rate of age-dependent change in mortality rate across treatments ($F = 30.51$, df = 3 and 252, $p < 0.0001$), and then to compare pairs of groups. All comparisons were highly significant after sequential Bonferroni correction. The value of G for the virgin males was significantly higher than all the other treatments, which when ranked in descending order were '7 day', 'always' and '29 day'.

Survival and thorax length data in this experiment were not normally distributed, so they were analysed non parametrically. There were no significant differences in survival ($X^2 = 4.01$, df = 4, $p = 0.41$, $\alpha = 0.05$) between males of differing vial of origin. Mean thorax length did not vary significantly either ($X^2 = 10.86$, df = 4, $p = 0.028$, $\alpha = 0.025$), although this result was marginal. Average thorax length did not vary significantly between treatments, either before ($X^2 = 1.62$, df = 3, $p = 0.65$), or after the removal of females from the '29 day' group ($X^2 = 2.10$, df = 3, $p = 0.55$). Two treatment groups showed a marginally significant positive correlation between body size and survival time;

the '7 day' (Spearmans Rho coefficient, r_s= 0.38, p = 0.05) and '29 day' (r_s= 0.34, p = 0.047) groups.

3.4.2. The Effects of Addition of Females on Male Survival and Fecundity (High Reproductive Rate)

In this experiment, 'never' males lived longer than continuously mated males (X^2 = 56.44, df = 1, p < 0.0001; see Figure 3.2). Following addition of females on day 19, the survival time of 'addition' males was significantly less than that of the 'never' males (X^2 = 19.37, p < 0.0001), and significantly higher than that of the 'always' males (X^2 = 18.88, p < 0.0001).

Estimates of the Gompertz parameters for these treatments are shown in Table 3.1b. The estimated values of G for the 'never' and 'addition' groups were not significantly different (F = 3.86, df = 1 and 73, p = 0.053) and both were significantly higher than the estimate for the 'always' group ('never' versus 'always', F = 31.74, df = 1 and 91, p < 0.0001; 'addition' versus 'always', F = 47.65, df = 1 and 73, p < 0.0001).

Survival times did not differ between males from different emergence groups or vials of origin (X^2 = 0.0023, df = 1, p = 0.962 and X^2 = 5.43, df = 5, p = 0.37 respectively). This data was analysed by Kruskal Wallis test because it did not conform to the assumptions of ANOVA. There was, however, significant variation in thorax length between males from different vials of origin (X^2 = 15.62, df = 5, p = 0.008), and males in the 'early' emergence group were significantly larger than those in the 'late' emergence group (X^2 = 11.17, df = 1, p = 0.0008). There were no significant correlations between life span and thorax length in any treatment, nor any significant differences in thorax length between treatments after addition of females on day 19 (X^2 = 3.27, df = 2, p = 0.20).

When fecundity was first examined on day 19, the 'always' treatment were nearly sterile, as a result of poor egg hatchability rather than low female egg-production. This cost to male fecundity was greater than the cost to male survival, since 'always' males showed about 80% survivorship when fecundity measurements commenced on day 19 (Figure 3.2). Fecundity of the 'addition' males was monitored until day 32 and declined significantly with each

consecutive fertility sample until day 25, by which time the males were almost sterile (Figure 3.3). The 'early' emerging group showed higher progeny hatchability than the 'late' group ($X^2 = 4.11$, df = 1, $p = 0.04$) following the first mating period on day 19, but not subsequently.

The mean RRV of the 'always' males following day 19 was 54.22, significantly lower than that of the 'addition' males, which was 1194.4 ($X^2 = 15.14$, df = 1, $p < 0.0001$). The previous reproduction of the 'always' males therefore induced a higher degree of ageing (Partridge and Barton, 1996). There were no significant associations between RRV and vial of origin, thorax length or emergence group among the 'addition' males.

3.4.3. The Effects of Addition of Females on Male Survival and Fecundity (Low Reproductive Rate)

Survival in the 'always' and 'never' treatments did not differ significantly in this experiment ($X^2 = 0.97$, df = 1, $p = 0.62$; see Figure 3.4) that involved a lower rate of reproduction for males than in previous experiments. On the commencement of mating, the 'addition' males did not differ significantly in subsequent survival to the 'never' or 'always' males ($X^2 = 0.21$, $p = 0.65$ and $X^2 = 0.04$, $p = 0.85$ respectively). The high p-values show that there was very little difference between these treatments indeed. Rates of the increase in age-dependent mortality, shown in Table 3.1c, did differ between groups, however. 'Never' males declined at a faster rate than the 'addition' males ($F = 46.14$, df = 1 and 117, $p < 0.0001$) and 'always' males ($F = 102.48$, df = 1 and 152, $p < 0.0001$). 'Addition' males also declined at a faster rate than the 'always' males ($F = 20.78$, df = 1 and 117, $p < 0.0001$).

'Late' emerging flies were significantly longer-lived than 'early' emerging flies in this experiment (see ANOVA details in Table 3.2), possibly because the food that they were reared on was fresher. Survival did not vary significantly with vial of origin (Table 3.2), but thorax length did (Table 3.3). Thorax length did not show any consistent differences between emergence groups. There were also no correlations between thorax length and death time in any treatment.

'Always' exposed males were sterile when first examined on day 34 . In contrast, 'addition' males had high fecundity at the same time because they had only just started to mate (Figure 3.5). At this point, 70% to 80% of the 'always' males were still alive (Figure 3.4). Figure 3.5 shows that after six days of mating, hatchability declined in the 'addition' group, so that eggs laid on day 40 were significantly less likely to hatch than eggs from previous matings. The 'late' emergence group showed significantly higher progeny hatchability after the second mating (day 36; $X^2 = 14.24$, df = 1, p = 0.0002), but there were no other such differences between emergence groups. The mean RRV of the 'always' males was 6.07 compared to 1051.3 for the 'addition' males, a highly significant difference ($X^2 = 33.97$, df = 1, $p < 0.0001$). Reproduction had therefore caused accelerated ageing in the absence of any effect on death rate. There were no significant associations of thorax length or emergence group with RRV in the 'always' or 'addition' groups.

3.4.4. The Effects of Mating on Sperm Number and Accessory Gland Volume

Both sets of data, sperm number and accessory gland volume, had to be log transformed to conform to the necessary assumptions of parametric analysis. Linear regression showed that 'always' males had significantly lower sperm number (F = 222.46, df = 1, $p < 0.001$) and accessory gland volume (F = 53.57, df = 1, p = 0.000) than 'never' males after just one day of mating, because the intercept of the regression equation for the 'never' group was significantly higher in both cases (see Figures 3.6 and 3.7). Sperm number in both groups increased significantly over time, since the slope of the regression equation was significantly greater than zero (F = 41.75, df = 1, $p < 0.001$), and increased at a slightly faster rate in the 'always' group because the interaction term was also significant (F = 5.44, df = 1, p = 0.02). Accessory gland volume increased significantly over time (F = 63.52, df = 1, $p < 0.001$) at a comparable rate in the two groups (interaction term, F = 1.3, df = 1, p = 0.26).

When 'removal' males were isolated from females on day 28, they did not differ in sperm number or accessory gland volume to the 'always' treatment in the following 11 days, when compared using ANOVA (F = 0.46, df = 1, p =

0.5 and F = 0.02, df = 1, p = 0.89 respectively, see Figures 3.8 and 3.9). When the fecundity of these males was tested on day 37, they were completely sterile. The addition of females on day 28 had a dramatic effect on the sperm number and accessory gland volume of 'addition' males (see Figures 3.8 and 3.9). By day 29, accessory gland volume had fallen dramatically, and the drop was significant at the 5% level using the Tukey Kramer HSD test to compare the means of the samples from the 'addition' and 'never' groups on that day. Accessory gland volume did not recover to pre-mating levels before day 39. Sperm number fell more gradually, with significant differences between the pre- and post-mating levels of days 27 and days 29 and also between days 29 and 31. In the same way as accessory gland volume, sperm number did not recover before day 39.

'Always' males were completely infertile when fecundity sampling started on day 30. Fecundity of the 'addition' males showed a significant drop from day 30 to 33, and again from day 33 to 35, by which time the males were virtually sterile (see Figure 3.10). Sterility of the 'addition' treatment, therefore, did not occur until after sperm number had dropped on day 34.

'Never' males showed a weak but significant positive correlation between body size and sperm number ($r = 0.25$, $p = 0.042$). A similar value was found for accessory gland volume ($r = 0.22$, $p = 0.045$). There was no correlation between thorax length and age of sampling ($r = 0.02$, $p = 0.85$), showing that these results were not an artefact of sampling larger flies later in the experiment.

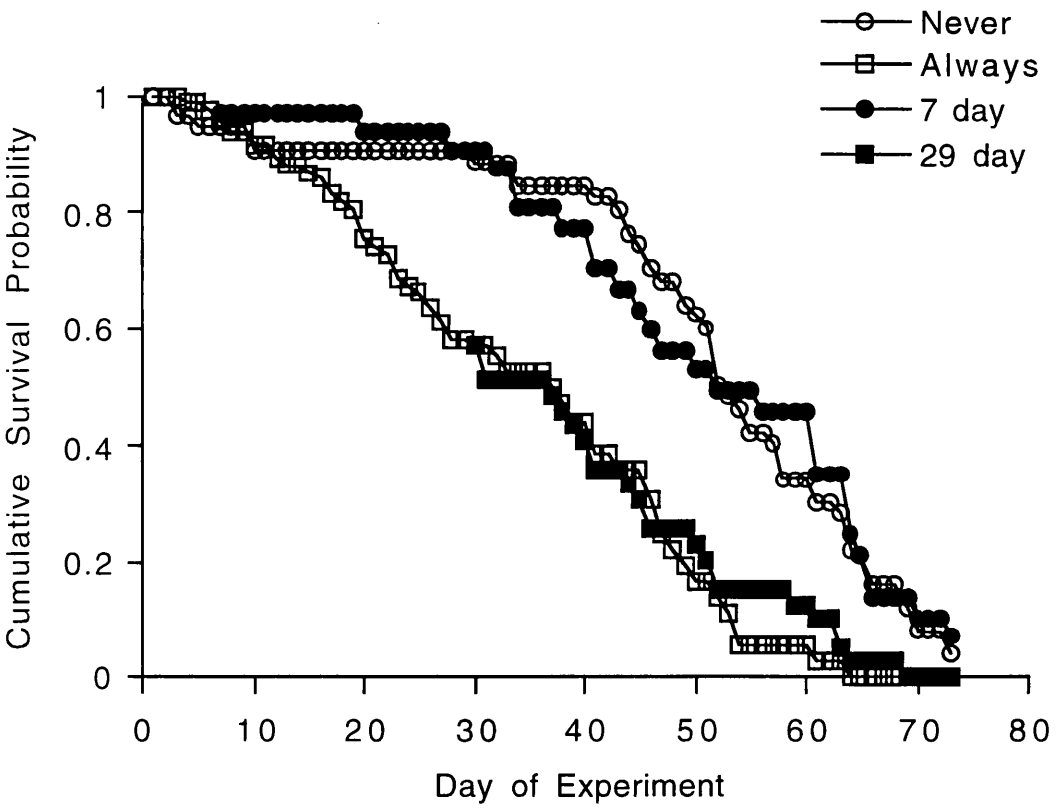

Figure 3.1. Cumulative survival probability against time for males in the removal experiment.

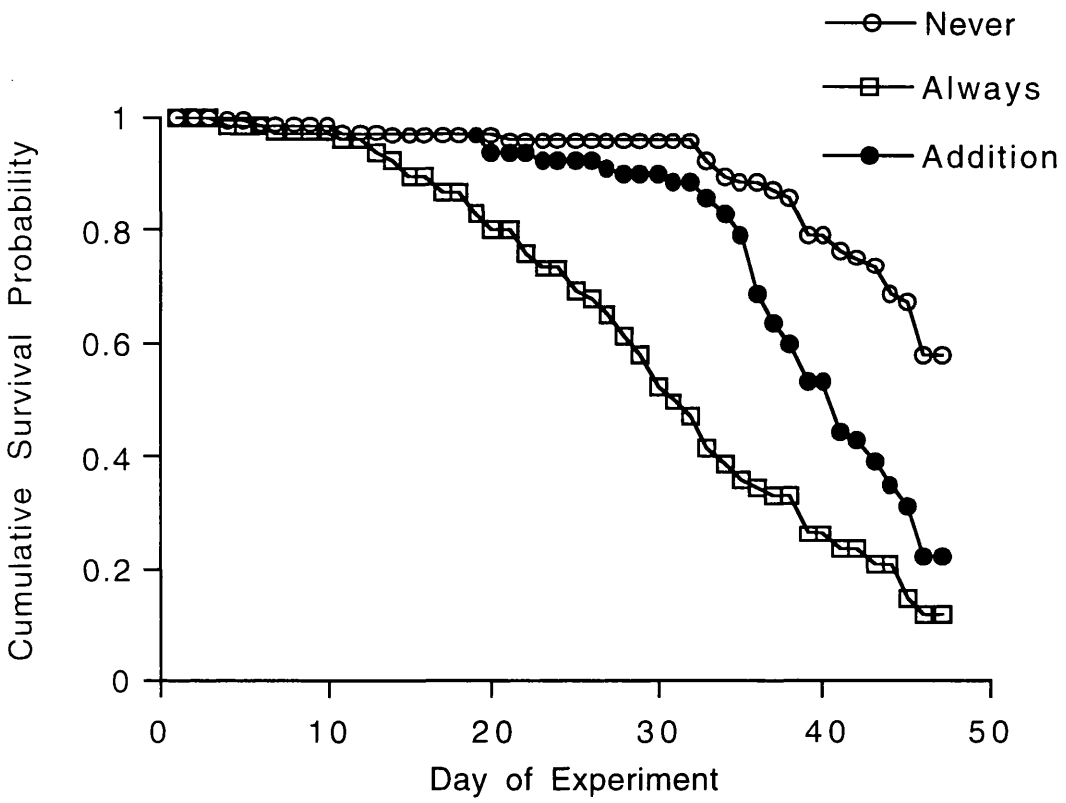

Figure 3.2. Cumulative survival probability for males in the first addition experiment (high reproductive rate).

Treatment	Age-Dependent Parameter, G	Age-Independent Parameter, A
Never	0.075	0.0009
Always	0.053	0.007
7 day	0.064	0.001
29 day	0.04	0.009

Table 3.1a. Removal experiment.

Never	0.129	0.0002
Addition	0.146	0.0003
Always	0.082	0.004

Table 3.1b. Addition experiment (high reproductive rate).

Never	0.098	0.0003
Addition	0.073	0.0009
Always	0.062	0.002

Table 3.1c. Addition experiment (low reproductive rate).

Table 3.1. Age-independent and age-dependent Gompertz estimates for treatments in the removal experiment (3.1a), the first addition experiment (high reproductive rate; 3.1b) and the second addition experiment (low reproductive rate; 3.1c). For details of the model and statistical analysis, see text.

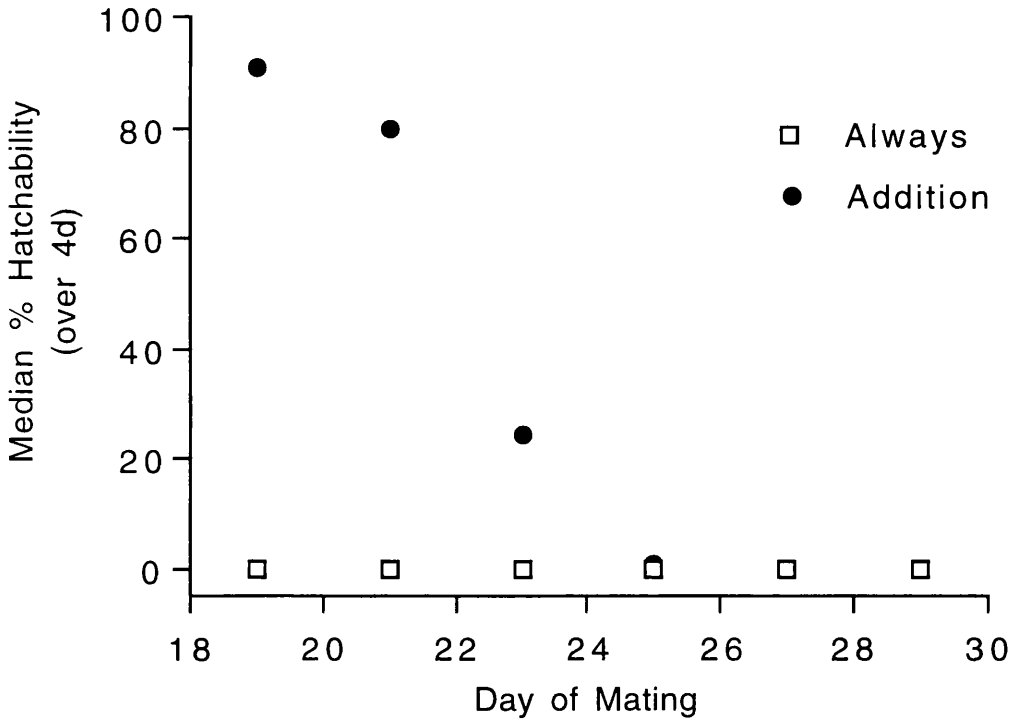

Figure 3.3. Median % hatchability of progeny of mated males in the first addition experiment (high reproductive rate). Each point refers to eggs laid over four days immediately following matings on the day indicated.

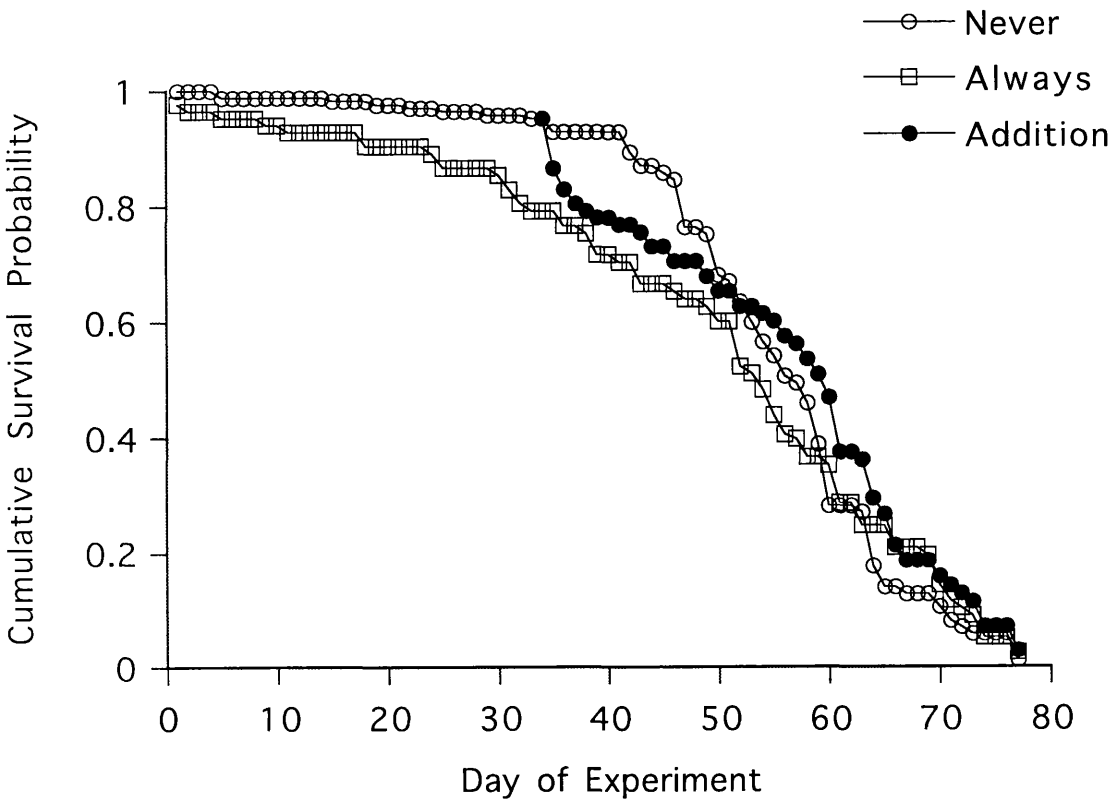

Figure 3.4. Cumulative survival probability for the second addition experiment (low reproductive rate).

Source	SS	MS	DF	F ratio	p value
Treatment	1535.306	767.653	2	3.082	<0.05
Emergence	2880.106	2880.106	1	85.41	<0.01
Vial	134.884	33.721	4	0.135	>0.05
Treatment*Emergence	764.753	382.376	2	1.804	>0.05
Treatment*Vial	1695.48	211.93	8	0.851	>0.05
Error	53793.79	249.045	216		

Table 3.2. Two-way nested ANOVA testing the effect of treatment group, vial of origin (both fixed effects) and relative emergence time (a random effect) upon survival in the second removal (low reproductive rate) experiment. SS = sums of squares, MS = mean squares, DF = degrees of freedom.

Source	SS	MS	DF	F ratio	p value
Treatment	3.341	1.671	2	2.682	>0.05
Emergence	13.785	13.785	1	5.23	>0.05
Vial	10.542	2.636	4	4.23	<0.01
Treatment*Emergence	3.948	1.974	2	3.095	>0.05
Treatment*Vial	5.102	0.6378	8	1.024	>0.05
Error	134.545	0.623	216		

Table 3.3. Two-way nested ANOVA testing the effect of treatment group, vial of origin (both fixed effects) and relative emergence time (a random effect) upon thorax length in the second removal (low reproductive rate) experiment.

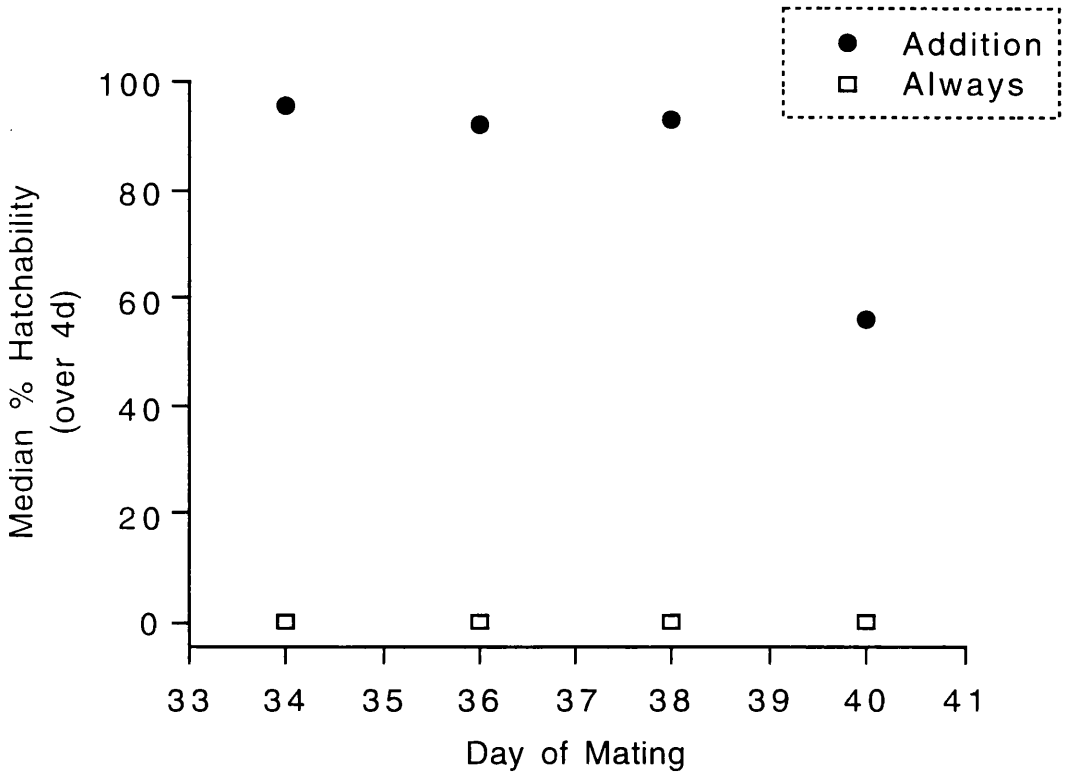

Figure 3.5. Median % hatchability of progeny of 'always' and 'addition' males in the second addition experiment (low reproductive rate). Each point refers to eggs laid over four days immediately following matings on the day indicated.

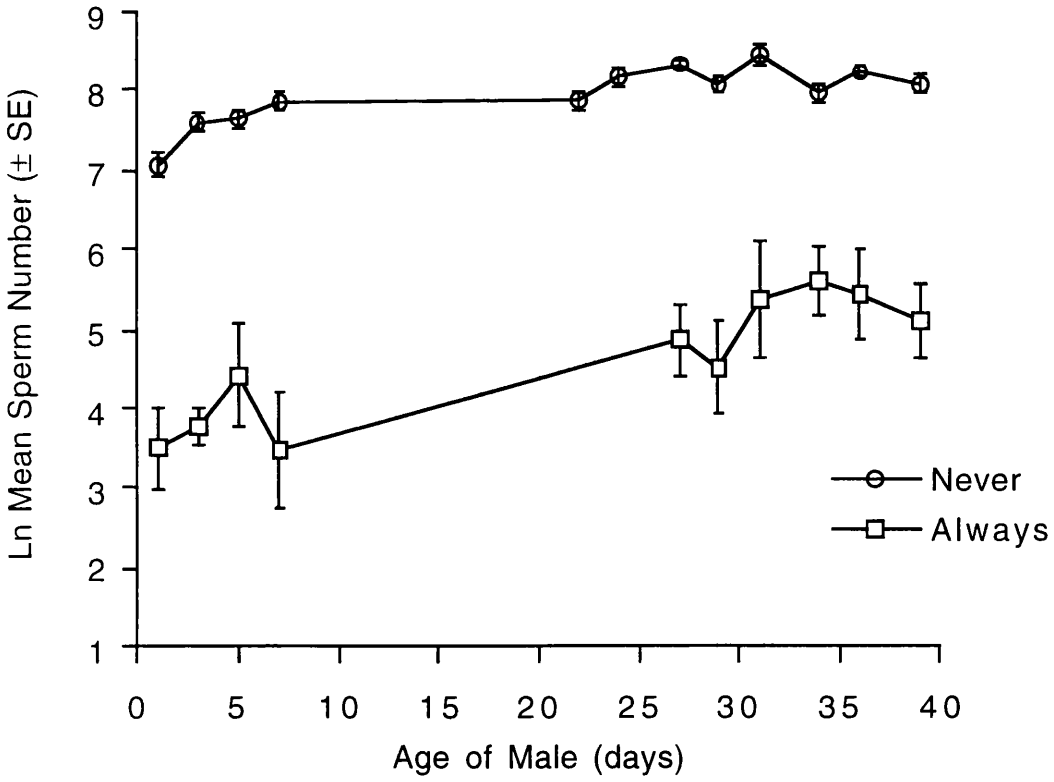

Figure 3.6. Mean ln sperm number (± standard errors) of males from the 'always' and 'never' treatments against time (days). The 'always' males started to mate on day 0.

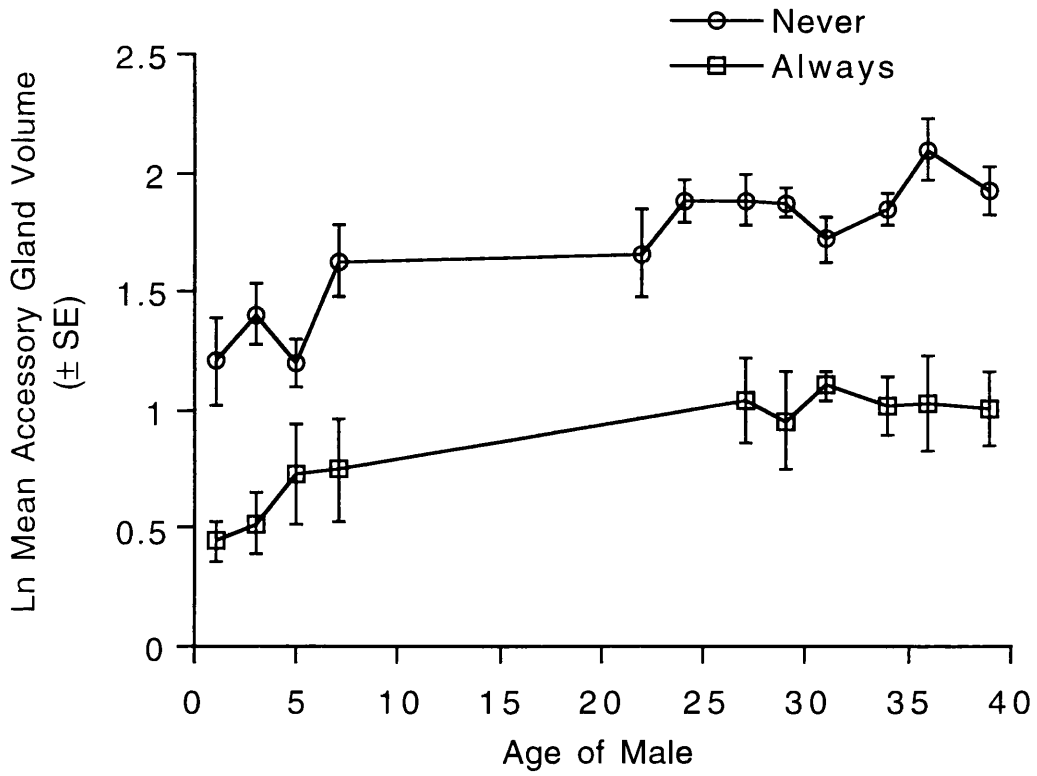

Figure 3.7. Mean ln accessory gland volume (± SE) of males from the 'always' and 'never' treatments against time (days). The 'always' males started to mate on day 0.

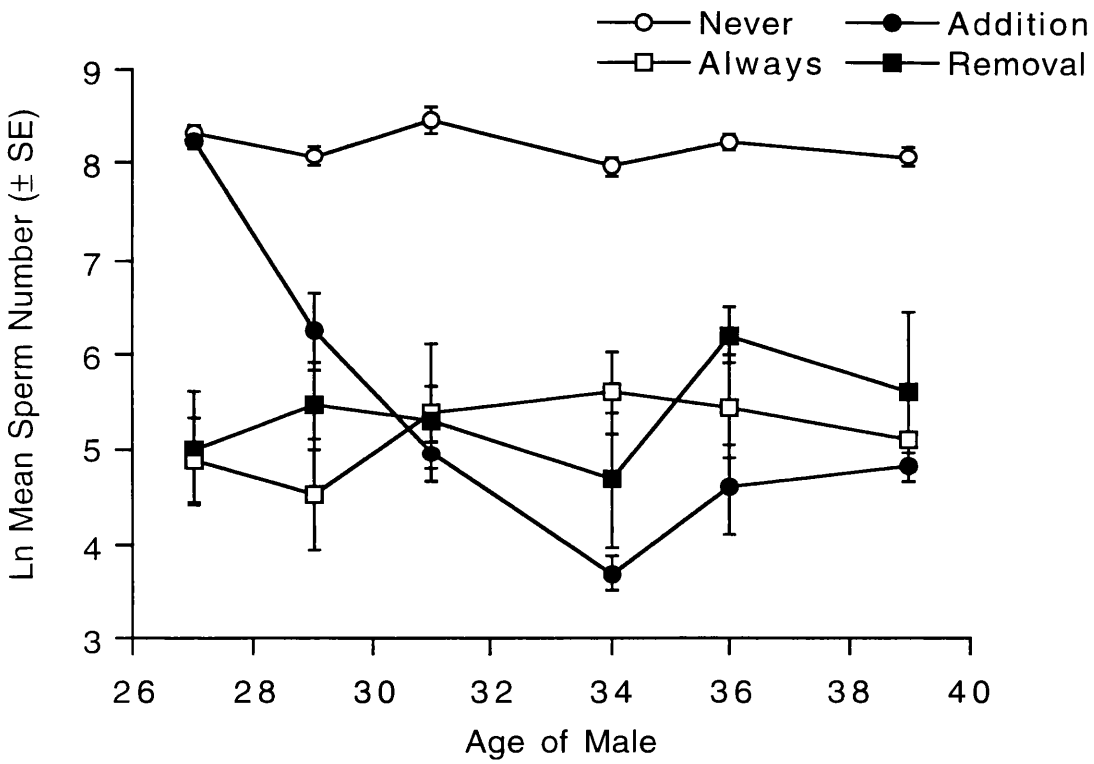

Figure 3.8. Mean ln sperm number (± SE) against time (days) of males from all treatments between days 27 and 39. The switch in mating status occurred on day 28.

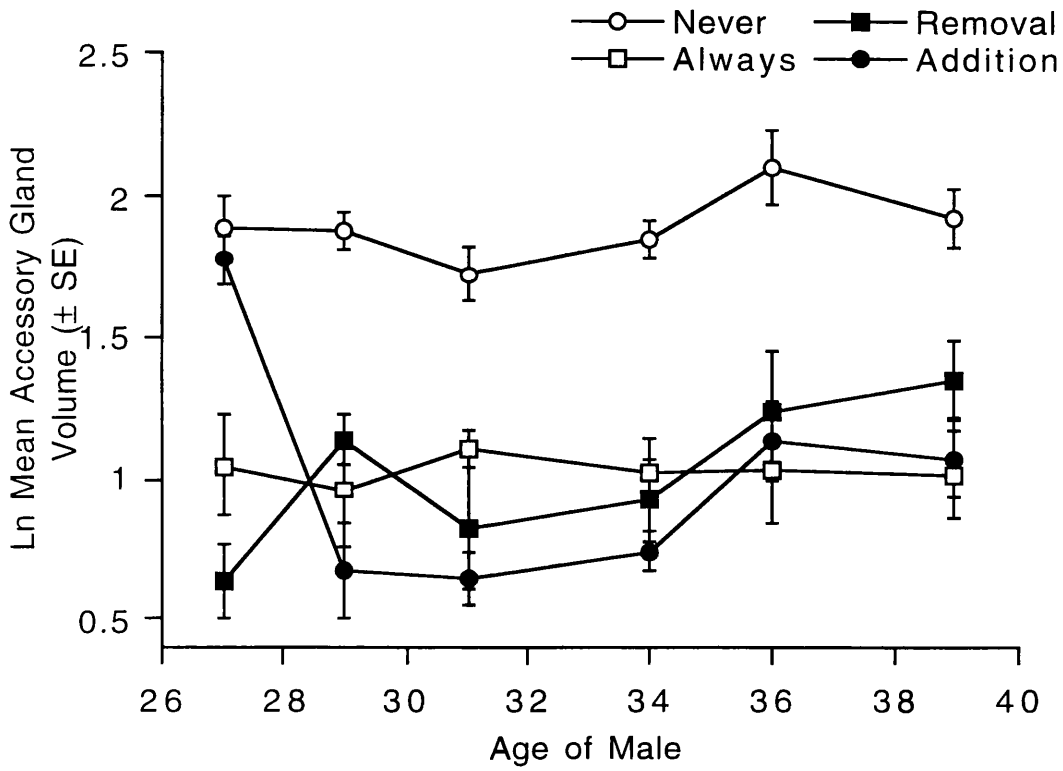

Figure 3.9. Mean ln accessory gland volume (± SE) against time (days) of males from all treatments between days 27 and 39. The switch in mating status occurred on day 28.

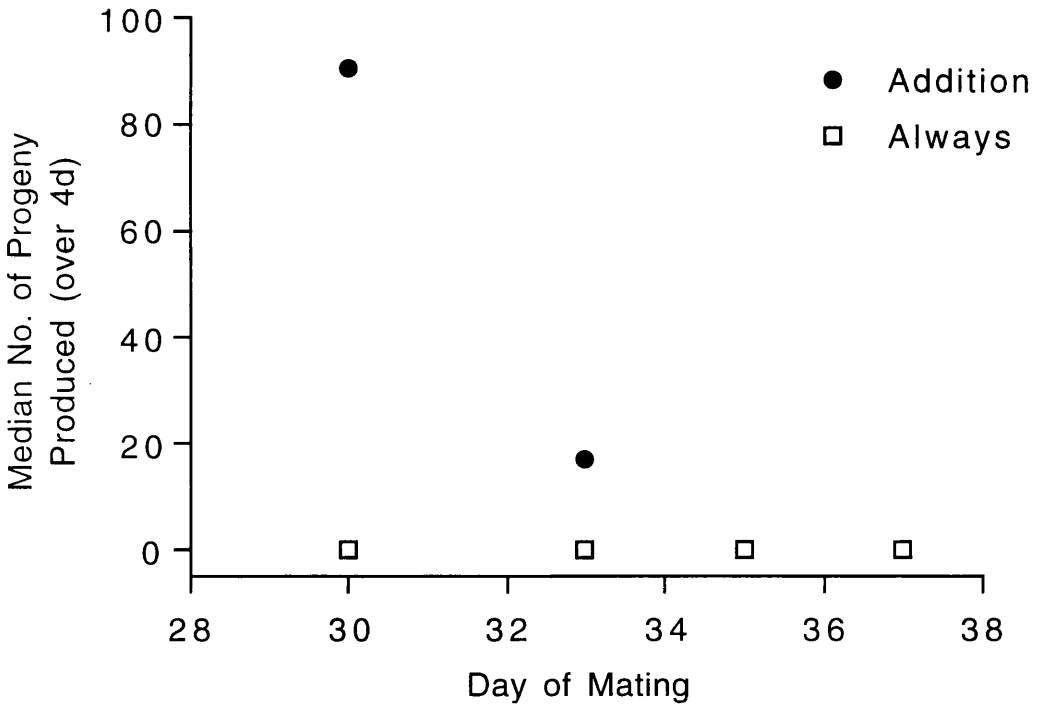

Figure 3.10. Median progeny production of males in the 'addition' and 'always' treatments of the sperm/accessory gland experiment. Each point refers to eggs laid over four days immediately following matings on the day indicated.

3.5. Conclusions

- Male *D.melanogaster* were found to experience a reproductive cost to fertility that was much greater than the corresponding cost to survival in the same males. This conclusion is based upon two findings: first, at a relatively low rate of mating a fertility cost was found when survival cost was not; and second, when the rate of mating was high enough to induce a survival cost, the cost to fertility occurred before survival was affected, because at the time the majority of males were still alive.
- Use of the Gompertz age-dependent parameter gave a misleading measure of senescence. RRV provided a much accurate description of ageing in these experiments.
- Reproduction caused an irreversible decline in both survival and fertility. Infertility as a result of exhaustive sexual activity was partly the result of a reduction in sperm count, although other factors were also involved.
- The effect of individual heterogeneity on the outcome of these experiments was difficult to assess because the indices of frailty used did not reflect the variation that had been introduced between individuals during development.

3.6. Discussion

3.6.1. Costs to Fecundity and Survival

In both addition experiments, reproductive costs to fecundity far outweighed those to survival in the same males, since 'always' males were already sterile when 'addition' males were still fertile, and between 70% and 90% of 'always' males were still alive. Indeed, no significant survival cost was incurred when males were exposed to one female per day, yet the fecundity cost in the same males was marked. Sterility made a much more important contribution to ageing, defined as the decline in residual reproductive value, than did death. Use of the Gompertz analysis alone would have suggested that the 'never' groups aged significantly more rapidly than the 'always' groups in all experiments, whereas the RRV data from both addition experiments showed

that the opposite was the case, since 'addition' males had residual fecundity that was entirely lacking in the 'always' males. Similarly, in the first addition experiment (high reproductive rate), Gompertz analysis would have suggested that the 'addition' group aged faster than the 'always' group, whereas the 'always' group were already sterile. Experiments that assess only death rates or the change in age-specific mortality rate as a measure of the rate of senescence can therefore miss the most important part of the process. In light of the magnitude of the permanent damage to fecundity, the overall decline in survival probability as a result of reproductive activity had relatively little impact on senescence. One interesting point is that the age-independent mortality rate estimates in 'always' treatments were an order of magnitude higher than those in 'never' treatments for all three experiments. This suggests that the differences in life span between mating and non-mating cohorts were caused by age-independent mortality rather than by differences in the rate of increase in age-specific mortality rates, which do not explain the decreased survival of mated males. This is also suggested by the shape of the survival curves for 'always' and 'never' groups, especially in the removal experiment (Figure 3.1) in which it appears that reproduction brought the onset of the decline in cohort survival forward by about 20 days.

The results also showed that reproduction can cause permanent damage to both survival and fecundity in male *D. melanogaster.* In the removal experiment, a short mating bout did not affect subsequent survival. In contrast, the survival of the '29 day' group did not recover after mating, remained significantly lower than that of 'never' males and did not diverge significantly from that of the 'always' males when a prospective comparison was made after day 29. Similarly in the first addition experiment (high reproductive rate), prospective comparison from the day that females were added showed that although survival probability of the 'addition' group was higher than the 'always' group, it remained significantly lower than the 'never' group. Partridge and Andrews (1985) found that males mated until day 16 showed a complete recovery of survival rate, whereas we found no such recovery in males that were mated until day 29. The length of the mating opportunity and the number of females supplied may both have affected the difference in the outcome of these experiments. In addition, there may have been more heterogeneity

between males in Partridge and Andrews' experiments, causing the survivors of reproduction to be of above average robustness.

3.6.2. Physiological Basis of Reproductive Costs.

The decline in the fecundity of 'addition' males was more closely paralleled by the decline in sperm number than of accessory gland volume. This is surprising, because Lefevre and Jonsson (1962) showed that serially mated *D. melanogaster* males that became sterile had empty accessory glands, whereas sperm, although reduced, were still present in the testes. We showed that fecundity declined only after sperm number had started to fall. One difference between our study and the previous one is that we did not mate our males to exhaustion in a short period. Hihara (1981) also mated males to exhaustion in a short time and attributed a decline in female oviposition to a loss of accessory gland fluid. It is interesting that sperm number and accessory gland volume did not decline in 'always' males with advancing age following their initial mating, and that the 'always' males in fact showed an increase in these traits over time. The result suggests that the decline in fertility in the 'always' males, and also in the other mated treatments, was caused by a parallel decline in something other than these two traits, perhaps the ability to replenish accessory gland fluid or sperm after mating. Alternatively, there may be a certain level that sperm has to recover to, following mating, in order for a male to remain fertile. In this instance, even though sperm number in 'always' males did increase over time, it was obviously not enough to restore fertility.

It would be interesting to know if sterility is a more important contributor to the decline in reproductive value than is the decline in survival rate, and any role of reproductive costs in causing ageing, under natural conditions. Two studies (Jones *et al.*, 1987; van Vianen and Bijlsma, 1993) have suggested that this species may mate as often as once per day in nature, so the results from the second addition experiment (low reproductive rate) may be the most relevant to nature. This also suggests that, just as fertility was a more important component of fitness in these experiments, this may be the case in the wild, since such low rates of reproduction in the wild may not be enough to affect male survival. Both environmental variables such as nutrition, which could

affect frailty, and the level of external hazard to survival will be different in nature, so data on the reproductive potential of field caught adults could be very informative.

Less clear is the role of manufacture of sperm or accessory fluid in elevating male death rate. Previous work has suggested that the main cost of reproduction for survival in male *D. melanogaster* is a cost of courtship (Cordts & Partridge, 1996). Some of the findings in the present study are consistent with this view. Although mated males rapidly became sterile, they were observed to mate, as in a previous study where males that were very likely sterile mated frequently for several weeks (Partridge and Farquhar, 1981). Courtship costs could account for the continuing cost of reproduction in males that could not produce sperm or accessory fluid. However, removal of females in the first experiment had no detectable effect on subsequent survival of males, despite the cost of courtship being removed. Of course, the lack of change in death rate could have meant that the maximum level of damage to survival had already been incurred. Just as 28 days exposure to receptive females was too long for fertility to be able to recover in the final experiment, so 29 days was too long for survival rate to recover in the removal experiment.

3.6.3. The Effect of Cohort Heterogeneity

There was little evidence for significant effects of individual heterogeneity in the experimental results. For instance, in the removal experiment, thorax length correlated significantly with life span in two experimental groups but there was no evidence that this source of variation significantly affected the outcome of the reversal experiment, because when the removals were made there were no even marginally significant differences in thorax length between the members of different experimental groups. In the first addition experiment (high reproductive rate), none of the indices of frailty showed any association with life span or residual reproductive value. In the second (low reproductive rate), although emergence group affected life span, there was no difference in the composition of the experimental groups for this variable by the time of reversal, and neither emergence group nor any of the

other indices were significantly associated with individual variation in residual reproductive value.

It is possible that individuals differed in frailty, but that the indices used to try and detect it were not appropriate. In addition, the power to detect any contribution for a measured index would have depended upon sample size. There is always a danger that the effects of cohort heterogeneity will be underestimated in this type of experiment. Nonetheless, since the findings showed permanent damage to '29 day' males despite a previous lower death rate in 'never' males, which should therefore have been, if anything, more frail, it can confidently be asserted that permanent damage to survival had occurred. Similarly, median life span of 'addition' males in the second experiment (high reproductive rate) was higher than that of 'always' males, which is evidence that the 'always' males had incurred permanent damage to survival as well as fertility. The effect of instantaneous risk is more difficult to infer with certainty because of the potentially confounding effects of individual variation. One indication that it played a role in shaping survival could be that in the third experiment (low reproductive rate), the survival probability of the 'addition' group appeared lower than that of the 'never' group immediately after addition, which would indicate a role for instant risk associated with reproduction. However, larger samples would have been needed to confirm that there was an immediate effect. '7 day' males did not show a decline in survival probability compared to virgins, which implies that the effect of reproduction was to reduce future survival prospects. Alternatively, it is possible that young flies are hardy enough to withstand the level of current risk to survival that mating involves, but that males mating for just seven days later on in life would have shown an effect. Indeed, this pattern is suggested by the sudden drop in survival of 'addition' males in the low reproductive rate addition experiment. Pulses of altered reproductive status, rather than long duration of exposure, may be a more effective method for detecting a role of risk.

Chapter 4. Responses to Selection on Age at Reproduction: Male Mortality and Competitive Reproductive Success

4.1 Summary

Populations selected for early ('young') or late ('old') life reproduction were set up prior to the start of this study by Partridge and co-workers. These lines were established with the aim of examining correlated responses to selection while avoiding covert selection for rapid pre-adult development and uncontrolled larval rearing densities. All comparisons were made with reference to the base stock. The responses to selection of male mortality and competitive reproductive success were examined. Male age-specific mortality rate diverged significantly between 'old' and 'young' regimes in the period of life when selection pressure differed between the two regimes. The subsequent rate of increase of male age-dependent mortality rate did not differ between lines, indicating that this period of the male life span did not show a correlated response to selection. 'Old' line male competitive reproductive success declined relative to the 'young' line and base stock males early in life. There was no significant divergence in male fertility later in life.

4.2. Introduction

Evolutionary theories of senescence provide a number of predictions that may be tested empirically. For example, Edney and Gill (1968) predicted that the artificial shortening of life should remove selection from the later part of the life history, resulting in the evolution of earlier senescence. Work with *Tribolium casteneum* provided support for this conjecture (Sokal, 1970; Mertz, 1975) and produced evidence for both the antagonistic pleiotropy and mutation accumulation theories of ageing. More recently, a number of experiments have provided a more stringent test of the pleiotropy theory by showing that selection for late life fitness results in a decline in some aspect of early life fitness (Williams, 1957; Partridge and Fowler, 1992). Artificial selection on age at

reproduction involves the propagation of populations using eggs laid by females at specific ages. Thus, genes conveying improved survival probability will be favoured when females are allowed to grow relatively old before contributing eggs. Control populations, propagated by eggs from younger females, can be used for comparison to assess how selection affects the life history. Using this method, a direct response in terms of extended life span has been successfully obtained in populations of *Drosophila melanogaster* (Rose and Charlesworth, 1981; Luckinbill *et al.*, 1984; Rose, 1984; Partridge and Fowler, 1992) and other species; *D. subobscura* (Wattiaux, 1968), *Acanthoscelides obtectus* (Tucic *et al.*, 1996) and *Bactrocera cucurbitae* (Miyatake, 1997). Selection upon life span has also produced a divergence in late life fertility between long and short lived populations (Rose and Charlesworth, 1981; Luckinbill *et al.*, 1984; Rose, 1984; Partridge and Fowler, 1992; Roper *et al.*, 1993; Tucic *et al.*, 1996; Miyatake, 1997). Late life fertility and longevity are not necessarily correlated, as shown by Zwaan *et al.* (1995b), who successfully selected on longevity without applying pressure on late fertility to increase and did not see a correlated change in the trait.

There are certain life history characters that have not shown consistent patterns of responses among independent selection experiments. A common correlated response to selection, a decline in early life fertility (Wattiaux, 1968; Rose and Charlesworth, 1981; Luckinbill *et al.*, 1984; Rose, 1984; Tucic *et al.*, 1996; Miyatake, 1997), has provided support for antagonistic pleiotropy by showing that Williams' prediction was correct. However, Partridge and Fowler (1992) did not obtain a decline in early fertility when selection was applied using a similar regime to previous studies. Patterns of pre-adult development time have been consistent in different populations selected for age at reproduction, since it was greater in both the Rose 'O' lines, selected for increased late life fitness, and the Partridge 'old' lines, also selected for fitness later in life, compared to their respective controls, the 'B' and 'young' lines (Partridge and Fowler, 1992; Roper *et al.*, 1993; Chippendale *et al.*, 1994). In contrast, pre-adult viability did not show agreement between populations; the 'O' lines were more viable as larvae than their corresponding 'B' lines (Chippendale *et al.*, 1994), but the Partridge and Fowler 'old' lines had lower pre-adult viability than their short lived controls (Partridge and Fowler, 1992; Roper *et al.*, 1993). In addition, the Partridge 'old' lines were larger than the 'young' lines (Partridge and

Fowler, 1992), as one might expect considering the longer development of 'old' regime flies, since size and development are positively correlated (Robertson, 1963; Partridge and Fowler, 1993; Roper *et al.*, 1996). In contrast, 'O' line females were lighter than 'B' line females (Rose *et al.*, 1984), which is contrary to expectations. These regimes should have shown very similar responses had selection been applied in an identical manner in the Rose and Partridge laboratories, but there are many conflicting factors that may have generated the lack of agreement between their respective results. Additionally, it is likely that certain factors were not controlled in each selection regime and that consequently, some of the responses seen were not the result of selection on life span. It is important to understand the basis of these problems so that the mechanisms of life span extension can be studied without confounding influences.

To study ageing using artificial selection the conditions must be properly standardised so that observed responses can confidently be regarded as real effects of the selection regime itself. Two problems need to be addressed. First, larval density needs to be controlled during selection and in experiments designed to measure correlated responses. The proportion of larvae in a culture that reach adulthood is governed in part by culture density, which affects the level of competition for food and the length of development. There is a minimum critical weight that a larva must attain in order to be able to pupariate, and as the number of larvae in a given amount of food medium increases, the fraction of larvae that are able to eat enough food to reach it declines (Bakker, 1961). It is in crowded cultures that variation in feeding rate and the efficiency with which a larva can turn food into biomass become important. This is because when food is limiting, a slight advantage in terms of faster feeding rate or efficiency can mean the difference between reaching the critical weight or not. Competition also reduces adult body size because the larvae that do pupariate do so at lower weights (Bakker, 1961). The length of time taken to reach adulthood also increases under such conditions.

Selection to increase life span that has been carried out at very low larval densities has failed in the past (Lints and Hoste, 1974; Luckinbill and Clare, 1985; Service *et al.*, 1988), so culture density needs to be high enough to get a response. The important point is not the exact density used during selection, but that it is standardised. Larval density has been allowed to vary considerably

within and between generations in some selection studies (Luckinbill *et al.*, 1984; Partridge and Fowler, 1992), although one group who have not controlled larval density claim that it has not varied "systematically" during more than 10 years of selection (Leroi *et al.*, 1994). It has been a problem for others, however, because Luckinbill *et al.* (1984) reported considerable variation in development time in their 'early' lines, selected for early life fitness, because of increased larval density due to the high fecundity of 'early' line females.

Systematic differences in culture density between selection treatments can lead to patterns that are not the result of selection on life span. Uncrowded rearing conditions allow the evolution of larger adults by an increase in development time (Roper *et al.*, 1996). In lines selected for large body size, development time was longer but larval viability was also lower than for controls (Partridge and Fowler, 1993). This shows that extended development has a fitness cost associated with it in terms of reduced pre-adult viability. In *Drosophila* populations selected in either high or low larval density environments, the low density flies took longer to develop and became larger adults than the high density flies, suggesting that at high density the fitness cost of extended development is greater than the advantage of being large (Roper *et al.*, 1996). This evidence provides an alternative explanation for the longer development (Partridge and Fowler, 1992; Roper *et al.*, 1993) and lower larval viability (Partridge and Fowler, 1992) of the 'old' lines, because 'old' line larval cultures were less crowded than the 'young' line cultures (Partridge and Fowler, 1992). This is in contrast to the original interpretation of those results, which was that selection for increased longevity caused 'old' flies to develop for longer to make a fitter adult (Partridge and Fowler, 1992). Whilst these two mechanisms could both explain the evolution of fitter adults, one is the result of the 'old' lines having benefited from lower rearing densities and the other is the result of selection on life span. Environmental factors such as density must therefore be standardised in future experiments to resolve this problem because the selection environment has a great effect on the expression of life history characters (see also Luckinbill and Clare, 1985) and on genetic correlations (Service and Rose, 1985).

The second problem with previous studies that needs to be addressed is that of inadvertent selection on pre-adult characters during selection experiments concerning adult traits. This is a potential problem because

selection on the pre-adult life history can also affect the evolution of adult characters. For example, populations selected for extended larval development time showed elevated fecundity early in life (Zwaan *et al.*, 1995a). In both the Rose and Partridge regimes selected for divergent age at reproduction adults that had not eclosed by a particular time were not used (Rose, 1984; Partridge and Fowler, 1992; Roper *et al.*, 1993; Chippendale *et al.*, 1994; Leroi *et al.*, 1994), which resulted in selection for rapid development. Such pressure was especially strong on 'B' and 'young' lines because of the requirement for them not only to have eclosed by a certain time but also to reproduce soon afterwards. In this respect females were placed under more intense selection pressure than males because of the need for them to feed as adults to become sexually mature, whereas males have mature sperm on eclosion (Roper *et al.*, 1993). Selection was intensified further in the Partridge lines because cultures of 'young' line larvae were especially dense due to the precocious fecundity of 'young' line females (Partridge and Fowler, 1992). This could easily explain why 'young' and 'B' line flies developed faster than their respective 'old' and 'O' lines (Partridge and Fowler, 1992; Roper *et al.*, 1993; Chippendale *et al.*, 1994) and also why 'old' line flies were larger than 'young' line flies (Partridge and Fowler, 1992), since development time and body size or weight are known to be well correlated (Robertson, 1963; Bierbaum *et al.*, 1989; Partridge and Fowler, 1993; Nunney, 1996; Roper *et al.*, 1996). At least, inadvertent selection on development time could be partly responsible for these results. Covert selection on development time has a knock-on effect for many aspects of the adult life history. The length of development affects adult body size, which is correlated with adult fitness components such as survival (Partridge and Farquhar, 1981, and 1983; Santos *et al.*, 1992), female fertility (Robertson, 1957; Tantawy and Vetukhiv, 1960; Santos *et al.*, 1992) and male reproductive success (Ewing, 1961; Partridge *et al.*, 1987a and c; Santos *et al.*, 1992). This illustrates why it is important to design artificial selection experiments carefully because selection can affect more than just the character in question.

It is clear that the effects of larval density and inadvertent selection on developmental rate must be considered when attempting to examine the true responses to selection on late life fitness. Measurements should also be made with reference to the original base stock, so that the direction of responses to selection can be determined (Roper *et al.*, 1993). The factors in consideration

here are especially important in the design of new experiments since one set of studies (Partridge and Fowler, 1992; Roper *et al.*, 1993) stands out from the others because its results did not fit the commonly observed pattern of early fecundity being traded off against late life fitness. It is possible that the response of late fertility observed in many other cases is a consequence of the way that selection on age at reproduction operates. In long lived populations, the individuals that contribute the most progeny to each generation are likely to be the most fecund, so genes for high fecundity are selected along with those for longevity. Alternatively, this pattern may represent a cost of reproduction in the short lived lines, expressed in terms of a decline in future fertility, that is the result of selection to reproduce early on in life. There are also major contradictions between the results from different laboratories concerning the evolution of pre-adult characters under such regimes. With these factors taken into consideration, it is necessary to re-examine the effects of selection upon life span while avoiding the problems that have been discussed.

Two regimes were established with the aim of selecting for late or early life fitness in *Drosophila melanogaster.* Pre-adult and adult densities were standardised and efforts were made to avoid covert selection on development by allowing flies to eclose and reach reproductive maturity with no time constraints. In addition, measurements were made with reference to the random bred Dahomey stock from which the selection lines were derived and which had been maintained under laboratory conditions for over 20 years. Responses to selection were measured in the selection environment whenever possible, since gene by environment interactions can seriously affect results (see Leroi *et al.*, 1994). Linda Partridge and co-workers established the selection regimes and maintained them for 18 months before I took over for a further two years while measuring direct and correlated responses to selection. This chapter reports the result of selection on age at reproduction in terms of mortality and reproductive success in males.

One does not expect males to respond to selection in exactly the same way as females because of the difference between male and female reproductive biology. For example, the intensity of selection on life span is not as strong for males as it is for females. For a female to contribute progeny to the next generation, she has to be alive on the day that eggs are collected. In contrast, a male could mate, die, and still sire progeny from eggs that are laid a

week later, because females can store viable sperm for a number of days. Selection on male life span is therefore not as intensive and, consequently, responses to selection should not occur as quickly in males. Males have, however, responded to selection on life span in previous experiments (Luckinbill *et al.*, 1984; Rose, 1984; Partridge and Fowler, 1992), and patterns of male competitive reproductive success have resembled those of female fecundity in the same lines. Where early female fecundity diverged, so did male competitive success (Service, 1993), but in a regime where early female fecundity did not diverge, neither did that of the males (Roper *et al.*, 1993). Mating physiology has shown some interesting patterns when examined in the Rose lines. 'B' males induced faster rates of female oviposition, and therefore increased progeny production, than 'O' males early in life, although the trend reversed later on (Service and Vossbrink, 1996). This matches the pattern of male competitive reproductive success shown by Service (1993) in the same lines. Early experiments involving selection on life span concentrated on females to study the evolution of fecundity under selection. The profound differences in reproductive biology between the sexes means that males should be studied in their own right.

4.3. Methods and Materials

4.3.1. Establishment and Maintenance of the New 'Old' and 'Young' Selection Regimes.

The Dahomey stock was used to generate flies for each selection regime. Bottles of Lewis medium were placed into a Dahomey population cage and removed when a sprinkling of eggs had been laid. These cultures were incubated at 25°C and the adults used to produce eggs on grape juice medium in laying pots. The first instar larvae were collected and placed in groups of 50 into culture vials. The resulting adults were the first generation of the selection lines.

Both selection regimes, 'old' and 'young', had five replicate lines that were treated in the same way. Adults from each line were kept in a population cage with a water source and a petri dish containing red grape juice medium with a

generous amount of yeast paste on top. The food was replaced every two days and for the 'old' lines the water was changed once a week. To establish the next generation, flies were allowed to lay eggs on fresh grape medium with yeast paste for three ('young' lines) or six ('old' lines) hours. In the 'young' lines this was carried out on the sixth or seventh day of adult life, whereas in the 'old' lines, eggs were collected as late as it was possible to collect the necessary amount of eggs in six hours, usually three to four weeks. Twenty four hours from the start of the laying period, first instar larvae were transferred into culture vials in groups of 50 on a mounted needle. The vials were then incubated at 25°C. For the 'young' lines, 15 vials per line were set up, with 25 vials each for the 'old' lines. In order to avoid covert selection for increased developmental rate, adult flies were not put into cages until every one had eclosed. The average egg to adult viability of the 'young' lines was estimated by counting the number of flies in two vials from each line. Approximately 250 flies of each sex were put into the cage for each line, the number of vials used was based on the viability estimate. For each 'old' line, 450 flies of each sex were counted and put into cages. The 'old' lines were maintained with greater precision because differences between replicate cages could be crucial late in life when eggs were collected. Survivorship differences in the 'young' lines were not so important because very few 'young' line flies would die prior to egg collection.

Once during the adult lifetime of the 'young' lines, and twice for the 'old' lines, each line was 'backed up' to ensure that losses in the main populations were not ruinous. Eggs were collected in the normal manner, the grape juice medium cut into pieces and placed into culture bottles, four per line. These cultures were incubated at 18°C and could be used whenever there were problems with the main stocks.

The generation of selection during which each of the following experiments was carried out is given for each regime. During selection some 'old' lines showed reduced adult viability. In order to try and keep selection on life span synchronous across lines, rather than take eggs from each line at a different time, the decision was taken to put some replicate 'old' lines through a short generation. This resulted in some 'old' lines going through more generations than others and so in some of the later experiments, the 'old' lines are described as being from a range of generations. This procedure was not deemed to be a problem because the difference in the number of generations of

selection that the 'olds' had been through compared to the 'youngs' was much greater that this small difference within the 'old' regime.

Care was taken to make sure that the same replicate of the Dahomey base stock was used for reference in each experiment. This was to avoid problems arising from slight genetic and environmental differences that may exist between Dahomey population cages.

4.3.2. Male Mortality

To measure male life span in the selection environment, eggs were collected from each 'young' (in generation 35) and 'old' (in generation 18) population cage, at a time when adults of both selection regimes were approximately the same age. Eggs were also collected from a Dahomey base stock cage. In all cases, the grape medium was cut up, placed into yeasted culture bottles and incubated. The emerging adults were allowed to lay eggs on grape juice medium in laying pot lids, from which first instar larvae were collected and placed in groups of 50 into culture vials. These standard density cultures produced the flies used in the experiment.

To monitor male mortality, 12 population cages were used, one for each selection line replicate and the base stock replicates. Males and females from each line were placed into a cage, 250 of each sex. The cages were fed and maintained in the same way as the normal selection lines. Every two days when the food was replaced, the cages were inspected for male corpses which were removed and counted. Females were replaced with younger ones four times during the experiment, and each time fewer females than before were used in order to keep their numbers approximately equal to the males. The density of flies in the cages therefore declined, as was the case in the selection regimes.

4.3.3. Male Competitive Reproductive Success

The aim of this experiment was to measure male reproductive success in a situation that mimicked the competition between males in the selection environment. Eggs were collected from 'old' and 'young' selection line cages

when the 'old' lines were in their 23rd generation and the 'youngs' were in their 44th. At the same time, eggs were also taken from a Dahomey base stock cage and from a cage of *scarlet* eyed mutants with a Dahomey genetic background. Sections of grape medium on which the eggs had been laid were placed into culture bottles at low density and incubated. Emerging adults from each replicate were put into a population cage to provide eggs for the experiment. All experimental, wild type and *scarlet* males were reared at a standard density of 50 larvae per vial. Bottle cultures were also used to provide females for the experiment.

Thirteen cages were established, one for each selection line replicate and three base stock replicates. Into each 250 *scarlet* virgin females, 175 *scarlet* males and 75 'old', 'young' or base stock males were placed. Male competitive reproductive ability was measured in comparison with that of the *scarlet* eyed males by periodically sampling fertility throughout the experiment. Egg samples were taken from the cages on days 2, 6, 9, 13, 16 and 24 of the experiment by placing a petri dish of fresh grape medium into each cages for five to six hours. The eggs were then incubated at low density in bottle cultures and the number and phenotype of emerging adults recorded.

Male mortality was monitored and taken into account in the calculation of competitive reproductive success. Differential mortality between wild type and mutant males would otherwise have biased the relative proportions of offspring, favouring the phenotype with the lowest mortality rate. The *scarlet* virgin females were replaced on day 15 of the experiment.

4.3.4. Statistical Analysis

Three different methods were used to analyse male mortality with reference to the base stock. It was desirable to examine the period of life span under selection in detail, something that a comparison of survivorship across the entire life span would not do. The age of final reproduction, day 7 for the 'young' lines and around day 26 for the 'old' lines, represents the time when selection pressure was effectively removed from each respective regime. Any deleterious age-specific effects would not be selected against if expressed after this time, and differences in survivorship following it are effectively correlated responses to

selection on the earlier period of the life span. Age-specific mortality rates were therefore calculated for two important periods in the life span; the first week of life, when both 'young' and 'old' line flies were alive in the selection environment, and the period between days 8 to 28, when just the 'old' line flies were alive. This method is described in section 2.4.2. The Kruskal Wallis test was used to analyse mean mortality rates for the base stock and selection regime replicates.

In addition to the examination of mortality rates during the period of life that was directly under selection, the rate of increase of age-dependent mortality was estimated for the period following the point of 10% mortality. This is the start of the 'senescent' period (Arking, 1987), and it finishes at the point of 90% mortality, after which sample sizes are too small to consider statistical comparison. These rates of mortality increase were estimated using the Gompertz survival model (Finch, 1990), which is described in detail in section 2.4.2. These rates were estimated for each replicate line and tested for heterogeneity within each selection regime and the base stock, which were then tested for between-regime variation using one-way ANOVA.

Entry of each replicate line into the senescent period of the life history was examined by comparing the time when each line reached the point of 10% mortality. By doing this it was possible to build up a clear picture of the schedule of mortality in each selection regime in conjunction with the estimates of age-specific mortality and the age-dependent Gompertz parameter. Regime means were compared by the Kruskal Wallis test.

The following formula was used to calculate an index of male competitive reproductive success that took into account the effects of selection regime and *scarlet* male mortality.

$$\text{Index} = \frac{(\text{wild type progeny} * \textit{scarlet}\ \text{males alive})}{(\textit{scarlet}\ \text{progeny} * \text{wild type males alive})}$$

This cross-product ratio was used as an index of wild type male competitive reproductive success for each replicate line, and meant that the actual competitive mating ability of males in each replicate line could be assessed without the relative male mortality in each cage introducing a bias in favour of the phenotype with the lowest mortality rate. The issue of interest was

whether selection on age at reproduction had produced a divergence in age-specific competitive reproductive success between 'old' and 'young' males with reference to the base stock. The indices of competitive success, calculated for each selection line and base stock replicate for the six sampling intervals, were combined into one of two blocks in order to analyse responses to selection in the early and later stages of the experiment. These divisions were intended to reflect the times when each selection regime was required to reproduce in order to contribute progeny to the next generation; therefore, samples taken on days 2 and 6, and those taken on days 9, 13, 16 and 24 were each grouped. Both blocks were then tested for variation between 'old', 'young' and base stock males using a one-way ANOVA. In cases where a significance difference was established, differences between selection regimes were further examined using the Tukey Kramer HSD.

4.4. Results

4.4.1. Male Mortality

Cumulative survival probability of 'young' and 'old' males is shown in Figures 4.1 and 4.2 respectively, each with the same two base stock replicates for reference. 'Young', base stock and 'old' regime males had median life spans of 37.7 days, 42.7 days and 44.5 days respectively.

There were no significant differences in age-specific mortality rate between groups in the first week of life ($X^2 = 2.84$, df = 2, p = 0.24). However, between days 8 and 28 there was a marginally non-significant difference between them ($X^2 = 5.25$, df = 2, p = 0.07) which warranted further investigation. The Kruskal Wallis multiple comparisons test showed that male mortality rate was significantly lower in the 'old' lines than in the 'young' lines (tested at the 5% level), but that neither group differed significantly from the base stock. The rates of age-specific mortality for each line and regime are shown in Table 4.1.

The overall chi-square that test for a difference between points of 10% mortality showed a marginally non-significant difference between groups ($X^2 = 5.58$, df = 2, p = 0.06). Multiple comparisons then showed that 'young' males,

which reached the point of 10% mortality after 28.8 days (± 95% confidence limits = 5.10), reached it significantly earlier than 'old' lines, which reached it after 34.4 days (± 3.60). Comparisons with the base stock (10% mortality after 33 days ± 8.60) were non-significant for both the 'olds' and 'youngs', a pattern which matches that of male mortality rates between days 8 and 28 (see above). The point of 10% mortality is given for each replicate line in Table 4.2, which also shows the regime and base stock means.

The age-dependent and age-independent Gompertz parameters were estimated for replicate lines within each selection regime and for the base stock replicates in the manner described in section 2.4.2. The estimates for each replicate line and selection regime, and for the base stock, are shown in Table 4.3. The base stock and the 'old' and 'young' regimes were each tested separately, by way of an F test, for heterogeneity between replicate lines for the age-dependent parameter, G. The 'old' and 'young' regimes both showed significant heterogeneity between lines ($F = 7.64$, $df = 116$ and 120, $p < 0.001$; $F = 4.82$, $df = 146$ and 150, $p = 0.001$, respectively). The two base stock replicates did not differ significantly in their rate of age-dependent decline in mortality ($F = 1.90$, $df = 72$ and 73, $p = 0.17$). A one-way ANOVA was then used to test the hypothesis that there was significant variation between either selection regime and base stock. The result of this was highly non-significant ($F = 0.31$, $df = 2$, $p = 0.74$). Therefore, the Gompertz parameter, G, varied significantly within selection regimes, but not between them or the base stock.

4.4.2. Male Competitive Reproductive Success

The mean indices of competitive reproductive success for males in each line and regime are shown in Table 4.4. The egg samples taken on days 2 and 6 of the experiment, used to calculate the index of male reproductive success described in section 4.3.4, were shown to vary significantly between selection regimes ($F = 6.96$, $df = 2$, $p = 0.004$). The Tukey Kramer HSD showed that 'old' line males had significantly lower reproductive success than both 'young' line and base stock males (tested at the 5% significance level), whereas the base stock and 'young' line males did not differ significantly. The second block, comprised of samples taken on days 9, 13, 16 and 24, also showed a significant divergence between the three groups ($F = 3.68$, $p = 0.03$). On closer scrutiny, the selection regimes were not found to differ significantly from each other, but 'young' regime males had significantly lower reproductive success than the base stock.

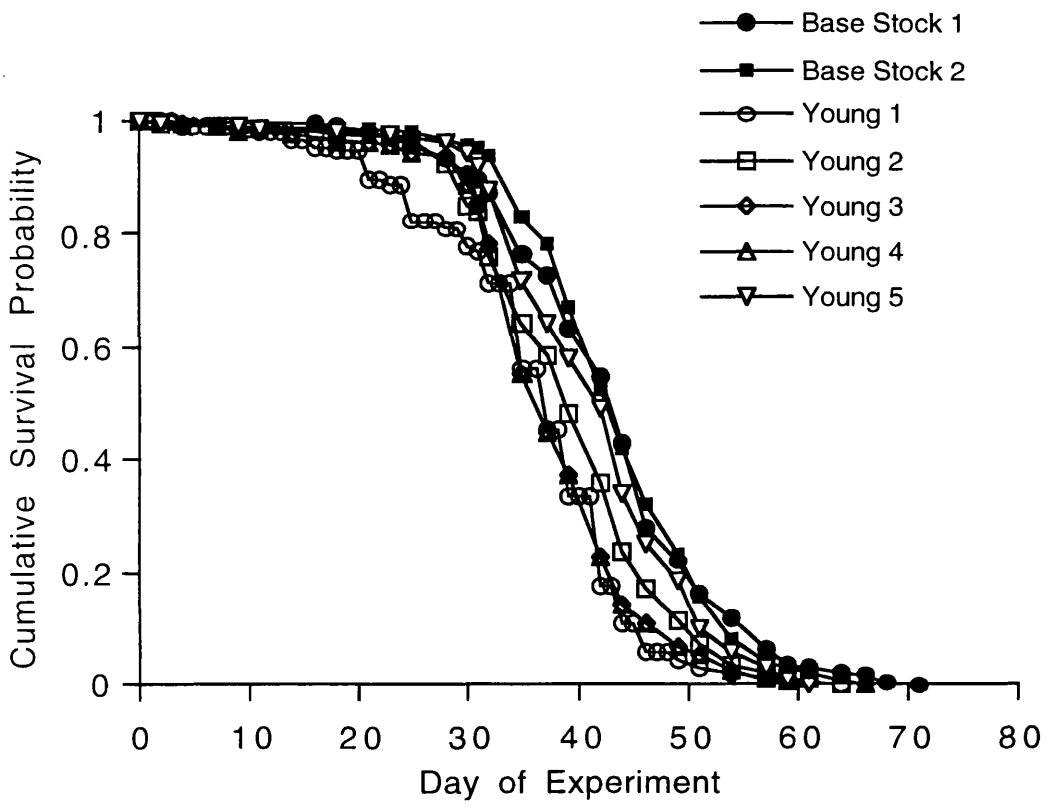

Figure 4.1. Cumulative survival probability of 'young' line and base stock males.

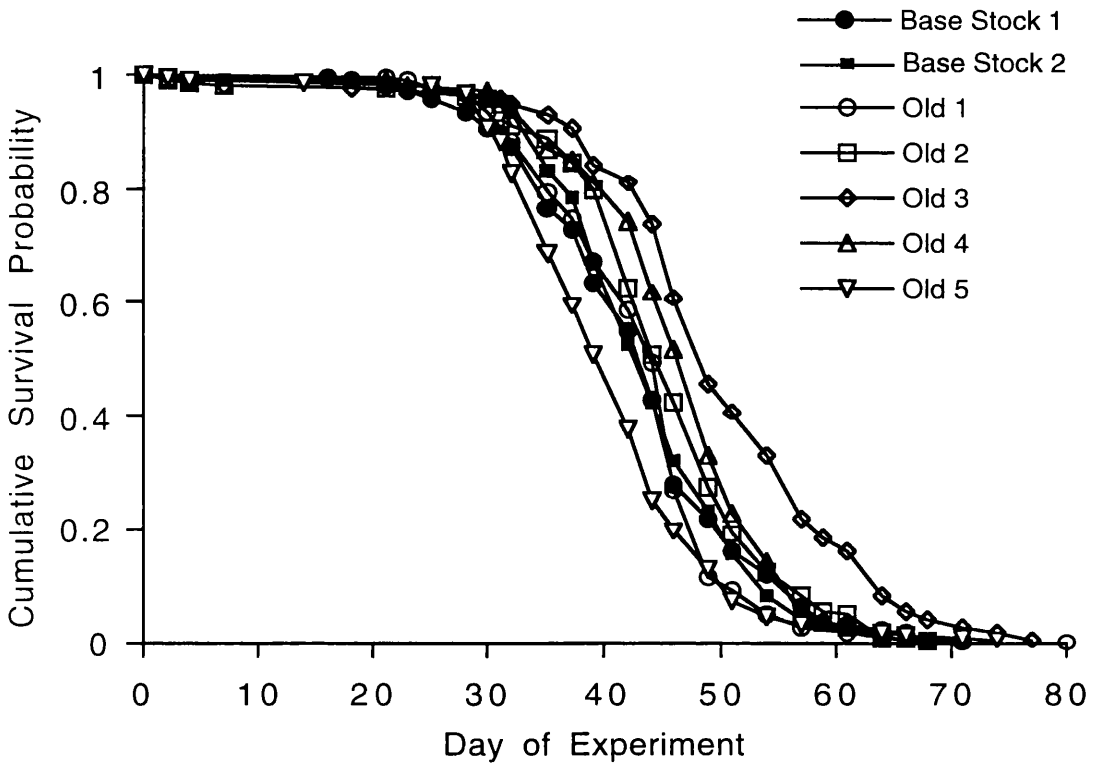

Figure 4.2. Cumulative survival probability of 'old' line and base stock males.

Selection Regime	Line	Mortality Rate (First week)	Mortality Rate (2nd-4th weeks)
YOUNG	1	0.013	0.178
	2	0.009	0.066
	3	0.009	0.050
	4	0.022	0.027
	5	0.009	0.027
Grand Mean (±95% CL)		0.013 (±0.007)	0.070 (±0.072)
OLD	1	0.000	0.033
	2	0.017	0.018
	3	0.019	0.014
	4	0.005	0.019
	5	0.010	0.024
Grand Mean (±95% CL)		0.010 (±0.009)	0.021 (±0.009)
DAHOMEY	1	0.000	0.065
	2	0.009	0.022
Grand Mean (±95% CL)		0.004 (±0.019)	0.043 (±0.091)

Table 4.1. Age-specific mortality rates for each replicate line and mean mortality rate for each regime for two periods during the life span, the first week and days 8 to 28. For details of calculation of mortality rates, see text. CL = confidence limits.

Selection Regime	Line	Point of 10% Mortality (days)
YOUNG	1	21
	2	30
	3	30
	4	31
	5	32
Grand Mean (± 95% CL)		28.8 (±5.10)
OLD	1	32
	2	35
	3	39
	4	35
	5	31
Grand Mean (± 95% CL)		34.4 (±3.60)
DAHOMEY	1	31
	2	35
Grand Mean (± 95% CL)		33.0 (±8.61)

Table 4.2. Point of 10% mortality (in days) for males in each replicate line and the grand mean and 95% confidence limits for each regime.

Selection Regime	Line	Age-Dependent Parameter, G	Age-Independent Parameter, A
YOUNG	1	0.1421	0.0005
	2	0.1426	0.0004
	3	0.1762	0.0002
	4	0.1762	0.0002
	5	0.1442	0.0003
Grand Mean (±95% CL)		0.1563 (±0.021)	0.0003 (±0.0001)
OLD	1	0.1648	0.001
	2	0.1513	0.0001
	3	0.1138	0.0003
	4	0.1561	0.0001
	5	0.1538	0.0002
Grand Mean (±95% CL)		0.1480 (±0.023)	0.0003 (±0.001)
DAHOMEY	1	0.1419	0.0002
	2	0.1534	0.0001
Grand Mean (±95% CL)		0.1477 (±0.025)	0.0002 (±0.0003)

Table 4.3. Estimates of male age-dependent and age-independent Gompertz parameters for each selection line and base stock replicate, and means and confidence limits for each regime and the base stock.

Selection Regime	Line	Early (± 95% CL)	Late (± 95% CL)
YOUNG	1	0.87	0.82
	2	0.89	0.88
	3	0.75	0.57
	4	1.16	0.79
	5	0.73	0.63
Grand Mean (±95% CL)		0.88 (±0.20)	0.74 (±0.15)
OLD	1	0.67	0.84
	2	0.61	0.74
	3	0.83	1.11
	4	0.56	0.77
	5	0.55	0.45
Grand Mean (±95% CL)		0.65 (±0.13)	0.78 (±0.27)
DAHOMEY	1	1.06	1.10
	2	0.95	1.15
	3	0.93	0.76
Grand Mean (±95% CL)		0.98 (±0.08)	1.00 (±0.24)

Table 4.4. Index of male competitive reproductive success for each replicate line and the regime means (± 95% CL) during the early (sampled on days 2 and 6) and later (days 9, 13, 16 and 24) stages of the experiment. For details of calculation of the index, see text.

4.5. Conclusions

- Male age-specific mortality rate diverged significantly between 'old' and 'young' regimes in the period following the termination of reproduction in the 'youngs', but neither differed significantly from the base stock. Examination of the points at which each regime reached 10% mortality showed that 'young' regime males arrived significantly earlier than 'old' regime males.
- Estimation of the age-specific increase of death rate using the Gompertz survival model showed that, following the point of 10% mortality, there was no consistent difference between the selection regimes or the base stock in terms of the age-dependent Gompertz parameter, G.
- There was a correlated response to selection in terms of decreased male competitive reproductive success early in life in 'old' line males. In marked contrast to other studies (Roper *et al.*, 1993; Service, 1993), no late life increase in male competitive reproductive success was observed in the 'old' regime relative to the base stock.

- Female age-specific mortality and fecundity were measured by Partridge and co-workers shortly before I started work on the selection lines, when the 'young' lines were in generation 31 and the 'old' lines were in generation 16. Female age-specific mortality rate showed a similar pattern to that of the males. Also in accordance with the males, early life progeny production declined in 'old' line females relative to base stock and 'young' line females, and the three groups converged later in life (taken from Partridge and Pignatelli, unpublished data).

4.6. Discussion

4.6.1. Novel Responses to Selection on Age at Reproduction

The pattern of responses and correlated responses to selection on age at reproduction in males that have been described here represent the first results obtained from a study that has intentionally avoided covert selection on pre-adult development and rigorously controlled larval rearing densities. In light of these

factors, the observed patterns of early and later life male competitive reproductive success and the schedule of male mortality under selection are particularly important.

Selection to increase late life fitness has never produced a reduction in early life fecundity without also producing an increase in late life fecundity. Late life fecundity did not increase in the present 'old' selection lines relative to the base stock. This was confirmed in both sexes. This suggests that previously observed patterns of late life fecundity in long lived lines have been, at least partly, the result of confounding aspects of each individual selection regime. In addition, selection has been shown to have produced a particular schedule of mortality in lines selected for early or late life fitness. By analysing age-specific mortality rates during the crucial times of each regime's lifetime, the times that they reach the point of 10% mortality and the rate of increase in age-dependent mortality following that point, it has been possible to show that selection has altered the timing of the onset of the decline in survivorship. However, the rate of decline after that onset was not affected.

4.6.2. Direct Responses to Selection: Mortality Rate

Selection produced a divergence in age-specific mortality rate between 'young' and 'old' regime males. Comparisons with the base stock were not conclusive, but it is clear from the median life spans that the majority of the response was in the 'young' regime and that 'old' line males showed a smaller response to selection. It could be argued that the use of only two base stock replicates reduced the statistical power of this analysis, and it is therefore not known if the base stock would have differed significantly from the selection regimes if more statistical power had been available. However, a similar inconclusive result was seen in a study of the females (Partridge and Pignatelli, unpublished data), in which five base stock replicates were used. Females from the selection regimes showed very similar patterns in terms of age-specific mortality rate, measured shortly before the males were examined. In the first week of life, 'old' and 'young' regimes did not differ, but between days 8 and 28 'young' line females died at a faster rate than 'old' line females (Partridge and Pignatelli, unpublished data). There are two other possible reasons why the

base stock did not differ significantly from either selection regime. The first is that selection may not have been applied for long enough to observe a significant response. A response in terms of overall survivorship has been observed in fewer generations (e.g. Rose and Charlesworth, 1981; Luckinbill *et al.*, 1984; Rose, 1984; Partridge and Fowler, 1992), but it may be that the intensity of selection used here was not enough to produce a significant divergence in mortality rate between each regime and the base stock. Age-specific mortality rate rather than cumulative survivorship was used in the first place to avoid analyses that take the whole life span into consideration, such as the Log-Rank test. The period under selection was the first week of life in the 'young' lines and the first four weeks of life in the 'old' lines. The rest of the life span was under no selection, as suggested by the lack of divergence between regimes with respect to the age-dependent Gompertz parameter. The second possible reason why no difference was seen is that there was a high degree of environmental variation between replicate lines in each regime. Indeed, one 'old' line, replicate 5, was evidently shorter lived than the base stock. It is possible that selection on the 'old' lines was not as effective as it was on the 'youngs' because there was a high degree of variation in adult viability between 'old' replicate lines resulting in reduced statistical power during the analysis and which may have caused the intensity of selection for longer life to be weaker than it has been in previous regimes.

The age-specific mortality rate differences between groups and the lack of variation between regimes with respect to the rate of acceleration of mortality, estimated using the Gompertz model, illustrate how selection has acted upon males from the 'old' and 'young' regimes. Mortality rates diverged between the 'young' and 'old' regimes after day 7, but since comparisons with the base stock were inconclusive, so it is unclear whether mortality declined in the 'old' regime or increased in the 'young' regime. However, it is reasonable to assume that 'young' regime mortality rose relative to that of the 'old' lines. This is because after day 7 and before day 28, deleterious alleles expressed in the 'young' lines would not have been selected against whereas in the 'old' lines they would have been, because 'old' line flies were yet to reproduce. It is interesting to note that once all cohorts had reached the point of 10% mortality, the acceleration rate of age-specific mortality did not differ between them, which fits the hypothesis that the later part of the life history was not under selection in either regime. This

aspect of the mortality distribution directly contrasts with the divergent mortality rates of the period between days 8 and 28, during which time 'old' males were selected for low mortality rate. These results suggest that selection altered the timing of the onset of senescence, as indicated by the significant difference between the times that each regime reached 10% mortality and the later similarity between regimes with respect to the rate of increase in age-specific mortality rate. An alternative model would have been for selection to have caused a gradual divergence in ageing rate throughout life, but this is not supported by the data. A pattern of age-specific mortality that agrees with the results presented here was shown in the 'O' and 'B' lines (Rose, 1984), where the 'O' lines had a consistently lower mortality rate than the 'B' lines up until day 50.

4.6.3. Reproductive Success: A Correlated Response to Selection

The observed pattern of male reproductive success indicates that 'old' line males definitely responded to selection on life span. In light of the inconclusive position of the base stock with respect to age-specific mortality rate, this result is important in showing that the 'old' line males did respond significantly to selection. The response to selection of the females in terms of fertility closely resembled that of the males. 'Old' line female fertility was significantly lower than the 'young' lines or base stock in the first week of life, and converged with the base stock and 'young' females later in life (Partridge and Pignatelli, unpublished data). This makes the conclusion that 'old' line females responded to selection as strong as for the males, despite the problems with the mortality rate comparison.

The reproductive schedule that has evolved in the 'old' and 'young' lines has a number of surprising features. The first is that in both sexes early life reproductive success was significantly lower in the 'old' lines compared to the base stock. This is in contrast to the results of Partridge and co-workers (Partridge and Fowler, 1992; Roper *et al.*, 1993), but supports Williams' (1957) prediction that selection for late life fitness would result in a decline in early life fitness. Similar patterns have been shown elsewhere (Wattiaux, 1968; Rose and Charlesworth, 1981; Luckinbill *et al.*, 1984; Rose, 1984; Tucic *et al.*, 1996;

Miyatake, 1997), although the similarity may be superficial because there may be another factor involved that is seldom considered. Many control populations are selected for early life fitness, including the Rose 'B' lines which were under extreme selection to develop early. Therefore, long lived lines may have responded to selection with decreased early fertility, but short lived lines may also have shown elevated early fertility. This pattern is likely to have occurred in a number of studies, but has gone undetected because of the lack of suitable control populations. Instead, a divergence in early fertility is taken to be a response to selection on the part of long lived lines. Reproductive success in the present 'young' lines did not differ with respect to the base stock in either sex. Therefore, this is the first study to illustrate the evolution of decreased early fecundity in both sexes as a correlated response to selection for extended longevity with reference to a reliable base stock control. As such, it provides evidence for pleiotropic gene action without any confounding factors.

Another striking result of these experiments was the lack of increase in late life reproductive success in 'old' flies of either sex. This has never before been reported in studies involving selection on age at reproduction. Indeed, the pattern more closely resembles the results of Zwaan *et al.* (1995b), who selected directly upon life span while avoiding selection on fertility (by keeping progeny isogenic lines at 15°C, measuring life span of lines at 29°C and selecting on the longest and shortest lived lines) than studies based upon selection on age at breeding. Zwaan *et al.* showed that in lines selected for extended life span fertility was suppressed throughout life, not just early on, strongly suggesting that in previous selection experiments longevity and late life reproductive success increased in parallel due to the nature of the experimental design rather than any genetic correlation between them. Life span is therefore negatively correlated with early fecundity (Zwaan *et al.*, 1995b). The present experiments support this conclusion and are the first involving selection on age at reproduction to demonstrate the same trade off in both sexes without late fertility confusing the issue. The question therefore remains as to why an increase in 'old' line late life fertility did not occur in response to selection.

The lack of response in 'old' line late life fecundity casts doubt upon previous studies that have reported an increase in late fecundity in response to selection. Female fecundity (Luckinbill *et al.*, 1984; Rose, 1984; Partridge and Fowler, 1992) and male competitive reproductive success (Roper *et al.*, 1993; Service, 1993) have diverged late in life in past experiments. The present study

shows that it is possible to select for genes enhancing life span by collecting eggs from old individuals without producing a response in late fertility. Therefore, what aspect of selection differed in the present study? First, it is possible that in past regimes, selection for extended longevity has also resulted in selection for increased late fecundity because females with the highest fecundity contribute the most eggs. It is possible that selection for extended life span in the present regime was not particularly strong, and so the selection for late fecundity that usually occurs under such conditions did not occur. The second reason why no late fecundity increase was observed is that the divergence in late fertility between the Rose 'O' and 'B' lines, and between other regimes, may have been partly due to selection on previous short lived lines for rapid development and early reproductive maturity. As discussed above, selection for early reproduction may partly explain previously observed divergences in early fecundity. It follows that the short lived lines enter adulthood in poorer shape in terms of both life span and late fecundity. It is therefore possible that the late life divergence in fertility reported by Rose (1984), Luckinbill *et al.* (1984) and Partridge and Fowler (1992) may not have been solely due to a direct response to selection in the long lived lines, but rather a correlated response to selection in the short lived lines. In the results described here, the 'old' lines were compared with the base stock, which was not under selection to mature quickly, so selection for rapid development was not a confounding factor. The avoidance of selection for rapid development and the control of larval densities will be discussed fully in the next chapter, which deals with the responses to selection of pre-adult characters and adult body size.

The trade off of life span against early fertility fits the antagonistic pleiotropy theory of ageing. There is some physiological and genetic evidence that the basis of life span extension involves a trade off between reproductive and somatic functions. It has been suggested that increased life span is correlated with an increase in stress resistance characters such as starvation resistance (Service *et al.*, 1985; Zwaan *et al.*, 1991; Hoffmann and Parsons, 1993) and levels of activity, both generally (Service, 1987) and in terms of flight (Graves *et al.*, 1992). Starvation resistance was found to be positively correlated with fat fraction (Zwaan *et al.*, 1991), as are other stress resistance traits (Service, 1987). Lipid metabolism is also very important for reproduction, especially in females (Geer *et al.*, 1970). This provides a possible physiological

basis for the negative correlation between early fecundity and starvation resistance (Service *et al.*, 1988; Hoffmann and Parsons, 1989) and therefore early fecundity and longevity (Zwaan *et al.*, 1995b; this study). It appears that selection on life span favours genes that enable the diversion of metabolites away from reproduction and into functions that enhance survival probability, in which the ability to resist environmental stress plays an important part. Some evidence for the genetic basis of this mechanism has been found in lines selected for late and early life fitness that have different forms of glucose-6-phosphate dehydrogenase or G6PD (Luckinbill *et al.*, 1990). G6PD mediates the allocation of sucrose derivatives to the glycogen and pentose phosphate pathways during glycolysis. The pentose phosphate pathway is important in lipid metabolism, so increased G6PD activity in the long lived lines (Luckinbill *et al.*, 1990) presumably leads to increased lipid synthesis. However, this does not account for the reallocation of lipids to different functions under life span selection, so there must be other genes involved. The physiological basis of life span extension in *Drosophila* fits in with expectations of life history theory. The optimality model of life history evolution, with the allocation of finite resources to different functions within the organism, predicts that this type of physiological trade off should be found. It highlights the conflict between survival and reproduction that is the underlying basis of the disposable soma model of senescence. Evidence that this mechanism is responsible for the extension of life span in the present 'old' lines is given by the reduction of 'old' line reproductive success early in life in both males and females.

4.6.4. The Use of a Base Stock Reference

Successful extension of life span has been reported before in *D. melanogaster* (Luckinbill *et al.*, 1984; Rose, 1984; Partridge and Fowler, 1992; Roper *et al.*, 1993), but this is the first time that a base stock has been used to monitor responses in males and females. Unlike the base stock used in this study, the controls used in some of the experiments performed in the early, 1980s were not particularly suitable for measuring some responses to selection. For example, in one laboratory life span was selected upon in both directions, but no base stock reference was used, so only the relative differences between

characters such as life span and fecundity could be determined (Luckinbill *et al.*, 1984). Another selected for late reproduction and used control lines that were kept in a manner similar to the original base stock, if not in every respect, certainly in terms of the 14 day generation time (Rose, 1984; Chippendale *et al.*, 1994; Leroi *et al.*, 1994). These 'B' populations were under abnormally strong selection pressure to eclose and mature rapidly as a result of this short life cycle, and the validity of such stocks as a control in life history experiments is questionable. It could be argued that the Rose 'B' lines were laboratory adapted and are, therefore, a stable control. However, the intense selection for pre-adult development in these lines may mean that they provided a rather extreme point of reference. The Dahomey base stock population from which the present selection lines were derived has been kept under constant conditions for more than 20 years with no artificial constraints on development. It can therefore be used as a reliable reference point because it is likely to be at equilibrium and not evolving in response to any form of directional selection. This means that the direction of evolution of particular characters in the selected lines can be determined (Roper *et al.*, 1993). The base stock used in the present experiments has been useful in reaching the conclusion that the response in early life fertility in both sexes was the result of changes in the 'old' lines alone.

4.6.5. Further Experiments

This study is important because it has shown unambiguous support for the antagonistic pleiotropy theory of senescence by a trade off between early fertility and longevity. The reason for its importance is the intentional avoidance of certain confounding features that have affected the results of previous regimes. The following chapter describes the experiments in which pre-adult and adult body size characters were measured. In the absence of confounding factors concerning these traits, the possibility that the correlated responses reported in previous studies were true responses to selection on life span was investigated. It is hoped that the avoidance of problems in the pre-adult period that were discussed in the introduction to this chapter will shed light on some outstanding questions about the ambiguous and apparently conflicting results of earlier studies.

Chapter 5. Correlated Responses to Selection: Pre-Adult Development, Competitive Ability, Adult Body Size and Weight

5.1. Summary

The effect of selection on age at reproduction on pre-adult development time, larval competitive ability, adult body size and adult dry weight was examined. Selection was applied with the specific intention of avoiding certain confounding effects that have previously made the interpretation of correlated responses difficult; these included inadvertent selection for rapid development and uncontrolled larval density. No evidence at all was found for the occurrence of these confounding effects in the present selection regimes. Neither selection regime differed significantly from the base stock in terms of larval competitive ability or adult thorax length. Patterns of pre-adult development time and adult dry weight did not fit the hypothesis that they had been affected by the confounding factors described. The results from the present selection lines were discussed in the context of previous studies.

5.2. Introduction

The present 'old' and 'young' lines were established with the specific aim of studying the correlated responses to selection on age at breeding in a regime where confounding forms of selection had not been applied. Many selection regimes of this kind have involved inadvertent selection for rapid development (Rose, 1984; Partridge and Fowler, 1992; Tucic *et al.*, 1996; Miyatake, 1997) and the results of some have been confounded by the effect of uncontrolled larval density (Luckinbill *et al.*, 1984; Partridge and Fowler, 1992). It is therefore difficult to interpret the evolution of certain characters in the pre-adult and adult life histories of these selected populations because they may not have been true correlated responses to selection, merely the result of these confounding elements. The aim of the present selection study was to avoid these problems

and then see whether the results observed by other authors still occurred as actual responses to selection on life span.

The evolution of mortality and fecundity as responses to selection were reported in the previous chapter. This chapter details experiments concerned with the measurement of adult body size and dry weight, pre-adult development time and larval competitive ability. These are all characters that one might expect to be directly affected by the confounding elements that have been discussed. They have also been presented as correlated responses to selection in a range of different studies (Luckinbill *et al.*, 1984; Rose *et al.*, 1984; Partridge and Fowler, 1992; Roper *et al.*, 1993; Chippendale *et al.*, 1994; Tucic *et al.*, 1996; Miyatake, 1997). It is hoped that these experiments will clear up any doubt about the way that selection on age at breeding shapes some important components of the *Drosophila* life history.

5.3. Methods and Materials

5.3.1. Egg to Adult Development Time

The first measurement of egg to adult development time was during 'young' line generation 38 and 'old' line generation 20. The second time was a year later when the 'youngs' were in generation 59 and the 'old' lines were in generations 30 to 32.

In the first development time experiment, eggs from each selection line cage and a Dahomey population cage were incubated at low density in bottle cultures. The emerging flies were put into cages at densities that were between five and ten times greater than during selection, one cage per line and one for the base stock. These high densities were used to ensure that enough eggs could be harvested for this development time experiment and the larval competitive ability experiment to follow. Two dishes of grape medium with a large amount of yeast paste were supplied and replaced every two days. Flies in the cages were aged for four to five days before they supplied eggs to the experiment.

Eggs were collected from each cage on yeasted grape juice medium for two to three hours. They were then incubated for 24 hours at 25°C until they

hatched. Forty first instar larvae were put into each of 10 vials for each selection line and two base stock replicates. The vials were checked on a regular basis until the first flies were seen to eclose, and then the vials were checked every 8 to 12 hours from the evening of the same day. All emerging flies were counted, sexed and some were retained for thorax length measurements.

In the second development time experiment, cages were again established using flies from low density bottle cultures. There were, however, five base stock replicates in this experiment. For each line, 16 culture vials were filled with 50 first instar larvae each, and their egg to adult development time monitored as in the first experiment.

5.3.2. Adult Thorax Length

The first time that adult body size was measured, flies from the first development time experiment were used. During two development time checks around the time of peak eclosion a total of five males and five females were taken at random from each replicate vial so that 50 flies of each sex were measured per line. These flies were scored for thorax length to the nearest 0.02 mm using a graticule lens fitted to a microscope eyepiece.

The second time that thorax length was measured was during 'young' line generation 60 and 'old' line generations 31 to 33. Adults from low density bottle cultures were placed into population cages. Eggs were collected, allowed to hatch and the emerging larvae placed into 10 culture vials per selection line including five base stock replicates, with 50 larvae in each. All of the emerging adults were collected, placed into micro-centrifuge tubes and frozen at -20°C until they were measured. The thorax length of 10 adults of each sex from each vial was measured to the nearest 0.01 mm using an eyepiece graticule. One hundred flies of each sex were measured per line.

5.3.3. Larval Competitive Ability

The aim of this experiment was to test larval competitive ability against a standard marked competitor at two different levels of larval density. The cages

used to produce larvae for the first development time experiment (see section 5.3.1) also provided base stock and selection line larvae for this experiment. In addition to wild type flies, large numbers of *scarlet* eyed mutant flies were reared by collecting eggs on Lewis medium in the *scarlet* population cage and rearing them at low density. Three cages of *scarlet* adults were established and maintained in the same way as the other populations used in this experiment. The reason for rearing so many *scarlet* flies was that larvae from all the other lines and base stock were to be tested against this standard competitor, so twice as many mutant larvae were needed as all other larvae put together.

In order to make the rearing conditions stressful so that competitive ability could be tested, the amount of food available to each larva was reduced. First instar larvae were placed into vials containing 3.5 ml of Lewis medium, half the volume used for rearing the selection lines. Densities of 150 larvae and 300 larvae per half vial were used, each with a 2:1 ratio of mutant to wild type larvae. The lower density treatment had 10 vials per replicate line, including two base stock replicates, whilst the higher density treatment had just seven vials for each replicate due to a shortage of mutant larvae. The number and phenotype of the emerging progeny were recorded and the percentage of wild type flies was used as a measure of pre-adult competitive ability.

5.3.4. Adult Dry Weight

This experiment was designed to test for any correlated response to selection in terms of adult dry weight. When it was carried out, the 'young' lines were in generation 67 and the 'old' lines were in generations 35 to 37. Eggs were collected from selection line and base stock cages when all selection line flies were less than two weeks old, incubated for 24 hours and the hatching larvae placed into vials at a density of 50 larvae per line. The emerging adults, which were collected from the selection line and base stock vials two days after the peak of eclosion, were sexed and then kept on ice for an hour. While still immobile, flies of each sex were placed in groups of five into empty glass vials that were incubated at 60°C for two hours. The groups of desiccated flies were then weighed using a Sartorius micro balance to the nearest 10 μg. Ten groups

of five males and 10 groups of five females were weighed from each selection line and base stock replicate.

5.3.5. Statistical Analysis

Prior to analysis all data sets were tested to see if they conformed to the assumptions of parametric analysis. Any data violating these assumptions were either transformed using an appropriate method or analysed by a suitable non-parametric method. Unless otherwise mentioned, it may be assumed that data subjected to parametric analysis had homogeneous variances and were normally distributed.

5.4. Results

5.4.1. Egg to Adult Development Time

i) First Measurement

Mean egg to adult development time was calculated for each vial in each replicate line. The mean and 95% confidence limits for each replicate line and selection regime are given in Table 5.1, and the selection regime means for each sex are shown in Figure 5.1. Differences within and between selection regimes and the base stock were tested using a two-way ANOVA (see Table 5.2). Replicate line was treated as a random effect and was nested within selection regime and crossed against sex, which were both fixed effects. There was no significant variation between 'old', 'young' and base stock regimes, although within-regime variation was significant. Females developed significantly faster than males.

ii) Second Measurement

Data from the second development time experiment were not normally distributed, nor were the variances of the vial means homoscedastic. However, for reference they are shown in Table 5.3. Transformation failed to alter these patterns, and so the Kruskal Wallis test was used as an alternative to ANOVA. The selection regime means are shown in Figure 5.2. There was significant variation in egg to adult development time between 'old', 'young' and base stock regimes for both sexes (X^2 = 74.43 and 67.45 for males and females respectively, df = 2 and $p < 0.0001$ for both). When tested further, the Kruskal Wallis multiple comparisons test revealed that males and females of the base stock developed significantly faster than those in the selection regimes, which did not differ significantly from each other.

5.4.2. Adult Thorax Length

i) First Measurement

Vial means for male and female thorax length were calculated and used to calculate the replicate line and selection regime means (see Table 5.4). In the ANOVA (Table 5.5) the sexes were analysed together as a fixed effect, and sex was crossed against selection regime, which was also a fixed effect. Replicate line was nested within regime as a random effect. Thorax length did not differ significantly between selection regimes or the base stock (see Figure 5.3), but there was significant deviation between replicate lines within each regime. Females were significantly bigger than males. There was also a significant sex by replicate line interaction.

ii) Second Measurement

The data from this experiment were not normally distributed, but the underlying error distribution was, and so the second set of thorax length measurements were analysed in the same way as the first, by ANOVA (shown

in Table 5.6). No significant differences were found between selection regimes, the means for which are shown in Figure 5.4, whereas variation between replicates was highly significant (see the line means in Table 5.7). Males were significantly smaller than females and, as with the first experiment, there was a significant line by sex interaction.

5.4.3. Larval Competitive Ability

The percentages of wild type progeny emerging from each vial were analysed in the same way as the first development time and both thorax length experiments, by two way ANOVA, but with rearing density substituted for sex (see Table 5.8). The viability of wild type larvae did not differ significantly between or within selection regimes. The selection regime means for each density are shown in Figure 5.5, and the selection regime and line means in Table 5.9. Rearing density did not affect the level of success that selection line or base stock larvae had relative to the standard competitor.

5.4.4. Adult Dry Weight

The data from the measurement of adult dry weight were normally distributed but also heteroscedastic. For this reason, a transformation was carried out that homogenised the within-regime variances (Dutilleul and Potvin, 1995). This meant that the variances of the selection regimes were made uniform, but that the relationship between the replicate line means was not changed. ANOVA could therefore be carried out on the transformed selection regime means, but not on the underlying replicate lines. The untransformed selection regime and line means are shown in Table 5.10, and the untransformed selection regime means are shown graphically in Figure 5.6. The sexes were analysed together, with sex crossed against regime in the ANOVA (see Table 5.11). There was a significant difference between regimes, and a highly significant difference between sexes. To investigate the between regime variation further, a single way ANOVA was carried out on each sex. This showed that the males had no significant variation between the 'youngs', 'olds' or base stock (F ratio = 0.156, df

= 2, p = 0.86) but that the females did vary significantly (F ratio = 6.24, df = 2, p = 0.02). The females were then tested using the Tukey Kramer Honest Significant Difference, which takes the number of different comparisons into account. At the 5% level, 'old' line females were significantly lighter than base stock females but not than 'young' line females, which were also not significantly different from base stock females.

Selection Regime	Line	FEMALES Mean (± 95% CL)	MALES Mean (± 95% CL)
YOUNG	1	11.05 (±0.35)	11.22 (±0.30)
	2	11.38 (±0.17)	11.56 (±0.19)
	3	10.71 (±0.11)	10.84 (±0.10)
	4	11.12 (±0.17)	11.21 (±0.17)
	5	11.16 (±0.08)	11.43 (±0.10)
Grand Mean		11.08 (±0.28)	11.25 (±0.31)
OLD	1	11.23 (±0.18)	11.42 (±0.17)
	2	11.13 (±0.15)	11.47 (±0.14)
	3	11.06 (±0.19)	11.25 (±0.16)
	4	11.23 (±0.16)	11.40 (±0.14)
	5	11.36 (±0.30)	11.52 (±0.29)
Grand Mean		11.20 (±0.13)	11.41 (±0.12)
DAHOMEY	1	11.21 (±0.35)	11.45 (±0.30)
	2	11.34 (±0.15)	11.56 (±0.18)
Grand Mean		11.27 (±0.27)	11.51 (±0.24)

Table 5.1. First development time line means (in days ± 95% confidence limits) for each sex.

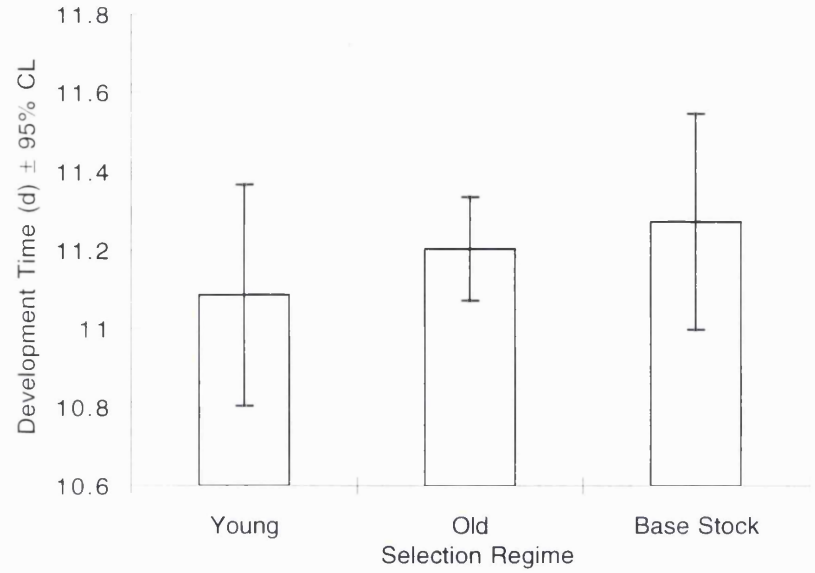

Figure 5.1a. First development time (± 95% CL) of females.

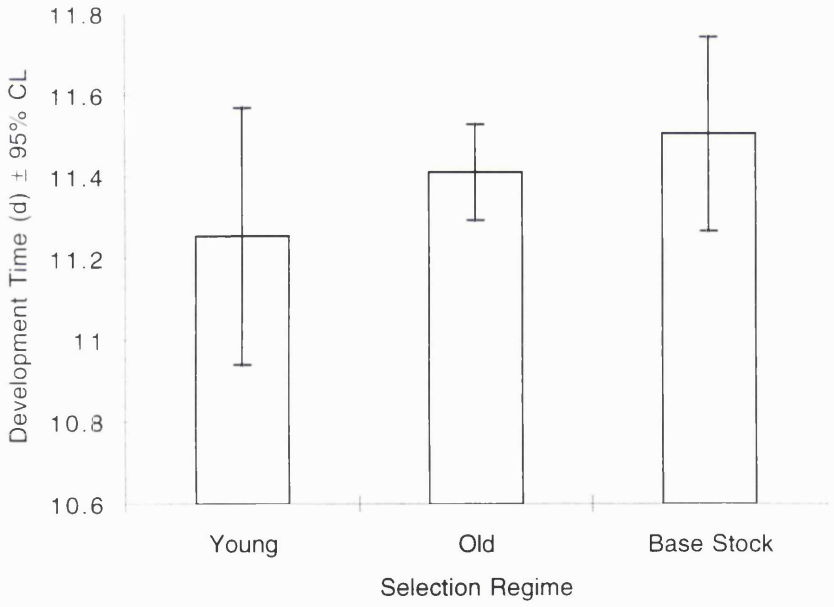

Figure 5.1b. First development time (± 95% CL) of males.

Source	SS	MS	DF	F ratio	p value
Regime	1.67	0.84	2	1.22	0.34
Line	6.25	0.69	9	30.66	<.0001
Sex	2.02	2.02	1	83.12	<.0001
Regime*Sex	0.04	0.02	2	0.74	0.50
Line*Sex	0.20	0.02	9	0.25	0.99

Table 5.2. Analysis of variance of vial means from first development time experiment. Line is nested within selection regime as a random effect, crossed against sex. Selection regime and sex are fixed effects. SS = sums of squares, MS = mean squares, DF = degrees of freedom.

Selection Regime	Line	FEMALES Mean (± 95% CL)	MALES Mean (± 95% CL)
YOUNG	1	11.70 (±0.21)	11.76 (±0.2)
	2	11.65 (±0.14)	11.72 (±0.18)
	3	11.34 (±0.09)	11.41 (±0.1)
	4	11.47 (±0.18)	11.60 (±0.19)
	5	11.20 (±0.13)	11.31 (±0.1)
Grand Mean		11.47 (±0.24)	11.56 (±0.22)
OLD	1	11.41 (±0.16)	11.50 (±0.13)
	2	11.36 (±0.11)	11.43 (±0.16)
	3	11.66 (±0.19)	11.70 (±0.19)
	4	11.65 (±0.24)	11.79 (±0.21)
	5	11.67 (±0.36)	11.72 (±0.37)
Grand Mean		11.55 (±0.18)	11.63 (±0.18)
DAHOMEY	1	11.21 (±0.13)	11.25 (±0.13)
	2	11.10(±0.14)	11.16 (±0.16)
	3	11.16 (±0.11)	11.19 (±0.11)
	4	11.03 (±0.07)	11.10 (±0.08)
	5	11.02 (±0.08)	11.09 (±0.08)
Grand Mean		11.11 (±0.09)	11.16 (±0.08)

Table 5.3. Second development time line means (in days ± 95% CL) for each sex.

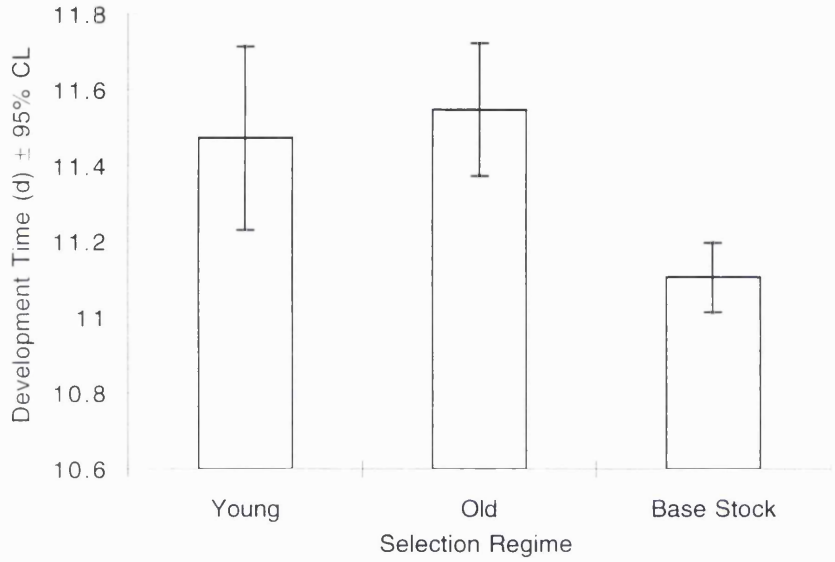

Figure 5.2a. Second development time (± 95% CLs) of females.

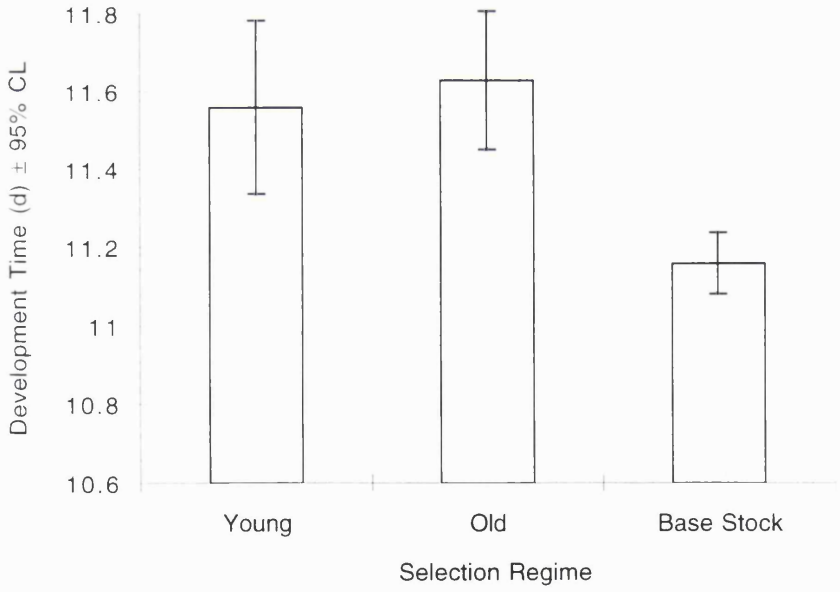

Figure 5.2b. Second development time (± 95% CL) of males.

Selection Regime	Line	FEMALE Mean (± 95% CL)	MALE Mean (± 95% CL)
YOUNG	1	1.007 (±0.02)	0.873 (±0.02)
	2	1.001 (±0.02)	0.865 (±0.02)
	3	1.024 (±0.02)	0.881 (±0.02)
	4	1.033 (±0.02)	0.882 (±0.02)
	5	1.01 (±0.02)	0.865 (±0.02)
Grand Mean		1.02 (±0.01)	0.88 (±0.01)
OLD	1	1.015 (±0.02)	0.886 (±0.03)
	2	1.032 (±0.02)	0.890 (±0.02)
	3	1.038 (±0.02)	0.885 (±0.02)
	4	1.006 (±0.02)	0.882 (±0.02)
	5	1.019 (±0.02)	0.872 (±0.02)
Grand Mean		1.02 (±0.02)	0.88 (±0.01)
DAHOMEY	1	0.999 (±0.03)	0.874 (±0.02)
	2	1.005 (±0.02)	0.865 (±0.02)
Grand Mean		1.00 (±0.01)	0.87 (±0.02)

Table 5.4. Line means (in mm) for each sex in the first thorax length experiment (± 95% CL).

Source	SS	MS	DF	F ratio	p value
Regime	24.82	12.41	2	2.33	0.15
Line	48.03	5.34	9	4.57	0.017
Sex	2721.81	2721.81	1	2329.65	<.0001
Regime*Sex	1.93	0.97	2	0.83	0.47
Line*Sex	10.51	1.17	9	2.06	0.03

Table 5.5. Two-way ANOVA of thorax length when measured for the first time. See Table 5.2 for details of abbreviations.

Source	SS	MS	DF	F ratio	p value
Regime	0.09	0.05	2	0.07	0.93
Line	7.62	0.63	12	14.59	<.0001
Sex	465.07	465.07	1	10689.90	<.0001
Regime*Sex	0.04	0.02	2	0.48	0.63
Line*Sex	0.52	0.04	12	1.85	0.04

Table 5.6. Two-way ANOVA of thorax length when measured for the second time. See Table 5.2 for details of abbreviations.

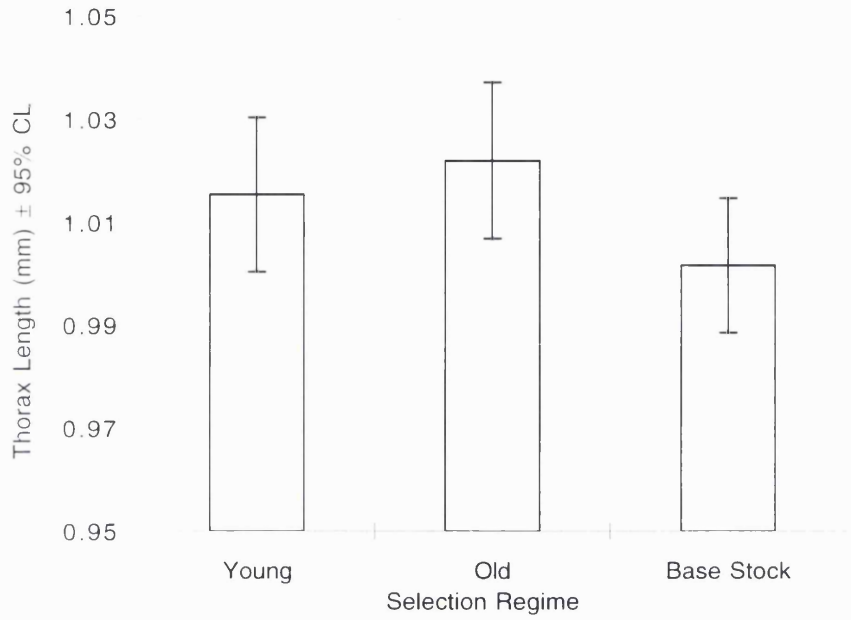

Figure 5.3a. First thorax length measurements (± 95% CL) of females.

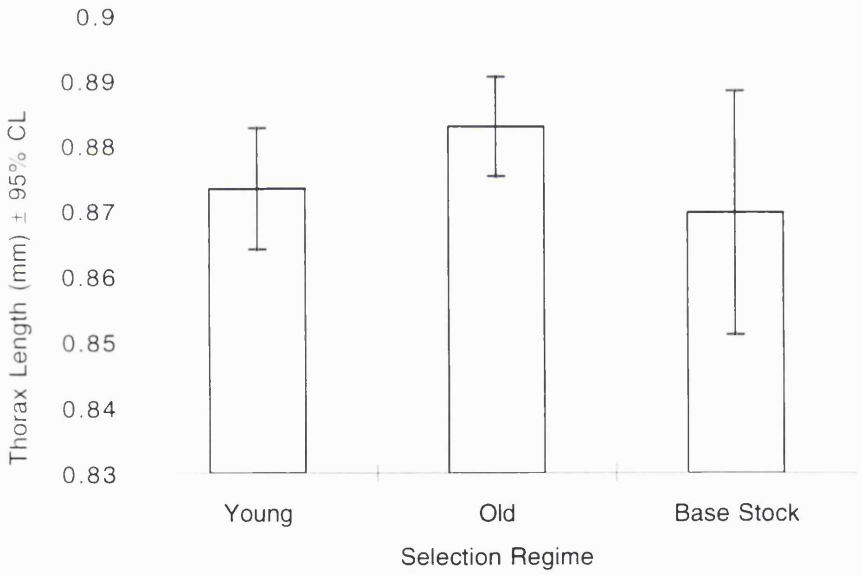

Figure 5.3b. First thorax length measurements (± 95% CL) of males.

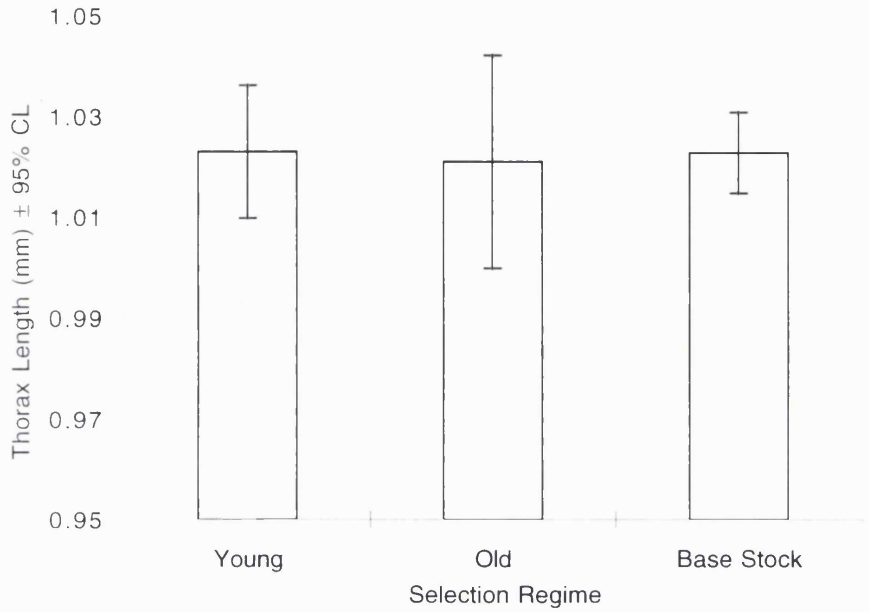

Figure 5.4a. Second thorax length measurements (± 95% CL) of females.

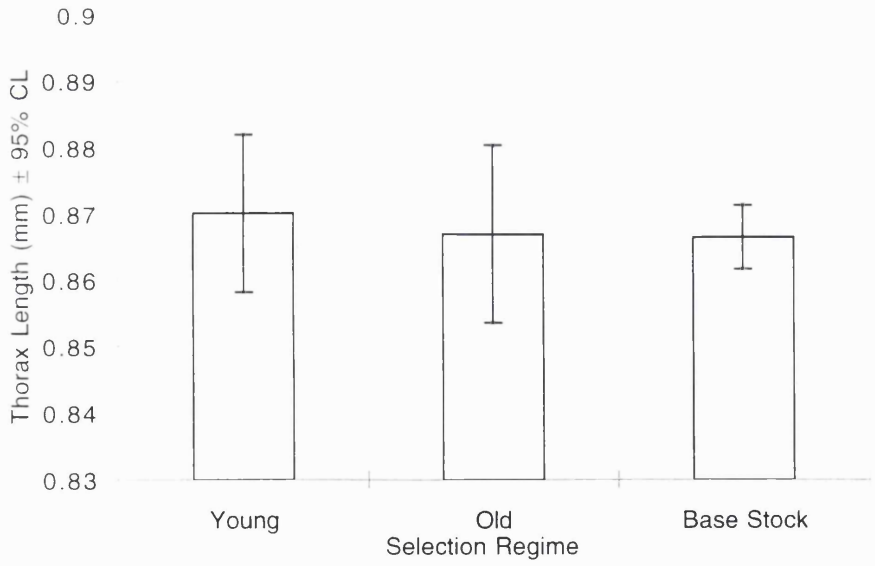

Figure 5.4b. Second thorax length measurements (± 95% CL) of males.

Selection Regime	Line	FEMALE Mean (± 95% CL)	MALE Mean (± 95% CL)
YOUNG	1	1.02 (±0.02)	0.865 (±0.02)
	2	1.034 (±0.02)	0.879 (±0.02)
	3	1.02 (±0.03)	0.868 (±0.02)
	4	1.035 (±0.02)	0.881 (±0.02)
	5	1.007 (±0.02)	0.857 (±0.02)
Grand Mean		1.02 (±0.01)	0.87 (±0.01)
OLD	1	1.029 (±0.03)	0.872 (±0.03)
	2	1.024 (±0.02)	0.874 (±0.02)
	3	1.022 (±0.02)	0.869 (±0.02)
	4	0.99 (±0.03)	0.846 (±0.02)
	5	1.04 (±0.02)	0.873 (±0.02)
Grand Mean		1.02 (±0.02)	0.87 (±0.01)
DAHOMEY	1	1.019 (±0.02)	0.866 (±0.02)
	2	1.025 (±0.02)	0.862 (±0.02)
	3	1.014 (±0.02)	0.863 (±0.02)
	4	1.033 (±0.02)	0.872 (±0.02)
	5	1.024 (±0.02)	0.869 (±0.02)
Grand Mean		1.02 (±0.01)	0.87 (±0.01)

Table 5.7. Line means (in mm) for each sex in the second thorax length experiment (± 95% CL).

Source	SS	MS	DF	F ratio	p value
Regime	36.35	18.18	2	2.95	0.10
Line	55.43	6.16	9	0.57	0.79
Density	9.45	9.45	1	0.88	0.37
Regime*Density	55.08	27.54	2	2.56	0.13
Line*Density	97.00	10.78	9	1.30	0.24

Table 5.8. Analysis of variance of larval competitive ability, with density crossed against selection regime and replicate line. Line is nested within regime as a random effect. For abbreviations see Table 5.2.

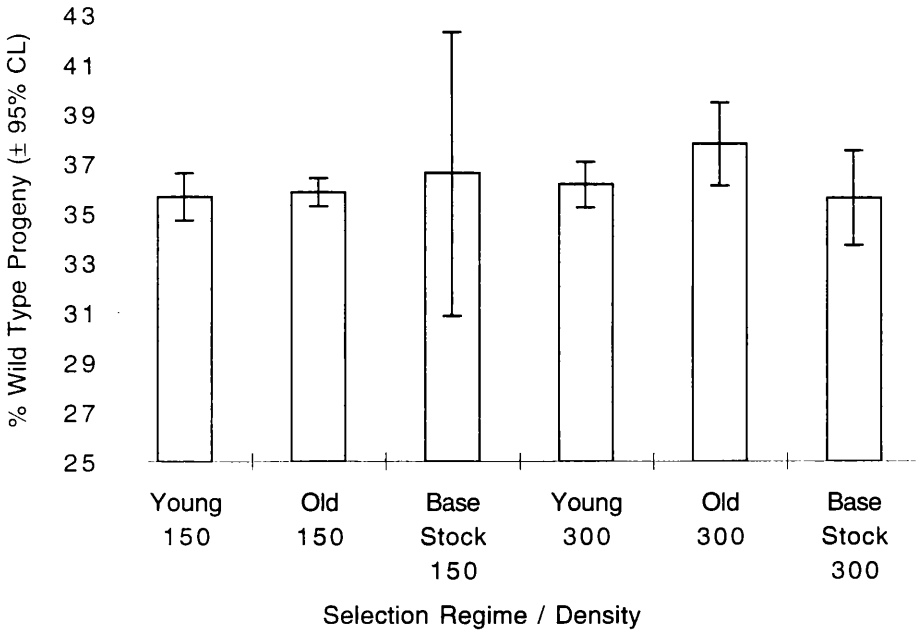

Figure 5.5. Percentage of wild type larvae emerging (± 95% CL) when reared with a marked competitor at densities of 150 and 300 larvae per vial (ratio = 2:1 competitor: wild type).

Selection Regime	Line	Density 150 Mean (± 95% CL)	Density 300 Mean (± 95% CL)
YOUNG	1	35.69 (±1.98)	35.64 (±1.80)
	2	35.28 (±3.32)	37.02 (±1.70)
	3	34.56 (±2.24)	35.78 (±1.28)
	4	35.76 (±2.47)	36.87 (±0.87)
	5	36.83 (±1.44)	35.25 (±1.77)
Grand Mean		35.62 (±0.95)	36.11 (±0.91)
OLD	1	35.19 (±1.57)	37.76 (±1.45)
	2	35.52 (±3.20)	36.85 (±1.12)
	3	36.00 (±2.68)	39.92 (±2.14)
	4	36.47 (±1.87)	36.05 (±1.53)
	5	35.89 (±1.79)	38.19 (±1.45)
Grand Mean		35.81 (±0.56)	37.75 (±1.69)
DAHOMEY	1	37.89 (±1.37)	35.13 (±2.05)
	2	35.24 (±2.34)	36.02 (±1.09)
Grand Mean		36.57 (±5.71)	35.57 (±1.90)

Table 5.9. Line means (± 95% CL) for the percentage of wild type larvae emerging at two densities, 150 larvae per vial and 300 larvae per vial, when grown against a standard marked competitor.

Selection Regime	LINE	FEMALE Mean (± 95% CL)	MALE Mean (± 95% CL)
YOUNG	1	0.46 (±0.02)	0.28 (±0.01)
	2	0.46 (±0.02)	0.27 (±0.01)
	3	0.49 (±0.02)	0.25 (±0.01)
	4	0.44 (±0.01)	0.25 (±0.003)
	5	0.43 (±0.01)	0.26 (±0.01)
Grand Mean		0.45 (±0.03)	0.26 (±0.01)
OLD	1	0.41 (±0.01)	0.24 (±0.01)
	2	0.43 (±0.02)	0.25 (±0.01)
	3	0.45 (±0.01)	0.28 (±0.01)
	4	0.43 (±0.01)	0.27 (±0.01)
	5	0.45 (±0.01)	0.28 (±0.01)
Grand Mean		0.43 (±0.02)	0.26 (±0.02)
DAHOMEY	1	0.47 (±0.02)	0.27 (±0.01)
	2	0.48 (±0.01)	0.26 (±0.002)
Grand Mean		0.47 (±0.02)	0.27 (±0.02)

Table 5.10. Untransformed mean dry weight (in mg ± 95% CL) for each sex in each replicate line. The data were transformed prior to analysis (see text for details).

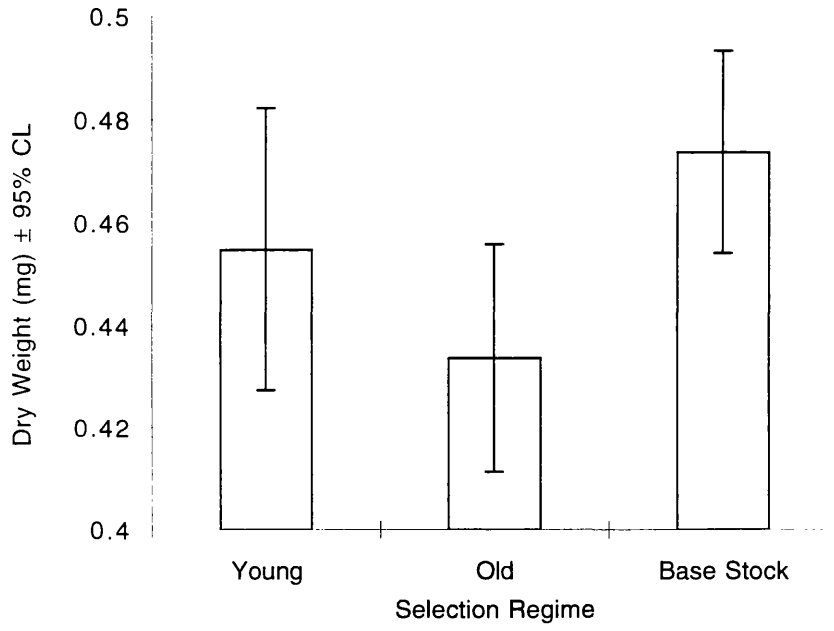

Figure 5.6a. Female dry weights (mg ± 95% CL).

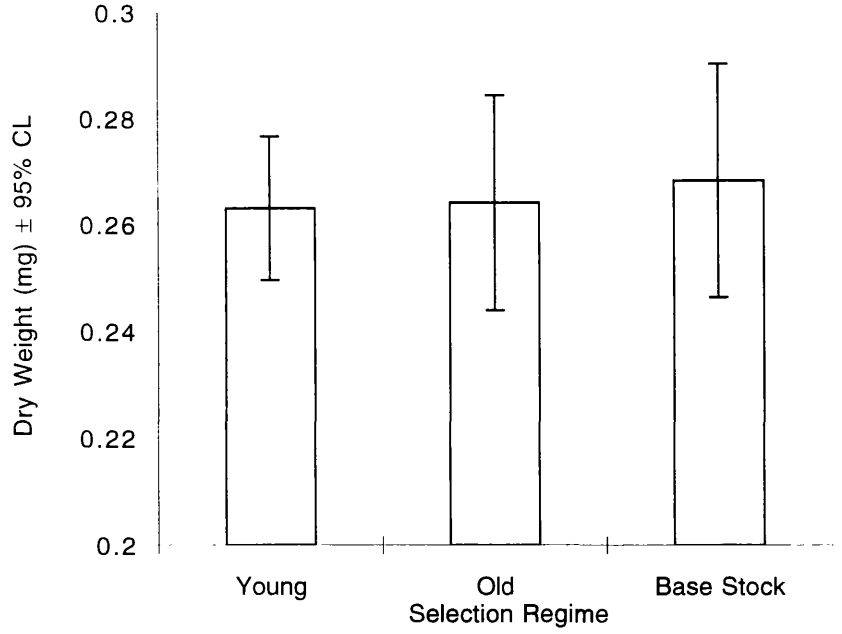

Figure 5.6b. Male dry weights (mg ± 95% CL).

Source	SS	DF	F ratio	p value
Regime	0.001	2	4.400	0.028
Sex	0.178	1	1059.000	<0.0001
Regime*Sex	0.001	2	3.370	0.057

Table 5.11. Two-way ANOVA performed on the transformed replicate line means from the dry weight measurements. For details of abbreviations see Table 5.2.

5.5. Conclusions

- There was no evidence that rapid pre-adult development was selected in the 'young' regime. This conclusion is particularly firm because development time was measured twice during selection with a year between experiments. In the second experiment, the base stock was faster than both regimes.
- In line with the lack of response in development time, adult body size also showed no response to selection, an equally firm result.
- There were no differences between selection regimes in pre-adult competitive ability at either density tested.
- There was a significant reduction in 'old' line female dry weight relative to that of base stock females. This contrasts with the lack of change in thorax length in the same selection regime.

5.6. Discussion

5.6.1. Avoidance of Confounding Elements During Selection

This is the first study involving selection on age at reproduction in *Drosophila melanogaster* to find no differences between selection regimes for egg to adult development time, larval competitive ability and adult body size. The 'O' and 'B' lines established by Rose (1984) have shown a consistent difference in egg to adult development time and viability, with the 'O' lines taking longer to develop and being more viable than the 'B' lines (Chippindale *et al.*, 1994). Lines selected for early life fitness reared by Luckinbill *et al.* (1984) showed variation in egg to adult development between generations, probably due to high, uncontrolled larval densities. Partridge and co-workers showed that in the previous 'old' and 'young' selection lines, 'old' line larvae took longer to develop, were less able to compete against a marked stock (Partridge and Fowler, 1992; Roper *et al.*, 1993) and became bigger adults (Partridge and Fowler, 1992) compared to 'young' line larvae. Although selection for a divergence in life span is always the primary objective, some studies have involved covert selection for rapid development in lines selected for early life fitness (Partridge and Fowler, 1992; Roper *et al.*, 1993; Chippindale *et al.*,

1994). This problem has also been encountered during similar experiments on two other species, the bean weevil (Tucic *et al.*, 1996) and the melon fly (Miyatake, 1997). Naturally, this has lead to a great deal of confusion as to which responses are the result of selection on life span and which are due to selection on other characters. The lack of response found in the present study for egg to adult development time, larval competitive ability and thorax length is undoubtedly because of a concerted effort to concentrate solely on selection for early or late life adult fitness and to avoid putting artificial selection pressure on other life history characters. It is also clear evidence that the patterns of observed responses in these characters in previous selection regimes were not entirely the result of selection on age at reproduction.

At first, some of the results presented here may appear anomalous to the general conclusion that no character was covertly selected. However, they do not fit the patterns that one would expect if they were the result of the problems that have been highlighted. For instance, the change in dry weight of 'old' line females is unlikely to have been due to inadvertent selection on characters other than longevity. Firstly, one would expect both sexes to have responded. Second, one would have also expected development time to have diverged, since there is a well-documented correlation between development and body weight (Hillesheim and Stearns, 1991; Nunney, 1996). These patterns were not observed, so whatever the reason for the decline in female dry weight, it is unlikely to be connected with the issues in question here, namely the avoidance of covert selection for rapid development and uncontrolled larval densities. Of course, it is possible that dry weight had only just started to diverge and that if the same characters were measured after another few generations of selection then a different pattern would be observed. However, it seems unlikely, considering the number of generations that had already passed, that any character for which there is undoubtedly sufficient additive genetic variation would start to diverge so late. Rose *et al.* (1984) showed a similar pattern in terms of adult weight in the early history of the 'O' and 'B' lines, with 'B' line females being heavier than 'O' line females, attributable to a difference in ovary weight. When measured recently, female weight was no longer significantly different between lines (Chippindale *et al.*, 1994), and neither was ovary weight (Leroi *et al.*, 1994). It seems prudent to examine this possibility as an explanation for the present result, a subject that will be discussed in the

following chapter. The other result that may cause difficulty with the general conclusion that there were no responses to inadvertent selection is the slow development of both selection regimes relative to the base stock in the second experiment. However, this does not suggest selection for rapid development. There may have been a slight change in the base stock itself or a gradual response to relaxed selection on development time in both 'old' and 'young' regimes. Cage adapted base stock populations are under some pressure to develop rapidly due to high larval densities. Larvae that do not eat quickly in such conditions may not reach the critical weight for pupariation (Bakker, 1961). The procedures during selection of allowing all flies to eclose before allocation to cages and not collecting eggs from the 'young' lines for at least six days may have resulted in the relaxation of the selection pressure that is normally experienced by the base stock population.

5.6.2. The Question of Pre-Adult Viability in Previous Studies

To examine the results of the present experiments in the context of the studies that lead to their conception, one must ask why were the Partridge 'old' line larvae were less viable and the Rose 'O' line larvae more viable than their respective controls? It is possible that this discrepancy was due to relative differences in rearing density and the intensity of the pressure placed on the 'B' lines to eclose. The 'old' line larvae reared by Partridge and co-workers grew under much lower larval density than the 'young' lines because 'young' line females were more numerous and more fecund when contributing eggs to the next generation (Partridge and Fowler, 1992). The 'old' lines were therefore selected at a lower density than the 'youngs' and they evolved a significantly longer developmental period and larger adult body size under those conditions. When the lines were tested at equal densities, the 'old' larvae were not able to compete against the faster developing 'young' larvae. There is a fitness cost associated with longer development (Roper *et al.*, 1996), possibly due to the chance of not attaining the critical weight for pupariation (Bakker, 1961). Larval density was controlled in the present incarnations of the 'old' and 'young' regimes by counting first instar larvae into culture vials. Therefore, problems relating to differences in larval density were not encountered in the present

study. This conclusion is supported by the absence of any response in terms of larval viability, even at high density.

The Rose 'O' lines may have been more viable than the 'B' lines for a number of reasons. The intense selection pressure on the 'B' lines to grow extremely quickly may have lead to growth rate being traded off against larval survival, which may be why more 'O' than 'B' line larvae reached adulthood. Additionally, 'B' line females were heavier than 'O' line females, a difference largely attributable to a divergence in ovary weight (Rose *et al.*, 1984). Larval competitive ability and adult body size are negatively correlated (Partridge and Fowler, 1993), but this is due to the fitness cost associated with taking longer to develop into a large adult. It is unlikely that 'B' line females took longer to develop than 'O' line females since they were required to develop rapidly and reach peak fecundity very soon after eclosion. Development time was not measured in these lines until the female weight difference had disappeared, but when it was, 'O' flies developed more slowly than 'B' flies (Chippendale *et al.*, 1994) as one would expect. 'B' females were therefore under pressure to invest in ovary development as well as develop quickly. It is surprising that in this situation they evolved greater adult body weight, but these effects could have resulted in the evolution of reduced larval viability as a fitness cost of having to grow extremely quickly and produce a larger adult.

The second half of this explanation, suggesting that the 'B' populations showed reduced larval viability because they were under pressure to produce larger ovaries, does not cover the males of these regimes. It is possible that males were under weaker or differing selection pressure. For example, an increase in ovary weight, presumably by an increase in the number of ovarioles, can increase female fecundity. Male fertility may be improved by totally different mechanisms, such as an increase of sperm competitive ability or the speed with which sperm or accessory gland reserves are replenished. A response in the males may, therefore, not have been seen using simple morphological measurements. Indeed, testis weight did not differ in the 'O' and 'B' lines (Rose *et al.*, 1984), whereas 'B' males did evolve the ability to increase their early life reproductive success (Service, 1993) by inducing faster oviposition in females (Service and Vossbrink, 1996). Thus, 'B' males and females responded to selection in different ways. Whether 'B' males suffered the same decline in larval survival rate as 'B' females is not known, but both males and females were

under pressure to reach peak reproductive output soon after eclosion so it is likely that both sexes would have incurred fitness costs in terms of larval viability.

5.6.3. Measures of Body Size Are Not Interchangeable

Body size and weight have often been seen as interchangeable in life history studies of the kind presented here. Body size and development time are correlated (Robertson, 1963; Bierbaum *et al.*, 1989), as are body weight and development time (Hillesheim and Stearns, 1991; Zwaan *et al.*, 1995a; Nunney, 1996; Tucic *et al.*, 1996). When thorax length is selected, development time responds (Robertson, 1963, Partridge and Fowler, 1993), as it does when dry weight is selected (Hillesheim and Stearns, 1991). In addition, those who have measured both traits in populations selected for age at reproduction have not observed a discrepancy between them, both when differences are detected (Partridge and Fowler, 1992) and when they are not (Chippindale *et al.*, 1994). None of these facts are surprising; larger size or greater weight requires longer development because larval growth rate does not respond to upward selection on thorax length (Robertson, 1963; Partridge and Fowler, 1993). In addition, one can easily imagine how a change of body size would affect weight, since it would involve an altered amount of exoskeleton around the body. The size of different morphological characters are generally highly correlated, especially when they originate from the same imaginal disc (Cowley and Atchley, 1990), so when directional selection is applied to a trait like thorax length, others should respond in the same direction. Thus, body weight should change in line with the size of a selected morphological trait. Often, either thorax length or wet weight is used as an indicator of size (e.g. Luckinbill *et al.*, 1988b; Bierbaum *et al.*, 1989; Roper *et al.*, 1993; Zwaan *et al.*, 1995a; Tucic *et al.*, 1996). However, a slight decline in dry weight, as shown here, would not necessarily be accompanied by a change in thorax length. This is because a change in body composition or the size of a particular organ would have little bearing in the length of the thorax, unless the change in weight was by a great amount.

As stated above, there is no reason to believe that the decline in dry weight of the 'old' line females is due to inadvertent selection on characters

other than life span and so an alternative explanation must be sought. The Rose lines showed a pattern very similar to the present one in terms of wet weight of 'O' females (Rose *et al.*, 1984), and it would be very interesting to know if thorax length in their lines had diverged too. However, when measured recently the 'O' and 'B' lines did not differ in thorax length or wet weight (Chippindale *et al.*, 1994), and the ovary weight difference to which the original divergence was attributed had also disappeared (Leroi *et al.*, 1994). In light of the apparent correspondence between the two sets of lines, the following hypothesis needs to be tested. Females in the 'old' lines may be putting fewer resources into reproduction in response to selection on life span, as already evidenced by decreased early fecundity. At the time that dry weight was measured the trait had not diverged significantly between 'old' and 'young' lines, but the trend was very strong since the 'old' line females were already significantly lighter than the base stock females. Potential differences in female reproductive morphology must be investigated.

5.6.4. The Effects of Pre-Adult and Size Characters on Fecundity

When selection on life span produces a correlated response in early life fecundity (e.g. Rose, 1984; Luckinbill *et al.*, 1984; Tucic *et al.*, 1996; Miyatake, 1997), it suggests that ageing is postponed in the 'old' lines by the selection of pleiotropic genes that enhance future survival probability at the expense of early fertility. Partridge and Fowler (1992) observed that since both life span and fertility respond to selection, one may be correlated with early fertility and the other enhanced by the reversal of mutation accumulation, thus invoking both theories of senescence. In contrast to other studies, Partridge and Fowler (1992) found no trade off between early and late fitness, concluding that life span had increased in their 'old' lines due to a reversal of mutation accumulation (Roper *et al.*, 1993). Several previous studies were confounded to some extent by selection on certain pre-adult characters; development time, and in some cases, viability. Presented here is the first study that has avoided these pitfalls, and it is clear that any characters showing a departure from the base stock have responded to selection on life span. As such, these results support the antagonistic pleiotropy theory of ageing.

It seems likely that the reason for the lack of response in early fecundity seen by Partridge and Fowler (1992) was due to the differences between their selection regimes in terms of larval density. The Partridge and Fowler 'old' lines evolved under lower larval density than their 'young' lines. They therefore evolved larger adult body size as a result of extended development time. It has been shown that genetically large females are more fecund early in life (Hillesheim and Stearns, 1992; Zwaan *et al*., 1995a), which is likely to be the result of selection increasing the size of a number of different body organs, including the ovaries (Hillesheim and Stearns, 1992). Thus, the Partridge and Fowler 'old' line females had enhanced early fecundity which masked any trade off between early fecundity and life span that might have otherwise been expressed.

5.6.5. Further Experiments

One interesting result of these experiments is the evolution of reduced female dry weight in the 'old' regime. Could this be the result of reduced investment in the reproductive organs? In light of this question and the issues discussed above concerning the force of selection on reproductive characters in populations selected on age at reproduction, the next step is to examine in detail some aspects of female reproductive biology. This is to include reproductive behaviour, morphology and examination of the role of mating costs in the evolution of extended life span. Survival and reproduction are very closely related in terms of both mating costs and the optimality approach to life histories and the evolution of senescence. Therefore, study of these characters in populations selected for extended life span is highly appropriate.

Chapter 6.
Responses to Selection on Age at Reproduction: Female Reproductive Biology

6.1. Summary

A number of features of female reproductive biology were examined in lines selected for age at reproduction, including the level of investment in sexual morphology, remating rate and the effect of non-virginity on age-specific mortality rate. The results suggested that the main mechanism for the extension of life span in 'old' line females was a reduction in early life fertility which was shown previously in these lines by Partridge and Pignatelli (unpublished data). This conclusion was based upon a number of different observations: no differences were found in female remating rate or ovary weight between selection regimes or the base stock; the pattern of ovariole number differences did not support the hypothesis of reduced reproductive investment in the 'old' line females and survival costs of non-virginity did not differ between regimes.

6.2. Introduction

The previous two chapters have described the effects of the application of selection on age at reproduction in an environment in which larval density was controlled and confounding forms of selection were avoided. This made it possible to examine adult and pre-adult characters for evidence of true responses and correlated responses to selection. A trade off between longevity and early fecundity was found in both sexes, but without any related changes in pre-adult development time, viability or adult body size, characters that have changed under previous selection regimes (Luckinbill *et al.*, 1984; Rose, 1984; Partridge and Fowler, 1992; Roper *et al.*, 1993; Chippendale *et al.*, 1994; Leroi *et al.*, 1994). It is clear that the evolution of these life history traits is not a prerequisite for the extension of life span by this mode of selection, but that a correlated response in early reproduction is needed. The effect of life span

selection on fecundity is well documented; females in lines selected for longer life have shown decreased early (Wattiaux, 1968; Rose and Charlesworth, 1981; Luckinbill *et al.*, 1984; Rose, 1984; the present study) or lifetime fecundity (Zwaan *et al.*, 1995b) in a number of separate populations of *D. melanogaster* and *D. subobscura*. The aim of the work in this chapter was to determine if any other aspects of female reproductive biology are altered by selection in such circumstances. The production of progeny by a female is the end product of a process that starts with the development of the gametes and sexual structures and then continues with adult pre-copulatory and copulatory behaviour, including elements such as courtship, mate choice, remating frequency and sperm competition. If fertility is under selection in the type of regime used here, then it follows that at least some of these stages could also be affected. Therefore, other features of female reproductive biology in the populations selected on age at reproduction were investigated in order to find out how selection had affected them.

The first aspect of female reproduction to be examined was the amount of investment that selected females made in their sexual morphology. It is possible that, given the evolution of reduced female fertility in response to selection for extended life span, fewer resources are allocated to the development of the ovaries. An organism must allocate available resources to somatic and reproductive functions. It is known that lipids are vital for reproduction (Geer *et al.*, 1970), including the production of fertile eggs (Doane, 1960), and that they also have an important role in the ability to resist starvation. Starvation resistance is correlated with fat fraction (Zwaan *et al.*, 1995b) and it has been suggested that populations selected for increased life span divert lipids away from reproduction and into maintenance roles, such as the tolerance of starvation, that enhance survival (Service *et al.*, 1988; Zwaan *et al.*, 1995b). 'O' females from the Rose laboratory once had smaller ovaries than 'B' females (Rose *et al.*, 1984), which fits the hypothesis that life span is extended by reduced allocation of certain resources to reproductive morphology. Lipids, being essential to both somatic and reproductive processes, could well be involved in this type of trade off. The ovary weight difference has since disappeared (Leroi *et al.*, 1994) along with the early fecundity difference in the

same lines. This may be the result of the adaptation of the 'O' and 'B' regimes to their respective selection environments over the long course of selection on these populations (Leroi *et al.*, 1994). The possibility that reduced investment in reproductive structures was a mechanism of life span extension in the present lines was investigated.

It is possible that female morphology was not the only target of selection. This is obvious when the observed patterns of fertility are considered. Early fecundity was reduced in the 'old' lines in the present experiments, but later progeny production did not differ from the base stock. A simple model of reduced reproductive capacity does not necessarily fit this pattern. This means that there may have been some other aspect of reproductive physiology or behaviour that responded to selection. Unlike ovariole number, which is fixed, this character would have to be flexible across the life span so that a rise in relative fecundity as life progressed would be possible.

The rate at which female *Drosophila* remate is potentially one such character. On mating, a female's receptivity to other courting males declines and her oviposition rate increases (Chen *et al.*, 1988; Kalb *et al.*, 1993). This behavioural response is caused by the transfer of main cell products from the accessory glands (Chapman *et al.*, 1995). Remating rate is dependent, to an extent, on the amount of sperm that a male transfers (Letsinger and Gromko, 1985), but also on the rate that sperm is used. Remating rate is an important component of female fitness because it is negatively correlated with female survival probability (Fowler and Partridge, 1989). It is this relationship with survival that suggests that the rate of a costly activity such as copulation frequency would be expected to change under selection, since reproduction should be optimised throughout life in response to selection on mortality rate (Charlesworth, 1994). Indeed, time to first mating increased in females of long lived lines relative to short lived females (Pletcher *et al.*, 1997). The effect that males from selected populations have on the remating activity of females has also been studied. 'B' line males induced a faster rate of oviposition, and therefore sired more progeny, than 'O' males early in life, and this difference reversed later on in life (Service and Vossbrink, 1996). The situation between males and females is dynamic; sexual behaviour evolves in each sex in

response to changes in the other (Rice, 1996). Remating rate should evolve to suit the long term interests of the female (Chapman *et al.*, 1995) because it affects survival. Therefore, the evolution of female remating rate in the present selected lines may represent another part of the mechanism that operates in response to selection on life span.

Does the evolution of altered life span involve a change in the level of, or response to, costs to survival incurred during mating? This might involve a change in the ability to resist mechanical damage incurred during copulation or the evolution of an altered behavioural response to male accessory gland products in terms of either oviposition or remating rates. The question does not just apply to the 'old' lines and selection for increased resistance to mating costs. The 'young' lines are also of interest because the evolution of shortened life span may result in an increased rate of reproduction, since future mating costs would present less of a threat to survival.

The evidence acquired thus far from selected lines on mating costs is inconclusive. In two sets of lines, the longevity difference between late and early reproduced lines is not dependent on mating status (Partridge and Fowler, 1992; Service, 1989). One regime, however, has shown a divergence in the survival cost of mating. Mated 'late' line females did not show as great a reduction in life span as mated 'early' line females when compared to virgins from the same lines (Luckinbill *et al.*, 1988b). This pattern was not seen in the males. Life span extension in these females was not solely due to a reduction in the cost of mating, however, because the difference between regimes persisted to an extent in virgins (Luckinbill *et al.*, 1988b). It is possible that egg laying plays a part in altered mating costs, because it is costly to females (Partridge *et al.*, 1987b). It would be informative to determine to what extent the divergence in mortality rates between lines is dependent on resistance to reproductive costs. To this end, the effect of mating and egg laying on female mortality was investigated.

This chapter details experiments designed to scrutinise some aspects of the reproductive biology of selection line females. The primary reason for performing the experiments was that there is a very important relationship between survival and reproduction because selection acts on a combination of

the two in order to optimise the life history in a given environment (Partridge and Barton, 1994; 1996). Therefore, it would be informative to know how selection on life span acts to shape female reproductive morphology, physiology and behaviour.

6.3. Methods and Materials

6.3.1. Female Reproductive Morphology

These experiments were designed to compare the degree of reproductive investment made by females in each of the selection regimes with reference to the base stock. Ovariole number and ovary weight were measured. When ovariole number was counted, the 'old' lines were in generations 34 to 36, and the 'young' lines were in generation 65. Flies from the selection lines were less than two weeks old when eggs were collected for standard density cultures, which were laid up at 50 larvae per vial. Dahomey flies for the three base stock replicates were reared from eggs collected from a cage filled with flies reared in low density bottle cultures.

Ovariole number is most easily determined in females that are not retaining eggs, because excess eggs fill up the ovarioles and make them difficult to count. Prior to dissection, therefore, the females were put onto new, yeasted Lewis food several times during a week in order to give them ample opportunity to oviposit. Ovaries were dissected out into a drop of PBS solution and the number of ovarioles in each ovary counted by teasing apart the ovarioles with a mounted needle. Twelve flies from each replicate line were sampled. A mistake was made with the labelling of the vials, so it was not possible to include 'young' and 'old' lines 3 in the analysis of this experiment.

Ovary weights were measured in females from generation 66 for the 'young' lines and generations 35 to 37 for the 'old' lines. Flies for the three base stock replicates were collected as for the ovariole counting. All measurements were carried out on four day old virgin females in order to standardise conditions as much as possible. Both ovaries were dissected out in a drop of

PBS solution, placed onto a pre-weighed piece of aluminium foil and dried at 60°C for an hour. Measurements were made to the nearest 100th of a milligram on a Sartorius micro balance, taking the weight of the foil into consideration. Ten females were sampled from each line.

6.3.2. Female Remating Rate

The aim of this experiment was to investigate female remating rate at the times when females in each selection regime were required to contribute eggs to the next generation. Eggs were collected from the cages that had been established for the second development time experiment described in chapter 5. These flies were reared from low density bottle cultures, including the base stock flies. The 'young' lines were in generation 60, and the 'old' lines were in generations 31 to 33. Females were allowed to lay eggs on grape juice medium and the hatching larvae reared in groups of 50 per vial to provide female progeny for the experiment. The only males required were standard Dahomey males that were reared in large numbers at low density in bottle cultures.

Virgin females were collected, aged for two to three days and then placed individually into shell vials with two Dahomey males. All vials, approximately 50 per line, were placed onto observation boards in a constant 25°C environment. The majority mated within minutes of being placed with males and those that were observed to mate during that day were used in the experiment. The few that did not mate at this point were discarded. The mated females were then kept with the same males and on the following days the vials were checked every 20 minutes between 10 am and 4 pm. Mating frequency was scored for each female during the first week of life, and again during the third and fourth weeks. This was in order to obtain data on remating activity during those periods when 'young' and 'old' lines usually have eggs collected to establish the next generation. During the interim week, females were held individually on sugar-yeast medium without males. At the end of this, each was again placed with two new males on Lewis medium. Matings were scored and

those females that mated within approximately eight hours were kept and had remating rate monitored again for six hours a day during the following week.

6.3.3. Costs to Survival of Non-Virginity in Females

Virgin and mated longevity was measured in selection line females. This was in order to compare the extent of the cost of non-virginity to survival in 'old' and 'young' line females. Eggs were collected from the selection line cages when both 'old' and 'young' flies were approximately a week old, the 'youngs' were in generation 64 and the 'olds' were in generations 34 to 36. At the same time, eggs were taken from a base stock cage that had been produced from low density bottle cultures. All larvae were reared at 50 per vial, and when the females eclosed two cages per line were established. For each line, one cage contained 500 virgin females, the other contained 250 virgin females and 250 standard base stock males. All cages were treated in the same way as the selection lines, but each time that they were fed corpses were collected and the number of females recorded. The density in each cage was allowed to decline in the same way as it would in the normal regimes.

Fecundity of the selection line females was also checked because egg laying is implicated in the female cost of mating (Partridge *et al.*, 1987b), and so in the event a of divergence in survival costs between the two selection regimes, information on the contribution of egg laying to this effect would be valuable. On days 7 and 34, fecundity was measured by placing grape juice medium with yeast paste into each cage for an hour and a half on the first occasion and for four hours on the second. The lids were then removed and the number of eggs counted. During analysis, female mortality and laying time was taken into account when comparing fecundity between cages.

6.3.4. Statistical Analysis

The morphology, mortality and fecundity data were tested for deviation from the normal distribution and equality of group variances. Depending on the outcome, data were then either analysed using ANOVA, suitably transformed so that ANOVA could then be used or analysed using the Kruskal Wallis test. Parametric analyses, it may be assumed, were only used on normally distributed, homoscedastic data. Female mated and virgin mortality was analysed in the same way as male mortality, by examining age-specific mortality rates, the point of 10% mortality, and by comparing the age-dependent Gompertz parameter. For details of these calculations, see section 4.3.4. For the comparison of these mortality parameters between selection regimes and mating treatments, whenever ANOVA was used, a two-way design was employed with selection regime crossed against mating status, both as fixed effects.

The remating data showed an extremely left-skewed distribution, could not be suitably transformed and so were analysed non-parametrically. Analysis of chi-square was used to examine the extent of between-regime variation present for remating rate in selection line and base stock females during the periods when eggs were collected from one or other of the selection regimes, the first week for 'young' females and the third or fourth weeks for 'old' females. The test also took the variation within each regime into account. The remating scores were split into two blocks, those collected during the first week of life (days 2 to 6) and those collected during the third and fourth weeks of life (days 16 to 22). For each of these two blocks of data, the number of days on which each fly mated and the number on which each fly did not, out of the total number of days on which they were observed (four for the first block and six for the second), were placed into a contingency table. The following chi-square values were then calculated, based upon the null hypothesis that there were no differences between regimes or replicate lines with respect to remating frequency. For each block of data, the selection regime X^2 (based upon a contingency table in which all data within each respective regime, including the base stock, were grouped to calculate a chi-square value reflecting the

between-regime variation) and the replicate line X^2 values (with a contingency table for each regime that examined within-regime variation) were calculated. Degrees of freedom were equivalent to n-1 in each case; two degrees of freedom (3-1) for the selection X^2 and four degrees of freedom (5-1) for each replicate line X^2. The three values obtained for within-regime variation ('old', 'young' and base stock) were then added together. The resulting values were used to produce an F ratio in a way similar to an ANOVA. The F ratio was calculated as follows.

F ratio = (Selection X^2 / 2 df) / (Σ Replicate Line X^2 / 12 df)

The p value was then calculated from tables of F ratios, with the degrees of freedom from the above calculation as the numerator and denominator, as would be the case in a standard F test.

6.4. Results

6.4.1. Female Reproductive Morphology

The number of ovarioles from the left and right ovaries in each female were totalled for this analysis. The regime and line means are shown in Table 6.1, and the regime means are shown graphically in Figure 6.1. Analysis of variance was used to test for a divergence in ovariole number between and within regimes, with line nested within regime as a random effect (see Table 6.2). There was not any significant variation among replicate lines, but there was between regimes. This difference was tested further using t-tests. The three comparisons made were tested against Bonferroni-corrected significance levels. 'Old' line females had significantly more ovarioles than 'young' line females (t_1 = 2.73, p = 0.007, tested at the 0.017 level). The base stock and 'old' line females did not differ significantly (t_1 = 0.31, p = 0.76, tested at the 0.05 level), and neither did the 'young' line and base stock females (t_1 = 2.22, p = 0.029, tested at the 0.025 level).

Before the ovary weight experiment could be analysed parametrically, the data had to be squared in order to make them conform to the assumptions of ANOVA, since the original data set was not normally distributed. Replicate line was nested within regime as a random effect and the ANOVA showed that there were significant differences in ovary mass between replicate lines within each regime (see Table 6.3), but not between either regime or the base stock (see Figure 6.2 for the regime means). The untransformed regime and line means are shown in Table 6.4

6.4.2. Female Remating Rate

Remating rate was analysed in two blocks, as described in section 6.3.4. The mean daily remating rates for females in each regime, on the days that they were observed, are shown in Figure 6.3. The analysis tested for differences between regimes in the number of days that each female spent mating out of each block. The first block, incorporating data collected on days 2, 4, 5 and 6 of life, produced an F ratio of 2.49 (df = 2 and 12). When tested, $p = 0.12$. The second set of observations were made on days 16, 17, 18, 20, 21 and 22 of adult life. For this analysis, F ratio = 1.91, df = 2 and 12, $p = 0.19$. Therefore, no differences in remating rate on a day by day basis were found between 'old', 'young' or base stock females prior to the times when 'young' or 'old' line females were expected to produce eggs for the next generation, i.e. leading up to the end of the first week of life for 'young' females, and the third and fourth weeks of life for the 'old' females.

6.4.3. Costs to Survival of Non-Virginity in Females

Female cumulative survival probability for each mated and celibate 'young' line is given in Figure 6.4, and the 'old' lines are shown on the same scale in Figure 6.5. As can be seen from these graphs, the mated 'old' lines (median life span = 46 days) are not as distinct from the virgin 'old' lines (53 d)

in terms of survival probability as the mated and virgin 'young' lines (36 d and 51 d respectively) are from each other. The median life spans also indicate this fact.

The question to be addressed by this analysis was, what was the effect of copulation on the mortality patterns of each selection regime? The effect of selection on the 'old' and 'young' regimes with respect to the base stock has been examined elsewhere. Age-specific mortality rate was calculated for two periods during the experiment, before day 8 and days 8 to 29, representing the respective periods during which the 'young' and 'old' flies were alive and then when just the 'old' flies were alive. Age specific mortality rate was calculated as described in section 2.4.2. For the first week of life, mating status did not have a significant effect on mortality rate (see Table 6.5a for details of the ANOVA), but selection regime did. There was no significant interaction effect. For the second to fourth weeks of life, the age-specific mortality data had to be natural-log transformed to make it normally distributed (Shapiro-Wilk $W = 0.94$, $p = 0.26$). During this period of life, the selection regimes did not differ significantly in terms of mortality rate (see Table 6.5b for details), but mated females died at a significantly faster rate than virgin females. Again, there was no interaction effect. The untransformed means for each replicate line and the selection regime grand means for both periods for which mortality was analysed are shown in Table 6.6.

The point of 10% mortality was compared between mated and virgin females and between each regime. It was reached by 'old' virgins on day 38, mated 'old' females on day 33, 'young' virgins on day 37 and mated 'youngs' on day 26. The points of 10% mortality for each replicate line and selection regime are shown in table 6.7. Selection regime did not have a significant effect on this mortality parameter (see Table 6.5c), but mating status did. There was no significant interaction effect.

Gompertz parameters were estimated for the period between 10% and 90% mortality, the senescent period. Parameters were estimated for each set of five replicate lines within the four groups; mated 'old', mated 'young', virgin 'old' and virgin 'young'. In each group, there was highly significant heterogeneity between replicates for the rate of increase in mortality rate ($F = 16.58$, 19.67,

19.36 and 20.45 respectively, $p < 0.0001$ in every case). The values of G from each of the four groups were then analysed with a two-way ANOVA of the same design that had been used to analyse age-specific mortality and the points of 10% mortality, described above. No significant differences were found between selection regimes or mating status, or as an interaction in terms of estimates of G (see Table 6.5d for the ANOVA). The estimates of both Gompertz parameters for each replicate line in both treatments are shown in Table 6.8.

When fecundity was sampled on days 7 and 34 of this experiment, it was found that the same pattern was evident in both regimes. The replicate line and regime means are shown in Table 6.9. The data from these samples were not homoscedastic or normally distributed, and no suitable transformation could be found to correct the two problems at the same time. Therefore, selection regimes were analysed separately using Kruskal Wallis. Mated 'old' and 'young' females had significantly higher fecundity on day 7 than their respective virgin counterparts ($X^2 = 6.82$, $p = 0.01$ for both tests). Fecundity on day 34 did not differ significantly for either regime ('old', $X^2 = 0.53$, $p = 0.46$; 'young', $X^2 = 0.1$, $p = 0.75$). One between-regime test was performed. Fecundity of mated 'old' females was compared with that of mated 'young' females for both sampling intervals. On neither day did fecundity differ significantly (for days 7 and 34; $X^2 = 0.88$ and 0.10, $p = 0.35$ and 0.75 respectively).

Selection Regime	Line	Mean Ovariole Number per Female	95% confidence limits
YOUNG	1	29.75	1.9
	2	30.33	2.08
	3	-	-
	4	31.33	2.92
	5	32.08	2.0
Grand Mean		30.88	1.44
OLD	1	32.17	2.38
	2	32.58	1.75
	3	-	-
	4	33.0	2.94
	5	33.67	1.7
Grand Mean		32.85	0.89
DAHOMEY	1	31.75	2.06
	2	33.25	2.13
	3	32.83	2.32
Grand Mean		32.61	1.42

Table 6.1. Replicate line and selection regime means (± 95% CL) for ovariole number.

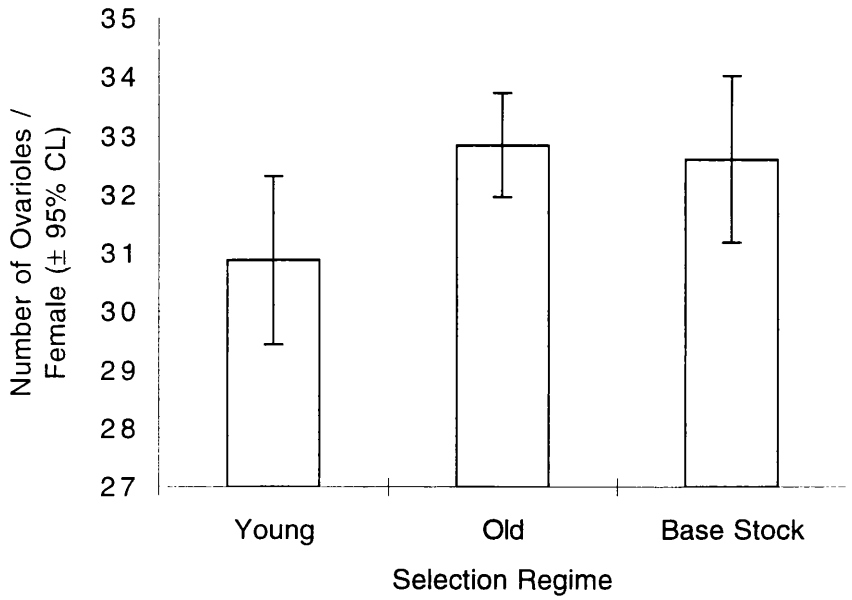

Figure 6.1. Mean ovariole number per female (± 95% CL) for each selection regime.

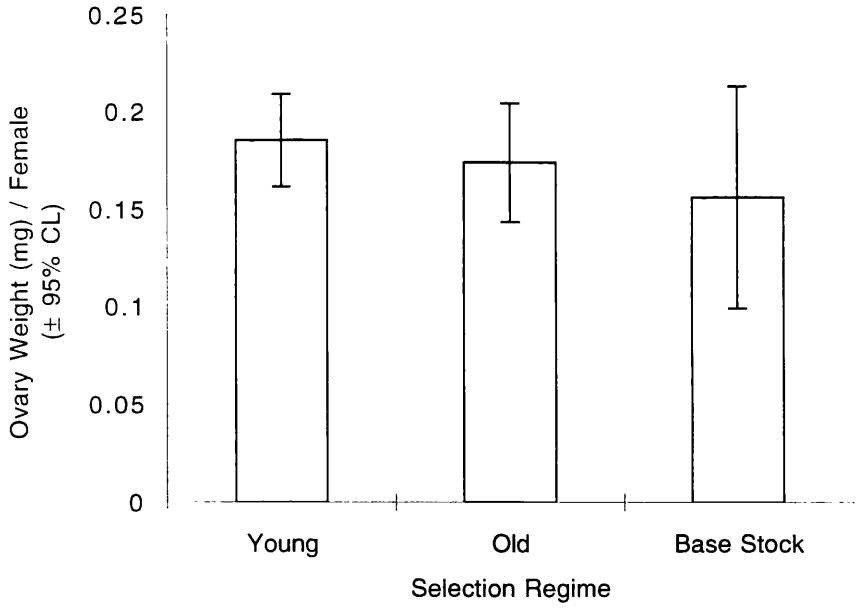

Figure 6.2. Untransformed ovary weight (mg) per female (± 95% CL) for each selection regime.

Source	SS	MS	DF	F ratio	p value
Regime	108.6	54.3	2	4.31	0.016
Line	67.87	8.48	8	0.67	0.71

Table 6.2. ANOVA of ovariole number per female, with replicate line nested within regime as a random effect. SS = sums of squares, MS = means squares, DF = degrees of freedom.

Source	SS	MS	DF	F ratio	p value
Regime	0.0015	0.0007	2	0.96	0.41
Line	0.008	0.0008	10	4.66	<0.0001

Table 6.3. ANOVA of transformed ovary weight data. Replicate line is nested within selection regime as a random effect.

Selection Regime	Line	Mean Ovary Weight (mg)	95% confidence limits
YOUNG	1	0.221	0.02
	2	0.172	0.03
	3	0.184	0.02
	4	0.173	0.03
	5	0.175	0.01
Grand Mean		0.19	0.02
OLD	1	0.16	0.02
	2	0.168	0.02
	3	0.211	0.03
	4	0.142	0.03
	5	0.187	0.02
Grand Mean		0.17	0.03
DAHOMEY	1	0.174	0.04
	2	0.12	0.02
	3	0.173	0.01
Grand Mean		0.16	0.06

Table 6.4. Untransformed regime and line means (± 95% CL) from the ovary weight experiment.

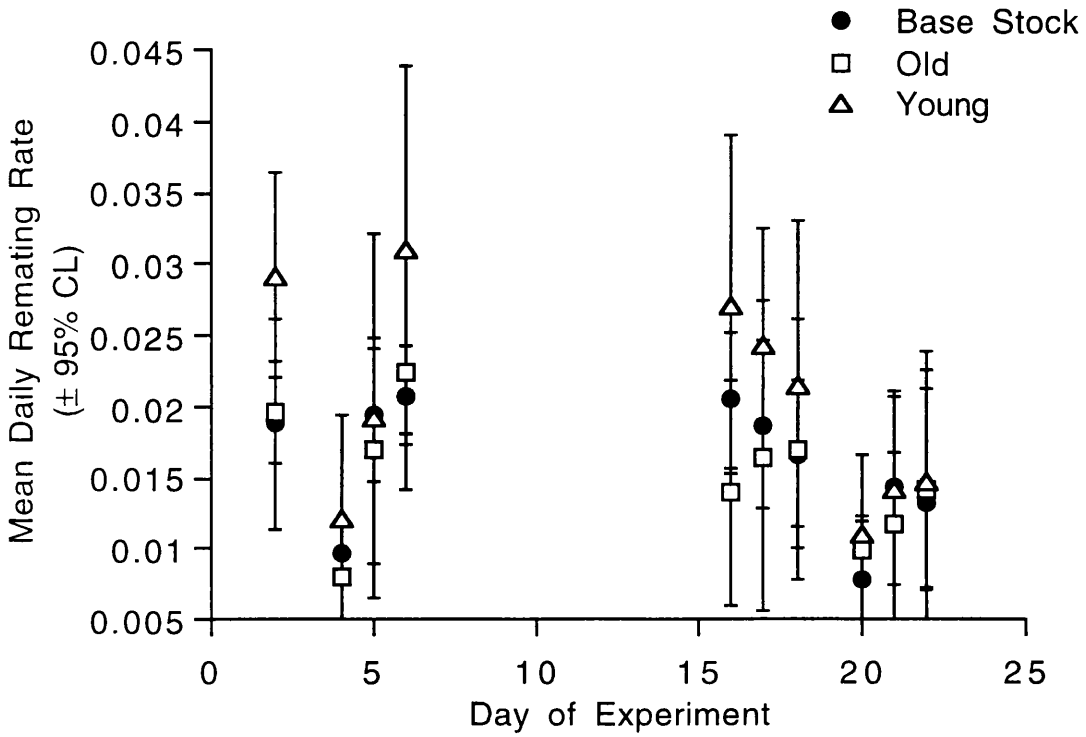

Figure 6.3. Mean daily remating rate (± 95% CL) for the selection regimes and base stock. See text for details of statistical analysis.

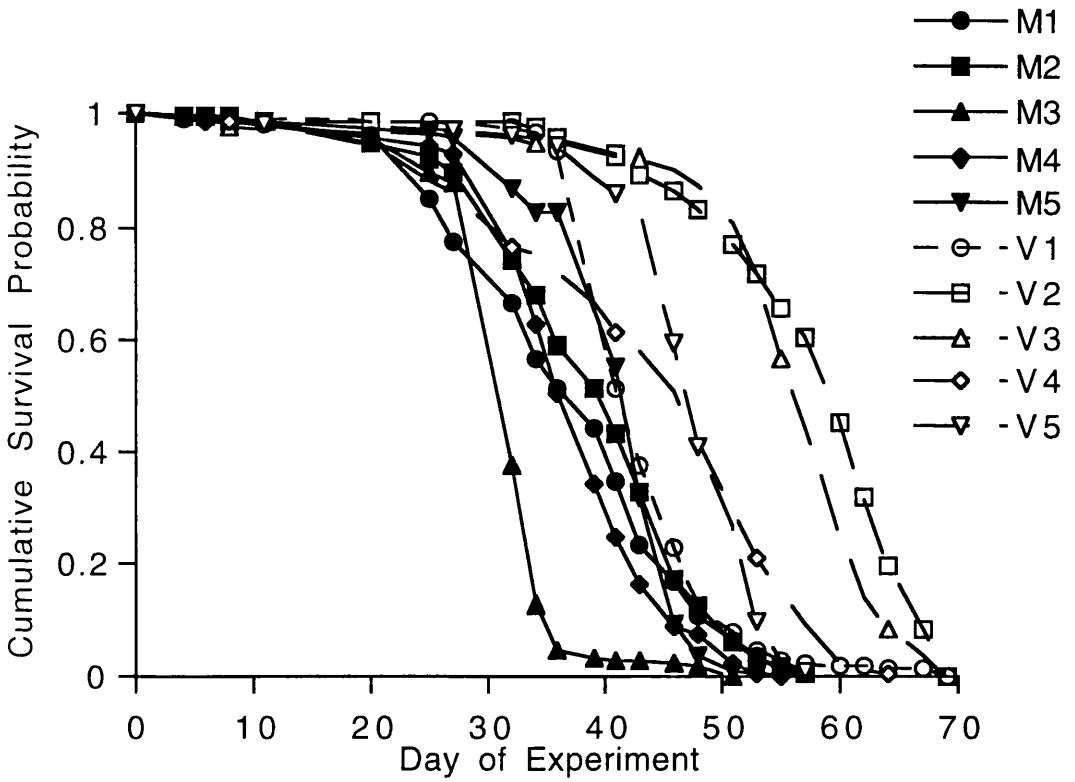

Figure 6.4. Cumulative survival probability for mated and virgin 'young' line females. M = mated, V= virgin, numbers in the key indicate replicate 'young' lines.

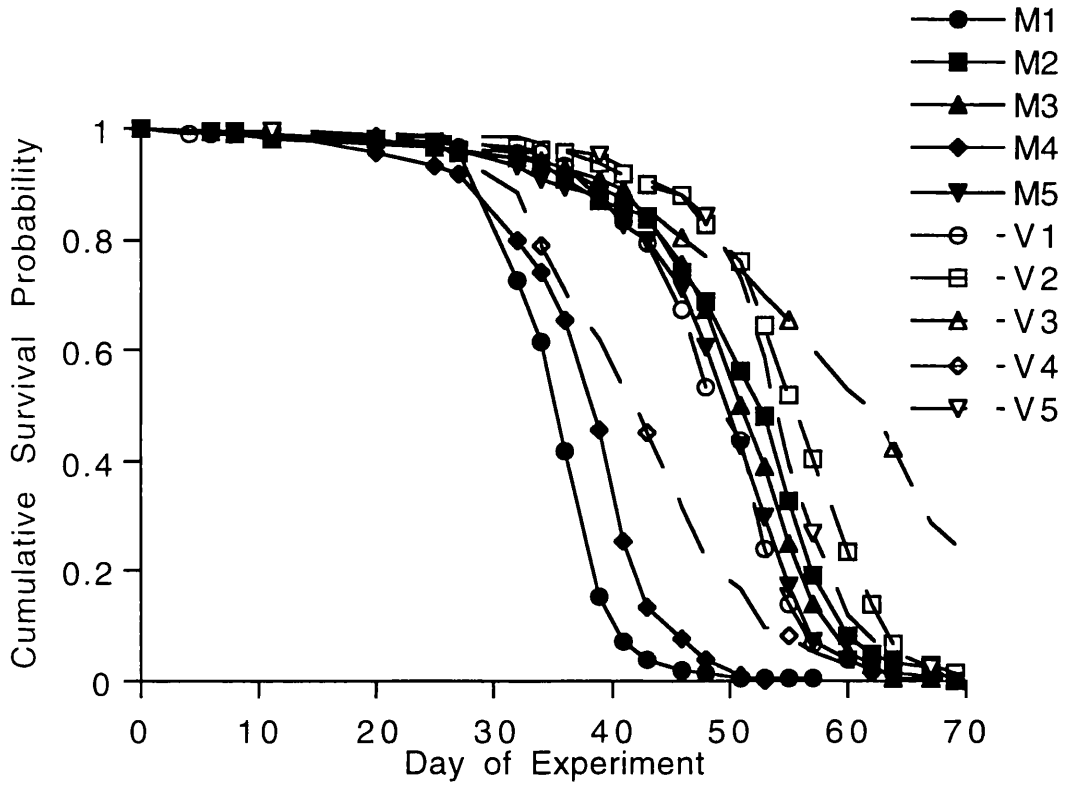

Figure 6.5. Cumulative survival probability for mated and virgin 'old' line females. M = mated, V= virgin, numbers in the key indicate replicate 'old' lines.

Source	SS	MS	DF	F ratio	p value
Regime	0.0002	0.0002	1	9.67	0.01
Mating Status	0.000001	0.000001	1	0.01	0.94
Interaction	0.00001	0.00001	1	0.51	0.49

Table 6.5a. Age-specific mortality up to day 8.

Regime	0.81	0.81	1	1.17	0.30
Mating Status	8.20	8.20	1	11.86	0.003
Interaction	1.41	1.41	1	2.04	0.17

Table 6.5b. Age-specific mortality days 8 to 29.

Regime	81.04	81.04	1	2.37	0.14
Mating Status	321.60	321.60	1	9.42	0.01
Interaction	41.64	41.64	1	1.22	0.29

Table 6.5c. Points of 10% mortality.

Regime	0.14	0.14	1	1.14	0.30
Mating Status	0.19	0.19	1	1.51	0.24
Interaction	0.02	0.02	1	0.14	0.72

Table 6.5d. Age-dependent Gompertz parameter.

Table 6.5a-d. Analysis of variance of age specific mortality in the first week (6.5a) and from days 8 to 29 of life (Table 6.5b), points of 10% mortality (6.5c) and estimates of the age-dependent Gompertz parameter, G (6.5d).

Selection Regime	Line	Mortality Rate (First week)	Mortality Rate (2nd-4th weeks)
YOUNG MATED	1	0.014	0.245
	2	0.005	0.175
	3	0.014	0.208
	4	0.014	0.080
	5	0.010	0.069
Grand Mean (± 95% CL)		0.011 (±0.005)	0.155 (±0.089)
OLD MATED	1	0.004	0.145
	2	0.005	0.032
	3	0.010	0.038
	4	0.005	0.093
	5	0.009	0.028
Grand Mean (± 95% CL)		0.007 (±0.003)	0.067 (±0.059)
YOUNG VIRGIN	1	0.007	0.012
	2	0.008	0.008
	3	0.022	0.017
	4	0.012	0.181
	5	0.016	0.019
Grand Mean (± 95% CL)		0.013(±0.007)	0.048(±0.075)
OLD VIRGIN	1	0.012	0.028
	2	0.007	0.021
	3	0.003	0.021
	4	0.005	0.071
	5	0.000	0.012
Grand Mean (± 95% CL)		0.005 (±0.005)	0.031 (±0.026)

Table 6.6. Line and regime grand means (± 95% CL) for female age-specific mortality rates from each reproductive treatment.

Selection Regime	Line	Point of 10% Mortality (days)
YOUNG MATED	1	23
	2	27
	3	25
	4	28
	5	30
Grand Mean (± 95% CL)		26.58 (±3.29)
OLD MATED	1	28
	2	37
	3	40
	4	28
	5	35
Grand Mean (± 95% CL)		33.49 (±6.23)
YOUNG VIRGIN	1	37
	2	43
	3	46
	4	24
	5	38
Grand Mean (± 95% CL)		37.49 (±9.73)
OLD VIRGIN	1	38
	2	43
	3	38
	4	31
	5	44
Grand Mean (± 95% CL)		38.63 (6.02)

Table 6.7. Points of 10% mortality for females in each replicate line and reproductive treatment, and the regime means (with confidence limits).

Selection Regime	Line	Age-Dependent Parameter, G	Age-Independent Parameter, A
YOUNG MATED	1	0.1087	0.0001
	2	0.1264	0.0006
	3	0.3787	0.000002
	4	0.1741	0.00001
	5	0.2309	0.00001
Grand Mean (± 95% CL)		0.2038 (± 0.125)	0.00002 (± 0.00003)
OLD MATED	1	0.2555	0.00002
	2	0.1454	0.00006
	3	0.1677	0.00003
	4	0.196	0.00008
	5	0.1616	0.00004
Grand Mean (± 95% CL)		0.1852 (± 0.05)	0.00005 (±0.00003)
YOUNG VIRGIN	1	0.2271	0.00002
	2	0.1404	0.00002
	3	0.1795	0.00001
	4	0.0911	0.0011
	5	0.2173	0.00001
Grand Mean (± 95% CL)		0.1712 (± 0.065)	0.00002 (± 0.0006)
OLD VIRGIN	1	0.1686	0.00003
	2	0.1632	0.000003
	3	0.0924	0.00002
	4	0.1290	0.00004
	5	0.1982	0.000003
Grand Mean (± 95% CL)		0.1503 (± 0.047)	0.00002 (± 0.00003)

Table 6.8. Estimates of female Gompertz parameters for replicate lines in each reproductive treatment, and the regime means and 95% confidence limits.

Selection Regime	Line	Fecundity/Female on day 7	Fecundity/Female on day 35
YOUNG MATED	1	10.33	9.47
	2	10.73	3.27
	3	7.52	3.48
	4	3.43	0
	5	4.35	1.64
Grand Mean (± 95% CL)		7.27 (±3.84)	3.58 (±4.12)
OLD MATED	1	10.96	0.13
	2	15.33	3.03
	3	5.19	3.10
	4	23.43	2.65
	5	1.38	4.24
Grand Mean (± 95% CL)		11.26 (±9.95)	2.63 (±1.75)
YOUNG VIRGIN	1	0.21	2.80
	2	2.60	6.17
	3	0.11	1.83
	4	1.48	3.11
	5	1.52	0.56
Grand Mean (± 95% CL)		1.18 (±1.20)	2.89 (±2.39)
OLD VIRGIN	1	0.48	2.41
	2	0.05	0.52
	3	0.08	2.61
	4	0.24	0.71
	5	0.01	4.87
Grand Mean (± 95% CL)		1.07 (0.22)	2.22 (±2.02)

Table 6.9. Fecundity per female in replicate cages, and the regime grand means (± 95% CL) on days 7 and 34 of the female survival experiment.

6.5. Conclusions

- 'Young' line female ovariole number showed a significant decline with respect to 'old' line females, but not the base stock. Ovary weight did not differ significantly between regimes.
- Female remating rate did not differ significantly between either selection regime or the base stock when it was sampled in the first, third and fourth weeks of life.
- The cost of non-virginity to survival did not differ significantly between 'old' and 'young' regime females. Both regimes exhibited a cost of non-virginity that did not appear to alter the rate of increase of mortality rate following the point of 10% mortality. Instead, mating bought that point forward compared to celibate females.

6.6. Discussion

6.6.1. The Effect of Non-Virginity on Mortality Rate

No evidence was found to suggest that part of the response to selection for extended life span was a reduction in the survival costs of non-virginity in 'old' line females. A cost of non-virginity was shown in both selection regimes after the first week of life in terms of age-specific mortality and the time taken to reach the point of 10% mortality. No evidence was found for an acceleration of the rate of increase in mortality following this point, as indicated by the lack of divergence in the age-dependent Gompertz parameter between mated and celibate treatments. This indicates that reproduction altered the onset of the decline in mortality rate, but did not cause an acceleration of the rate of that decline. To an extent, this parallels the effects of mating on wild type *Drosophila* males described in chapter 3, which also suggested that in certain cases the acceleration of mortality rate is not changed by reproduction, but that the onset of the decline in survivorship is bought forward in time.

Non-virginity did not affect mortality until after the first week of life, as indicated by the lack of divergence between mated and virgin treatments early in life. This suggests either that non-virginity did not have a survival cost associated with it during the first week of life, insofar as recently eclosed females are hardy enough not to be injured during mating, or that early life reproduction caused an accumulation of damage that only affected survival after the end of the first week. This also reflects results presented in chapter 3; reproduction in Dahomey strain males was found to affect future survival probability. Likewise, in the present experiments mating during the first week of life did not elevate the current risk of dying, and it is possible that copulation in the first week affected subsequent survivorship.

In the first week of life, 'old' line females died at a lower rate than 'young' line females, a pattern that was not seen in the males (see chapter 4) and has not been seen previously in the females (Partridge and Pignatelli, unpublished data). The reason for this is unclear, since at this point in life both regimes would have not yet passed eggs on to the next generation so selection to maintain mortality rate at low levels would have been acting on both regimes. It could mean that the continuing effect of selection has produced a reduction in the base-line level of mortality in the 'old' females. The lack of divergence in age-specific mortality rate between regimes following the end of week one is also a puzzle, but an inspection of the regime means for the mortality rate in weeks two to four (Table 6.6.) suggests an answer. Whereas the two-way ANOVA on this data did not show a significant divergence between selection regimes, the means suggest that there was a difference, but that the within-regime heterogeneity that existed confounded the result of the analysis. These data highlight the fact that the present selection regimes were prone to a large amount of variation between replicate lines. No particular replicate line showed consistently high or low fitness in terms of the life history traits that are discussed in this thesis. Therefore, the variation is probably not attributable to genetic inbreeding effects. It is far more likely that the basis of the variation was environmental, and could have arisen from slight differences between replicates with respect to handling, food or some aspect of the culture environment. For example, if temperature had varied between different areas of

the controlled temperature rooms that the selection regimes were kept in, different replicate lines would have experienced within-generation temperature differences. This is unfortunate, and illustrates the need for extensive replication within selection regimes, rigorous standardisation of experimental procedures and culture environment, and complete randomisation of the position of replicate cages within the controlled temperature rooms.

The patterns of fecundity that were found in the present experiments support the conclusion that costs of non-virginity did not differ significantly between selection regimes. Early-life mated fecundity did not differ between regimes, but it was greater in both regimes than the respective celibate treatments. However, when Partridge and co-workers measured female progeny production, samples taken on three consecutive days during the first week of life showed that 'old' line fertility was less than that of the base stock and the 'young' regime (Partridge and Pignatelli, unpublished data). The differences in the way that fecundity was sampled on the two occasions may have lead to a disagreement between the results. For instance, fecundity was sampled on three consecutive days in the earlier experiment, and on just one day for each of the two samples taken in the later experiment. In addition, a smaller amount of yeast paste was put onto the grape juice medium in the later experiment. This would have lead to lower overall fecundity in females from both regimes in the second experiment, as well as a shorter sampling time, which might have accentuated sampling error and environmental differences between replicate lines. Therefore, the later result, while agreeing with the mortality data, does not accurately reflect fecundity differences that have been previously shown to exist between selection regimes.

6.6.2. Remating Rate and Reproductive Morphology in Females

Remating rate was examined in these selection lines because reproduction is a costly activity for *Drosophila* females (Malick and Kidwell, 1966; Partridge et al., 1986) and an increase in remating rate reduces female life span (Fowler and Partridge, 1989). It was hypothesised that 'old' regime

females might mate less often as part of a response to selection to extend life. No evidence was found for this in the present experiments. However, there is a possibility that the experimental conditions were not conducive to the measurement of a character that is easily affected by environmental factors. For example, the presence or absence of live yeast greatly affects the rate of oviposition in female *Drosophila* (Leroi *et al.*, 1994). Females that are exposed to yeast will oviposit at a greater rate and so will use up stored sperm at a faster rate. Therefore, the absence of yeast in the vials used in this experiment will have reduced the rate of oviposition, making the rate of sperm depletion slower. There is some debate as to whether the decline in sperm numbers actually triggers remating; in experiments where intermittent exposure to males occurred, sperm number mattered (Manning, 1962; Letsinger and Gromko, 1985), but when continuous exposure was used, it did not (Newport and Gromko, 1984). However, there is certainly a period that follows mating during which a female is not receptive, and it is likely that the level of sperm has some effect. Therefore, the decision not to include yeast granules in the vials in which remating was observed may have lead to a suppression of fecundity and a consequent reduction in the rate that each female remated. The fact that remating rate was not sampled in a cage environment may also have resulted in any remating differences that have evolved between regimes during selection not being expressed. Prior to the experiment, it was envisaged that observation of copulating pairs in vials would be less prone to experimental error than observation of many mating pairs in a cage environment. However, this may have lead to a change in the expression of the phenotype that was being studied.

The relative investment in reproductive morphology made by 'old' and 'young' females was investigated because the previously observed reduction in female fecundity may have been accompanied by a reduction in other costly activities. When ovariole number and ovary weight were measured, no evidence was found for reduced investment in reproductive morphology on the part of 'old' line females. A reduction in ovary weight has been shown in another set of selection lines (Rose *et al.*, 1984). Indeed, it was also hinted at in the present lines when female, but not male, dry weight was found to be

significantly lower in 'old' line females compared to the base stock females. However, ovary weight did not show any differences between base stock and females from either selection regime. Furthermore, 'old' line females had significantly more ovarioles than 'young' line females, suggesting that, if anything, the long lived lines were putting more resources into reproduction. However, this does not seem very likely, since a trade off has been shown between early fertility and life span in these lines, suggesting that extended survival probability occurs at the expense of reduced reproductive output, not that more resources are put into it. It is possible that the difference in ovariole number does not have any biological significance. An inspection of the regime means shown in Table 6.1 reveals that there is a great deal of overlap between the 'old', 'young' and base stock females in terms of ovariole number. The difference between 'old' and 'young' females may therefore have been the result of sampling error caused by relatively small sample sizes. In addition, one replicate was lost from each selection regime during this experiment due to a mistake made while labelling vials of flies, so the statistical power was reduced below what it otherwise would have been.

The female dry weight difference between 'old' lines and the base stock may have been due to fecundity differences between the regimes at the age of sampling. Dry weight was measured in females early in life, the time when 'old' line fecundity was shown to be significantly lower than that of base stock females (Partridge and Pignatelli, unpublished data), so the difference in the number of eggs being carried by flies in each regime may have caused them to be different weights. However, fecundity in the 'old' lines was also found to be depressed below that of the 'young' lines in the same experiment, which does not fit the hypothesis that the patterns of dry weight were due to fecundity differences because 'old' and 'young' females did not differ significantly in terms of dry weight. In addition, and perhaps more importantly, if this hypothesis was correct, one would have expected ovary weight to have also reflected the differences in fecundity, which it did not, even though it was also measured in young flies. This leads to the conclusion that either the dry weight differences were also an artefact, or that they were the result of a divergence in some aspect of morphology other than that of the sexual organs. It is difficult to believe

that the first explanation is true because the patterns of dry weight in males and females were distinctly different. Would an effect of sampling error not be seen in both sexes? Therefore, it is possible that selection on the 'old' regime produced a change in an aspect of female physiology or morphology unrelated to reproduction that reduced 'old' regime dry weight relative to the base stock.

The investigations of female remating rate and reproductive morphology have shown that copulation frequency and reduced investment in reproductive morphology were not mechanisms by which 'old' line females lived longer. Similarly, no evidence has been found for 'young' regime females increasing investment in reproduction. This is not surprising, because early life fecundity in 'young' line females was not shown to be any greater than that of the base stock females. These findings are in contrast with other studies that have been carried out upon populations selected on age at reproduction. Females in the Luckinbill populations selected for short life span showed a decreased time to first mating early in life (Pletcher *et. al.*, 1997) and increased survivorship costs of non-virginity (Luckinbill *et al.*, 1988b) compared with the long lived lines. The Rose populations showed a relative reduction in 'O' regime ovary weight when measured early in the history of the lines (Rose *et al.*, 1984), whereas the longevity difference in females between 'O' and 'B' regimes was not affected by mating status (Service, 1989). Female remating rate has not been measured in the Rose lines, but it has been found that males in the 'B' populations have evolved the ability to induce a faster remating frequency than 'O' males early in life (Service and Vossbrink, 1996). The way in which selection has acted upon these different sets of lines, and on others such as the Partridge and Fowler lines (1992), is varied. It would be surprising to get agreement between regimes because the selection environment differed between laboratories. These results illustrate how apparently subtle differences in the details of each selection regime can bring about totally divergent responses in terms of reproductive behaviour in order to extend life span. In addition, the current populations have been selected in conditions that have avoided covert selection on rapid development and incorporated standardised larval densities. It is possible that certain patterns that have been observed in other regimes with respect to

divergent reproductive biology have not been responses to selection on age at reproduction, but the result of some confounding effect of a particular regime.

6.6.3. Reproduction and Selection on Life Span

It appears that the primary response to selection on age at reproduction in the 'old' lines was reduced early life fecundity. This would have caused a reduction in some costly element of reproduction that allowed 'old' females to live longer. No changes in female remating rate or reproductive morphology were found in the 'old' regime. Therefore, it is quite possible that females in the 'old' regime showed an decrease in age-specific mortality rate because they laid fewer eggs. Whereas the current fecundity data taken during the female mortality experiment do not support this, as previously discussed, there are reasons to believe that the results of Partridge and Pignatelli, showing decreased fecundity in the 'old' lines, are more reliable. Egg laying has been shown to be costly in its own right in *Drosophila* (Partridge *et al.*, 1987b) and in *Callosobruchus* (Tatar *et al.*, 1993). Other selected populations have shown a difference in early fecundity between early and late reproduced lines (Luckinbill *et al.*, 1984; 1987). These lines also showed a difference in the magnitude of their survival costs of non-virginity (Luckinbill *et al.*, 1988b). This is in contrast with the present results because whereas 'old' line females had lower early life fecundity, they did not show a reduction in their survival costs of non-virginity compared with the 'young' lines. Therefore, the divergence in mating costs shown by Luckinbill *et al.* was evidently not solely due to oviposition differences. In the present 'old' and 'young' lines, the extension of 'old' female survivorship was, apparently, solely the result of reduced oviposition rate.

Once again, these results highlight the value of a base stock reference, since previous studies that have monitored female reproductive characters have not been able to distinguish between responses in long and short lived lines relative to the original founder population. In this study it has been possible to see that the responses that have occurred as a result of selection have been in the 'old' regime only. Comparisons with the base stock have

confirmed that no changes were found in 'young' female fecundity or reproductive behaviour and morphology.

Chapter 7. General Discussion

7.1. Reproduction and Senescence in *Drosophila*

A number of experiments described in this thesis have shown that, in many cases, reproduction does not affect the level of instantaneous risk experienced during copulation in *Drosophila*, but that it generally contributes to a subsequent persistent decline in survival. Males allowed continual access to receptive females showed an irreversible decline in survivorship (see chapter 3). The permanent loss of fertility in the same males also contributed to an age-specific decline in fitness. In addition, mated and celibate selection regime females showed no divergence in age-specific mortality rate in the first week of life, but did diverge after that (see chapter 6), suggesting that a cost of non-virginity was expressed in terms of a decline in future survival rather than elevated risk. These findings are in broad agreement with theories of senescence that are based upon a trade off between survival and reproduction: evidence for such a trade off, between mortality and early life reproductive success, was shown in both males and females in populations selected for age at reproduction that had not been subject to confounding forms of selection on development time or the effects of uncontrolled larval rearing densities.

The theory of antagonistic pleiotropy predicts that selection for extended longevity will favour alleles with a beneficial effect late in life despite an earlier deleterious effect on fecundity. In contrast, the predictions of the disposable soma hypothesis are more explicit in terms of the physiological mechanisms that are affected by selection on life span. A trade off between survival and reproduction may occur on a number of different levels (Kirkwood and Rose, 1991): the direct metabolic level, whereby constrained characters are reliant on the same resource, such as lipids; the indirect metabolic level, whereby characters are constrained by a common resource that is not directly convertible between the two, an example of this being metabolic energy, and the non-metabolic or physical level. The latter type of trade off may involve injury or damage sustained during reproduction that subsequently affects survival probability. The hypothesis that survival is traded off against fecundity in both sexes is supported by the evidence presented here, but there is no evidence to either support or refute the suggestion that flies in the 'old' selection regime were

diverting metabolic resources or energy towards maintenance functions and away from reproduction. The level of investment in sexual morphology was examined in females from the 'old' and 'young' lines, but ovary weight was not found to differ between regimes, and the difference in ovariole number was not thought to have any biological relevance. It is quite possible that 'old' flies had a lower age-specific mortality rate simply because they had evolved lower fecundity. For males, this may mean that they copulated at a lower frequency than those in the 'young' regime and base stock, whereas females may have benefited directly from a lower oviposition rate by receiving less injury, as discussed in chapter 6. Therefore, in terms of the types of trade off that may have occurred between survival and reproduction in females in these regimes, there is evidence that one mechanism by which survival probability was enhanced was as a result of a non-metabolic trade off against the level of oviposition. This does not suggest in any way that this is the only way by which 'old' line female age-specific mortality declined below that of the 'young' lines.

However, that the trade off model fits the pattern of senescence in the selected populations does not rule out the possibility that mutation accumulation also contributed to changing the schedule of ageing in these lines. The theory of mutation accumulation has good empirical support (Kosuda, 1985; Hughes and Charlesworth, 1994; Charlesworth and Hughes, 1996) and may also have a role, alongside trade offs, in causing senescence. However, the assessment of trade offs in populations selected for age at reproduction does not provide a sound test of the mutation accumulation theory because its existence can only be inferred by negative results. It is therefore a weak method with which to try and detect mutation accumulation. Failure to find correlated responses to selection may simply be due to insufficient statistical power. A possible test of the mutation accumulation theory using artificial selection on age at reproduction would be to establish regimes in which selection was applied either to the early part of life, very much as it was in the 'young' regime, or to the whole life span so that the contribution of progeny to the next generation was uniform throughout life. Under the mutation accumulation theory, the accumulation of deleterious alleles at late ages, when natural selection is weak, has the effect of suppressing the optimal life history (Partridge and Barton, 1993). In these novel selection lines the mutation accumulation theory would be supported if late life fitness increased without an early-life or pre-adult decline in fitness. This would

indicate that a proportion of late-expressed deleterious mutations that had, under the theory, accumulated in the laboratory-adapted Dahomey strain had been removed as a result of selection on the later part of the life history. Examination of correlated responses to selection under this type of regime would have to be carried out with very large population sizes so that statistical power would not be called into doubt, since, as with selection on age at reproduction, it would require negative results to provide support for the theory. However, it does represent a direct test of mutation accumulation in a way that selection on age at reproduction does not.

7.2. Measuring Senescence

There has been some discussion surrounding the use of particular measures of ageing in wild and laboratory populations (Promislow, 1991; Tatar *et al.*, 1993; Gaillard *et al.*, 1994; Partridge and Barton, 1996). This has highlighted the problem that measures of ageing that concentrate exclusively on mortality can be misleading because they do not take into account the reproductive schedule of the organism (Partridge and Barton, 1994, and 1996). Natural selection acts on survival and fertility together because the cost of mating means that reproductive activity influences survival probability (Partridge and Barton, 1994). In addition, the schedule of senescence is shaped by both mortality and reproduction, since survival probability at age x, l_x, and fecundity up to that point, m_x, combine to produce a description of the change in the force of selection as it declines with age (Kirkwood and Holliday, 1979). Senescence should be regarded in these terms because it is defined as a decline in state, or fitness, with age. Measures of fitness generally include survival and fertility (e.g. the intrinsic rate of increase). Caution should therefore be taken in interpretation of the phenomenon of levelling off of the mortality rate increase with age observed in large cohorts of *Drosophila* (Curtsinger *et al.*, 1992), medflies (Carey *et al.*, 1992) and *Caenorhabditis* (Brooks *et al.*, 1994), because fecundity schedules are not given in these studies, so the pattern of ageing cannot be inferred (Partridge and Barton, 1996).

In chapter three it was demonstrated that using the Gompertz equation to estimate rates of senescence can lead to spurious conclusions about ageing in

different treatment groups. The residual reproductive value provided a sounder method with which to measure ageing because it took fecundity beyond age *x* into account. Mortality was a relatively unimportant aspect of senescence in those particular treatments, since fertility declined well before there was any serious increase in cohort mortality. It is therefore with great caution that the results of the Gompertz analyses in chapters 4 and 6 are presented. The rate of increase in age-specific mortality did not differ between mated males in either selection regime or the base stock during the senescent period, and neither selection regime nor mating status were found to cause a divergence between selection regimes in females during this period between 10% and 90% mortality. These patterns, and those of age-specific mortality described earlier in the life span of these flies, should not be used to make anything but the most general of statements about senescence in these populations. The Gompertz parameter, G, is an estimate of the rate of the increase in age-specific mortality, so firm conclusions can only be made about mortality and not senescence. Without more detailed information on the schedule of fecundity in the 'old' and 'young' populations, the pattern of senescence, as defined by a decline in survival and fecundity, cannot be known. However, it is still informative to know that selection on age at reproduction did not alter the rate of age-specific mortality during the period between 10 and 90% mortality. The results of chapter three showed that Gompertz estimates can give results that are contrary to expectations about the schedule of senescence, and demonstrated that there are occasions when they are not informative. The Gompertz equation should therefore be used with caution, with the knowledge that it can only be used as an approximate indicator of the pattern of senescence, but that it is a useful tool for monitoring the schedule of mortality.

7.3. Areas of Future Research

There are a number of avenues of research that could be pursued with the present 'old' and 'young' selection lines. The ones that are followed depend very much upon one's viewpoint and interests. In the short term, it would be interesting to know more about the reproductive biology of the males in these lines. How did characters such as courtship rate compare with similar costly

activities in the females? In addition, there are many questions surrounding the metabolism of these flies. It would be interesting to know more about the way in which metabolic energy is invested, the way in which nutrients were allocated to different functions and also the efficiency with which food was utilised. Insight into the metabolic aspects of somatic maintenance and reproduction would reveal the basis of the trade off(s) between survival and reproduction. Such experiments may also help to suggest a physiological basis of life span extension in these lines. Care must be taken in these experiments to ensure large sample sizes to avoid statistical problems related to the degree of variation between replicate lines. In addition, measurement within the strict confines of the selection environment is important, especially with respect to characters such as metabolic rate or male courtship that may be very sensitive to certain environmental factors.

Life history theory predicts multiple causes of ageing (Williams, 1957; Kirkwood and Rose, 1991; Kirkwood and Franceschi, 1992). Work on the Luckinbill populations, selected for age at reproduction, has suggested that there are many loci involved with the long lived phenotype (Luckinbill *et al.*, 1988a; Buck *et al.*, 1993). In addition, there has been some theoretical discussion about the action of various components of the cellular defense network to combat the causes of ageing (Kirkwood and Franceschi, 1992; Kowald and Kirkwood, 1996). Some work concerning anti-oxidant defense has been carried out in populations of flies selected for age at reproduction (Tyler et al., 1993; Dudas and Arking, 1995). However, more work is needed to identify possible mechanisms and candidate genes for life span extension. The systems included in the network theory of ageing would be an ideal starting point from which to decide the aspects of cellular defense that are worth considering. For example, investigation of the activity of scavenger proteins and molecular proof-reading enzymes, as well as the various aspects of anti-oxidant defense may prove fruitful. Other candidate genes are those coding for hormones, which each have the potential to fit the pleiotropy model of ageing because many hormones change roles over the lifetime (Finch and Rose, 1995). It is possible that certain pleiotropic alleles are connected with ageing in many species and may be candidates for "common" causes of senescence (Martin *et al.*, 1996).

Once candidate mechanisms are identified, the 'old' and 'young' populations would be a valuable genetic resource because they could be used

to map the candidate genes. Using lines selected for divergent mortality rate as a starting point, it would be possible to establish recombinant inbred lines and use mapping techniques such as quantitative trait loci analysis to map genes related to life span in *Drosophila* (Dixon, 1993). Fruit flies are an ideal organism for this type of research because of the ease of culture, the brevity of their generation time and the wealth of information on their genetic markers (Dixon, 1993). Problems concerning the effects of inbreeding depression need to be addressed, but it may be possible to identify strains that do not exhibit hybrid vigour when crossed, so that the effects of heterosis do not mask the genes being studied (Dixon, 1993). It may then be possible to elucidate the interactions between different genes and the role of epistasis in the expression of longevity. It is acknowledged that there are many loci that affect life span, but little consideration has been given to how the different genes may interact. Studies in *Drosophila* would undoubtedly highlight some potential areas of research in other model systems, possibly even in mammalian species that could have relevance to human gerontological research.

7.4. Concluding Remarks

The future holds great potential in terms of research into the causes of senescence and the characterisation of genes affecting life span (Curtsinger *et al.* 1995). Work that has already been carried out in a number of different areas has contributed to the understanding of certain aspects of ageing and longevity. These disciplines include evolutionary theory, molecular genetics, physiology and experimental biology. It has long been known that evolutionary theory predicts the evolution of senescence due to the decline in the force of natural selection with age. This tells us that we will not find 'ageing genes' as such, and that the mechanisms that need to be studied are those that affect life span in some way. The term 'cellular defence network' (Kirkwood and Franceschi, 1992) has been used to describe the collection of processes that exist to prolong life span.

There has already been some integration of knowledge of ageing and life span extension between disciplines: for example, the investigations of the patterns of expression of particular anti-oxidant alleles in *Drosophila* populations selected for age at reproduction (e.g. Dudas and Arking, 1995); or the use of mathematical modelling to examine the effect of the interaction of a number of the perceived causes of senescence (Kowald and Kirkwood, 1996). These studies highlight the need to examine systematically potential mechanisms

affecting life span. This approach should be multi-faceted; mapping and indentification of the genes involved should progress to their cloning and the characterisation of their function. On the other hand, cell biology and physiological studies could highlight mechanisms and proteins that can then be probed using genetical techniques.

The use of transgenic technology has great potential here, as has already been shown with investigations of anti-oxidant enzymes by Agarwal and Sohal (1994) and Orr and Sohal (1994), although there are certain pitfalls that must be avoided (see Kaiser *et al.* 1997). It is here that the genetical and cell biological approaches are combined, since the effect of a particular gene will be assayed in a controlled environment. It is important that the potential for interaction between genes involved in the maintenance of life span is considered, since a particular gene studied in isolation in a transgenic population may not be a realistic model. Sophisticated experiments need to be devised to examine the effects of more than one gene at a time.

Populations of *Drosophila* selected for age at reproduction provide a valuable resource to be used in the study of life-span-extension mechanisms. Whereas transgenic models involve the insertion of a gene of interest, and may not provide an accurate picture of the operation of a number of mechanisms, selected populations are likely to have evolved extended longevity as a result of selection for genes at a number of different loci. They therefore provide a basis for research into an integrated system as described by the cellular defence network. The knowledge of mechanisms such as anti-oxidant defence provide a basis for the characterisation of the physiology of the extended longevity phenotype.

In carrying out such studies, whatever the approach, it is important that isolated causes are not championed to the exclusion of others, since there is no single mechanism of senescence (Kirkwood and Rose 1991) and the known contributing factors interact to a great extent (Kowald and Kirkwood 1996). In isolation, there is a danger that research on ageing in *D. melanogaster* will make no contribution to the knowledge of senescence in other species. Model systems such as *Drosophila* and *C. elegans* each have their merits, and in particular are useful for testing evolutionary and genetical hypotheses and for examining the basic principles and common causes of ageing. As discussed, there needs to be integration between disciplines, but this is also required between species. Study of mammalian species to study pathological effects (Kirkwood 1996) would be desirable. It might then be possible to build up a wider understanding of senescence.

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