# SENESCENCE AND REPRODUCTIVE PERFORMANCE IN THE EUROPEAN SHAG (*PHALACROCORAX ARISTOTELIS*)

Margaret Emily Hall

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Division of Environmental & Evolutionary Biology
Institute of Biomedical & Life Sciences
Faculty of Science
University of Glasgow

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## CANDIDATE'S DECLARATION

I declare that the work recorded in this thesis is entirely my own unless otherwise stated, and that it is of my own composition. No part of this work has been submitted for any other degree.

Margaret Hall

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*If the young knew...*
*If the old could...*

*French proverb*

# CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# Abstract

Senescence is rarely observed in wild populations. This is due to a combination of *(a)* high extrinsic mortality rates, for instance acting through predation or food limitation, which mean wild animals rarely die of old age, *(b)* the inevitably small numbers of old individuals, which limits sample size, and *(c)* the difficulties of studying known age individuals across appropriate time periods. Motivated by reports of lower survival in old European shags *Phalacrocorax aristotelis*, this project aimed to determine whether reproductive senescence or declines in adult condition also occur in old birds of this species breeding on the Isle of May, Scotland. A large proportion of the shags at this site are known age as a consequence of long-term ringing effort. Telomere length, which plays a role in cellular senescence and organismal ageing, was also measured among birds of different age, and in serial samples taken from the same individuals, in order to assess factors affecting rates of telomere attrition.

The correlation between the ages of breeding partners was found to be weak in old shags, and the age difference in pairs increased in relation to the age of the female. Therefore, in the oldest shags the age of one pair member does not provide a reliable estimate of the age of its partner. Old female age was confounded with young male age in pairs, and thus with relatively newly established partnerships. A pattern of smaller eggs laid by old females was attributed to declining egg size within individuals. However, at least on a population level, this did not translate into lower hatching or fledging success, and old shags do not fledge fewer chicks than middle-aged birds. There was a tendency for complete breeding failure to be associated with a large age difference between breeding partners. There was no difference in the quality of the nest site occupied by old shags, and old birds also did not commence breeding later than expected. With the exception of a decline in number, and increase in size, of red blood cells, no differences in the physical attributes of adult shags were observed in old age, despite adequate samples of old individuals. This may in part reflect biases in the sample of birds that could be measured, and the years in which the study was carried out.

Telomere length declined within individuals between the nestling and adult stage. However, in adults there was no relationship between age and telomere length. Environmental conditions experienced as a chick were related to subsequent telomere shortening in these birds, suggesting that early conditions, possibly through effects on oxidative stress, may play a role in telomere attrition, and potentially in the longevity of individuals.

# General introduction

## *A definition of senescence*

In the following introduction, and throughout this thesis, the terms ageing and senescence are used interchangeably, to refer to processes that accompany increasing age. Comfort (1960) suggested ageing is "an increased liability to die, or an increasing loss of vigour, with increasing chronological age". Similarly, Maynard Smith (1962) defined senescence as processes "which render individuals more susceptible as they grow older to the various factors, intrinsic or extrinsic, which may cause death". Arking (1998) identified four characteristics of senescent change: the process is cumulative, progressive, intrinsic and deleterious. Senescence includes declining survival, declining reproductive performance, and deteriorating condition in old age, though the prevalence and strength of each of these factors may vary among organisms.

## *The causes of senescence*

As senescence involves a progressive loss of function, accompanied by falling fertility and greater mortality rates as age advances, it is clearly bad for the individual. This raises the question of why and how it has evolved. A huge number of theories have been proposed to explain the occurrence of senescence (reviewed by Comfort 1979, Rose 1991, Arking 1998, Kirkwood and Austad 2000, Martin 2002). Medvedev in 1990 suggested the number of theories for ageing at that time stood at over 300. These range from ultimate, evolutionary explanations to detailed descriptions of potential proximate mechanisms. It is not the purpose of this thesis to test hypotheses for the evolution of senescence, but an understanding of these is required to interpret the results and set them in context. Therefore, this section summarises the main concepts behind currently prominent hypotheses for ageing (table 1.1).

### 1. Evolutionary explanations for senescence

The first evolutionary theories of ageing were based on group selection (Wallace 1865, Weismann 1889, 1891), whereby the programmed death of individuals prevented competition with their progeny. Although the main focus has now shifted away from these theories, they have not been fully rejected, as recent modelling work suggesting that programmed death can evolve in spatially structured populations (Travis, 2004) shows. However, most evolutionary thought on ageing now takes the view that organisms are selected for survival rather than programmed for death. Thus, the question underlying current theories is why is senescence not selected against? This is based on the premise that, all other things being equal, a long-lived individual will leave more offspring than a short-lived one (Williams, 1957). One reason why senescence may persist despite this is the declining force of selection with increasing age (Bidder 1932, Haldane 1941, Medawar 1952, Comfort 1956, Williams 1957). This can be explained by falling reproductive potential as age increases, due to the cumulative probability of death (even in the absence of senescence). By an age when survivorship has declined to very low levels, the force of selection is too weak to oppose the accumulation of mutations with late-acting deleterious effects.

Given that selective pressures are weaker at older ages, processes that are favourable early in life but disadvantageous later can be selected for, even if the early benefits are small and the effects late in life lead to senescence and death (Medawar 1952, Williams 1957). This is known as antagonistic pleiotropy, and forms the second main strand of current evolutionary theories of senescence. Genes with antagonistic pleiotropic effects have been identified, for instance *sch9* mutants in yeast have a threefold increase in lifespan, but grow at a slower rate and produce smaller colonies (Longo and Finch, 2003). This introduces the concept of a trade-off, in this case between fitness early and late in life.

The disposable soma theory also involves a trade-off, between the resources allocated to somatic maintenance and those allocated to reproduction (Kirkwood 1977, 1996). Particularly when levels of extrinsic mortality are high, very few individuals in the population will benefit from investing in self-maintenance, which is costly. This theory suggests that under these conditions resources are optimally

utilised in reproduction. Damage can therefore accumulate in the soma, and lead to ageing.

These three concepts are complimentary explanations for why ageing occurs. They also provide an explanation for the variation in lifespan among species. So, when levels of extrinsic mortality are high the probability of survival in the wild is low and has a large cumulative effect with advancing age. Therefore, the force of selection quickly drops off with age, deleterious genes are able to accumulate at comparatively early ages, and there is very little selection for somatic maintenance. Under these circumstances, organisms will evolve to be short-lived. In contrast, if extrinsic mortality is low, the attenuation of the force of selection will be more gradual, and there will be selection for a higher level of somatic maintenance. In that case, the organism will evolve to be long-lived. The trade-off between self-maintenance and reproduction also means short-lived species will display higher reproductive rates than long-lived species. These fundamental principles probably underlie both mortality and reproductive ageing, although there are some additional hypotheses for the evolution of particular patterns of reproductive senescence, such as the menopause (see Kirkwood and Austad, 2000).

### *2. Proximate causes of senescence*

Mechanisms for how ageing occurs on a proximate level range from molecular changes, through cellular processes, to alterations in tissues and systemic decline (see Masoro and Austad, 2001). Two inter-related mechanistic explanations for senescence, which have recently received increasing attention, are the free radical theory of ageing and telomere shortening. The free radical theory (Harman, 1956) emphasises the role of reactive oxygen species (ROS), which are produced during aerobic respiration, in causing damage to tissues and so contributing to ageing and death. There is now considerable evidence that implicates the generation of ROS, and corresponding responses to oxidative stress, in the ageing process (Beckman and Ames 1998, Finkel and Holbrook 2000).

Telomeres are repetitive DNA sequences that cap eukaryotic chromosomes and prevent the loss of coding DNA at cell replication, by themselves undergoing shortening (in the absence of telomerase, refer to chapter 6). Cellular senescence is triggered by short telomeres, and via this process telomere length has been linked to

tissue and organismal ageing. Oxidative stress accelerates telomere attrition (von Zglinicki, 2002). Thus, effects on telomere length are one means by which ROS could influence the rate of ageing.

### *Age-dependent patterns of survival and reproduction*

Reproductive success and survival are strongly associated with age in many iteroparous organisms, including birds (Lack 1966, Clutton-Brock 1988, Newton 1989, Wooller, Bradley and Croxall 1992, Martin 1995). In birds with moderate to long lifespans, age-related changes in reproduction are predicted to follow a quadratic function, increasing early in life and decreasing later, after a period of peak performance during experienced adulthood (Fowler, 1995). Most research on age-specific productivity in birds has focussed on the increase in reproductive success between the first breeding attempt and the next few attempts. Younger age classes often perform poorly in several aspects of breeding, but age-related improvements in reproductive performance are usually evident (e.g. kittiwake *Rissa tridactyla:* Coulson and White 1958, fulmar *Fulmarus glacialis:* Ollason and Dunnet 1978, great skua *Catharacta skua:* Hamer and Furness 1991, barnacle goose *Branta leucopsis:* Forslund and Larsson 1992, merlin *Falco columbarius:* Espie *et al.* 2000, tree swallow *Tachycineta bicolor:* Robertson and Rendell 2001, and northern wheatear *Oenanthe oenanthe:* Pärt 2001, review Saether 1990). European shags *Phalacrocorax aristotelis* also show improvements over their first few breeding attempts (Potts 1966, 1969, Coulson, Potts and Horobin 1969, Potts, Coulson and Deans 1980, Aebischer 1985, 1986, 1993, Daunt *et al.* 1999, Daunt 2000). Most studies of age-related performance make comparisons among individuals of differing age, rather than tracking single individuals from year to year, since the latter is difficult to achieve in the wild.

There are three main hypotheses for the poorer performance of younger age classes of birds. Firstly, low breeding success may relate to phenotypic variation among age classes, as a result of selection having removed more poor quality individuals from older age groups. Differences in the age of first breeding by birds of varying quality (better performance being associated with a later onset of breeding) could also lead to variation in phenotypic distributions among age classes. Alternatively, the poor performance of young birds may be linked to reproductive

restraint, or constraints at young ages (Curio 1983, Martin 1995, Forslund and Pärt 1995). Within this broad classification, numerous proximate mechanisms have also been proposed, such as increasing foraging experience with age.

After a period of peak reproductive success in middle age, performance may fall again in old birds. This decline can be a consequence of senescence-related constraints, or restraint in line with declining condition in old age. Alternatively, as with the improvements in reproductive performance among young breeders, declining productivity among old birds could reflect a process of selective mortality (if reproduction is costly). If individuals that invest less time and energy in reproduction have a higher probability of survival, the oldest age class will contain a large proportion of these individuals. However, declining reproductive success is not the only possible outcome of advancing age and reduced survival. In fact, life-history theory predicts that increasing reproductive effort will occur towards the end of life, as residual reproductive value falls (Williams 1966*b*, Gadgil and Bossert 1970, Stearns 1976). An increase is also expected if only the highest quality individuals survive into old age, i.e. if there is covariation between survival and reproductive ability. Lastly, as increasing age generally means an individual has greater experience of breeding, and possibly of co-ordinating the breeding attempt with the same partner (for species with stable long term pair bonds), this could explain increasing reproductive success with age (Fowler 1995, Cézilly and Nager 1996).

### *Senescence in birds*

Early studies on avian population dynamics (Nice 1937, Lack 1943) led to the conclusion that wild birds were exceptional among animals because they did not show lower survival in old age. After maturity, survival was thought to remain constant with age in birds (Lack 1954). It was assumed that the levels of extrinsic mortality in the wild were so high that no individuals survived long enough to experience senescence (Comfort 1979, Rose 1991). However, bird survival curves in captivity resemble those of mammals (Comfort 1962, Eisner and Etoh 1967), with reduced survival in old age. Botkin and Miller (1974) argued, based on data from captive populations as well as on theoretical grounds, that survival rates must decline in old birds. Subsequent work by Nesse (1988) and several long-term population

studies (see Newton 1989) demonstrated that this was the case, and that significantly lower survival rates do occur in old, free-living birds. This has been shown for a number of seabirds, including the kittiwake (Aebischer and Coulson, 1990), common eider *Somateria mollissima* (Coulson, 1984), short-tailed shearwater *Puffinus tenuirostris* (Wooller *et al.* 1990), wandering albatross *Diomedea exulans* (Weimerskirch, 1992), and European shag (Harris *et al.* 1994*b*).

In line with these findings of falling survival in old age, there have been an increasing number of reports of declining reproductive success towards the end of life. However, the number of studies of performance in the oldest age class of birds has been limited by the inability to age mature birds on structure or plumage, so that long-term ringing programmes are required to establish a known age population. In addition, the sample size of the oldest birds is often prohibitively small. For these reasons, significant reproductive senescence effects have been demonstrated in only a comparatively small number of cases. Nisbet (2001) reviews the challenges associated with measuring senescence in wild birds.

Newton (1989) listed four avian species in which a senescence effect on reproductive performance is seen (sparrowhawk *Acipiter nisus:* Newton 1989, great tit *Parus major:* Perrins 1979, arctic tern *Sterna paradisaea:* Coulson and Horobin 1976, and Tengmalm's owl *Aegolius funereus:* Korpimaki 1988). Coulson and Fairweather (2001) added seven further species to this list, all of which were seabirds, although in several of these studies the decline was not statistically significant. The most convincing reports of reproductive senescence in birds have come from long-term longitudinal studies, in which the breeding performance of individuals is monitored over at least several years. Longitudinal comparisons overcome a number of the drawbacks of cross-sectional analyses among individuals of differing age (see chapter 6 introduction, Arking 1998). Thus, there is convincing evidence for declining reproductive performance within old individuals in glaucous-winged gulls *Larus glaucescens* (Reid, 1988), female sparrowhawks *Accipiter nisus* (Newton and Rothery, 1998, 2002), red-billed choughs *Pyrrhocorax pyrrhocorax* (Reid *et al.* 2003), and white-tailed ptarmigans *Lagopus leucurus* (Wiebe and Martin, 1998). These studies make possible discrimination between age-related patterns due to changes within individuals, and those due to selective mortality. For example, Nielsen and Drachmann (2003) carried out a longitudinal analysis of reproductive

performance in northern goshawks (*Accipiter gentilis*) which showed the mean population trend of reduced fledgling production by old females could be attributed to age-related trends within individuals.

### *Study site and species*

Reproductive performance, condition and telomere length were studied in known age European shags *Phalacrocorax aristotelis* breeding on the Isle of May (Firth of Forth, Scotland 56°11'N, 02°33'W). Approximately 25% of the breeding birds at this colony are of known age, having been ringed as chicks as part of a long-term ringing programme (since the 1960s). Minimum ages are known for another 25% of the birds, ringed as adults. Nest counts on the Isle of May in the years of this study (2001, 2002 and 2003) showed there were between 676 and 968 breeding pairs present in each year (Scottish Natural Heritage unpublished records).

The European shag displays life-history traits that are characteristic of all seabirds (Hamer, Schreiber and Burger, 2002): they are long-lived, with delayed sexual maturation and breeding, and low annual reproductive rates. The longevity record for European shags is 30 years (Clark *et al.* 2002), but the oldest individual recorded on the Isle of May during this study was a 23-year-old. Overall, 90% of male and 17% of female shags breed for the first time at the age of two years, and the remainder are assumed to recruit at three years of age (Aebischer, 1986). So, first-time breeding males are generally two-year-olds, which can be recognised by their pale plumage, while the majority of first-time breeding females are three years old. By the age of three birds can no longer be aged on plumage characteristics. The sex of adult shags is determined by their overall size, males being on average larger than females, by behaviour, and by voice, as only male shags vocalise. Females lay between two and four eggs, with three eggs the modal clutch size (Snow 1960). Shags are socially monogamous, and both sexes take part in incubation and chick rearing. However, extra-pair matings do take place, with up to 18% extra-pair paternity on the Isle of May (Graves *et al.* 1992). Incubation lasts approximately five weeks, hatching is asynchronous (Amundsen and Stokland, 1988), the chicks that hatch are altricial, and they fledge at seven weeks of age (Snow 1960).

Previous work on long-term retrap and recovery data from the Isle of May has shown that a significant fall in survival occurs after 13 years of age in the European

shag (Harris *et al.* 1994*b*). This is suggestive of a senescence-related increase in mortality in old age, but whether this is matched by declines in reproductive performance or individual state has not previously been specifically studied.

### *Thesis content*

The main aims of this study are to determine whether European shags exhibit reproductive senescence, and if so at what stage of breeding this occurs. Whether they show senescence-related changes in adult condition is also investigated. In addition telomere shortening, which is implicated in ageing processes, is explored in known age birds.

The relationship between the ages of breeding partners in the European shag is addressed in the following chapter (**Chapter 2**), in order to assess whether the age of one pair member is a reliable estimate of the age of its mate. **Chapter 3** investigates a single aspect of breeding performance, egg size, with respect to the age, size and state of the laying female. Performance in the subsequent stages of reproduction are then examined in old birds (**Chapter 4**). Whether old shags exhibit physical or physiological signs of ageing is investigated in **Chapter 5**. Finally, telomere length and the amount of telomere shortening in individual shags is explored in relation to age and environmental conditions (**Chapter 6**). The appendix provides a summary of the results of a clutch exchange experiment, designed to distinguish between effects on reproductive success mediated by differences in egg quality from those mediated by differences in the rearing ability of middle-aged and old females. The findings of this thesis are discussed in a general context in **Chapter 7**.

| **Evolutionary explanations** |
|---|
| 1. The force of selection declines with increasing age. |
| 2. Antagonistic pleiotropy. |
| 3. The disposable soma theory of ageing. |
| **Mechanistic (proximate) explanations** |
| 1. The free radical theory of ageing. |
| 2. Telomere shortening and cellular senescence. |

**Table 1.1** Theories for the causes of senescence outlined in the introduction.

# The relative ages of breeding partners in the European shag


## ABSTRACT

A close relationship between the ages of the members of breeding pairs is a common pattern among birds that form stable, socially monogamous partnerships. As a consequence, when only one pair member is of known age, its age may be used to estimate that of its partner. However, relative age in pairs has most often been studied in young breeders, and the relationship may not hold in old birds, particularly if mortality patterns differ between the sexes. Therefore, the age of the male and the female in known age pairs of shags that bred in 2001, 2002 or 2003 were examined. Overall, there was a correlation between the ages of pair members, but this relationship was weak in older birds, and disappeared completely in females over nine years of age. Irrespective of age, in more than half of the pairs the female was older than the male. The difference in age between partners also increased in relation to the age of the female, and the oldest females were paired with males only three years old. This pattern is discussed in relation to the age of recruitment and mortality of each sex, as possible causative mechanisms. In the oldest shags the age of one pair member is not an accurate estimate of the age of its partner.

## INTRODUCTION

The European shag is a socially monogamous species, in which pairs form or re-form at the breeding colony each spring (Snow 1963, Potts 1966). The stability of the pair bond from one year to the next varies from approximately 20 to 60% (Potts, 1966). A close, positive relationship between the respective ages of partners has previously been reported in the shag (Potts 1966, Aebischer 1985, Daunt *et al.* 1999, Daunt 2000), as well as in other bird species (e.g. arctic tern *Sterna paradisaea*: Coulson and Horobin 1976, kittiwake *Rissa tridactyla*: Coulson and Thomas 1980). Though these studies have largely been concerned with determining age or measuring reproductive performance among young breeders, they also provide some evidence that suggests the correlation between the ages of breeding partners may be weaker in old birds. For instance, Aebischer (1985) reported that two-year-old male shags were paired with females of all ages, and discrepancies in his correlation between male and female age included a pair in which the male was two and the female 19 years old. Potts (1966) also observed that the oldest shags pair with relatively younger mates. A similar situation was reported in the arctic tern (Coulson and Horobin, 1976) and kittiwake (Coulson 1966, Coulson and Thomas 1980), where there is a tendency for old females to mate with younger males. Daunt (2000) reported that female shags were generally older than their mates, although in that instance no difference in the age gap was found between pairs in which the male was two years old and pairs containing an older male. The age gap was not compared with respect to the age of the female in the pair. In a small proportion of cases (1-2% of known pairs on the Isle of May, 4% on the Farne Islands), polygynous relationships are established among shags, with two females and one male forming a trio at the nest site (Potts 1966, Potts, Coulson and Deans 1980, Aebischer 1985). However, since these cases are very unusual they will not be considered here.

The present study is concerned with the potential effects of old age and senescence on reproductive performance. If male and female age in the pair are consistently closely related, then the age of one pair member can be used as an indicator of the partner's age (e.g. Aebischer 1985, Daunt *et al.* 1999, Daunt 2000). This is particularly useful if only one member of a pair is of known age (i.e. ringed as a chick or as a two-year-old). In contrast, if there is a large difference in the ages of the partners, either member's age may be influencing the breeding success of the

pair. If mate change in old age is a consequence of divorce rather than mortality, this process itself may be linked to the aged phenotype of pair members. Hence, the relationship between the age of the male and female in pairs, and whether this varies with the age of either sex, is of crucial importance, and is explored here among known age pairs of shags recorded on the Isle of May in 2001, 2002 and 2003. The age of recruitment and mortality with respect to sex could affect pair formation and change, and current knowledge regarding these factors in the shag is introduced below. General mechanisms of pair formation in birds, and the reasons for and consequences of mate change are also briefly summarised.

### *The age of recruitment and mortality in relation to sex in the shag*

Male shags first breed at a younger age than females (Snow 1963, Potts 1966), with 90% of males and 17% of females recruited in their second year on the Isle of May (Aebischer, 1986). It is assumed that the remainder in both sexes recruit in their third year. The earlier recruitment age of males may contribute to the tendency of young males breeding for the first time to pair up with older females (Potts, 1966). Whether the sexes differ in their post-fledging survival to breeding age is not known. There is equivocal evidence concerning adult survival in the European shag with respect to sex. Two published studies report no difference in survival between the sexes (Potts, Coulson and Deans 1980, Harris *et al.* 1994*b*). However, Potts (1969) reported a difference in Farne Islands birds, with a survival rate on average 5% higher for males than females in the same age class. Recent re-analysis of Isle of May data has also suggested higher survival of males, though this difference was not significant (survival estimates: males 0.891, females 0.816, $p = 0.07$, B. Morgan unpublished data). Sex-specific mortality may be important as a cause of mate change (when the partner dies), and could bias towards one sex the available mates from these pairs.

### *Mechanisms of pair formation, and the causes and effects of mate change in birds*

The way in which partnerships form, are maintained, and change in birds differ somewhat between resident and migratory species, and between species which form continuous or part-time pairings (review Ens, Choudhury and Black, 1996). The

shag falls into the category of birds with migratory part-time partnerships, so this pair type is the focus of the introduction. The late arrival of young birds at the breeding grounds probably contributes to the correlation between the ages of mates in the majority of birds in this category (Ens, Choudhury and Black, 1996). For instance, in common terns *Sterna hirundo* the failure of an established pair to re-form is often a consequence of asynchronous arrival at the breeding grounds (González-Solís, Becker and Wendeln, 1999). This seems a likely mechanism in shags, as older individuals breed earlier within the season than young birds (Coulson, Potts and Horobin 1969, Aebischer 1985, 1986, 1993, Daunt 2000). However, there are alternative mechanisms by which pairing with respect to age could occur, or be maintained. For instance, young birds may pair up together simply because there are very few birds of any other age available, as older individuals will already have an established mate. Another possibility is that age correlates with some other trait, such as differences in nest or nest site quality, by which individuals choose their mates (see Manning, 1985).

Once formed, pairs can be maintained by individual recognition or by site tenacity. Tinbergen (1953) showed that herring gulls *Larus argentatus* recognise their mates on the basis of the call, and pairs form prior to occupying their nesting territories. Similarly, in the kittiwake forced changes of breeding site have shown that the annual re-forming of pairs is not a result of nest site tenacity, but involves individual recognition (Fairweather and Coulson, 1995). In contrast, in the Manx shearwater *Puffinus puffinus* and Leach's storm petrel *Oceanodroma leucorhoa* recognition of the nesting burrow is considered the only way in which individuals can renew contact with their mate at the beginning of the season (Lockley 1942, Morse and Kress 1984). The benefits of familiarity with the current partner, and limited opportunities or high costs involved in acquiring a new mate can favour mate retention.

Mate change is the result of replacement of a mate who has died, or divorce, which includes all other circumstances of separation in bird pairs (after Potts, 1966). Divorce can be the consequence of one partner deserting the other, one partner being ousted by another bird, or an established partner being pre-empted by a new bird that arrived earlier. Mate changes that are forced upon an individual due to the death of its mate are not expected to result in improved reproductive success, especially in

those species where performance improves with experience of a particular mate. In the great skua (*Catharacta skua*) on Foula, Shetland death is responsible for three times as many pair break-ups as divorce, and direct costs of mate changes are reflected in later laying of the new pairs (of experienced birds) and the rearing of fewer chicks (Catry, Ratcliffe and Furness, 1997). In addition, in this species 26% of individuals did not breed in one year after mate loss. On the other hand, pair members may initiate divorce as a consequence of low breeding success, and in those cases performance may improve for the initiator of the divorce with their new mate (e.g. blackbirds *Turdus merula*: Streif and Rasa 2001, oystercatchers *Haematopus ostralegus*: Ens, Safriel and Harris 1993, Heg, Bruinzeel and Ens 2003). Recent reviews of divorce in birds (Choudhury 1995, Cézilly *et al.* 2000, Dhondt 2002) have highlighted the large number of hypotheses for divorce, but the limited number of empirical studies. However, they agree that divorce represents an adaptive strategy by which individuals maximise their own fitness. There has been a recent shift in emphasis from considering divorce as advantageous for both partners, towards regarding this process as an active choice by only one member of the pair (e.g. Ens, Safriel and Harris, 1993). The role of females in maintaining or breaking the pair bond has also received increasing attention (Cézilly *et al.* 2000).

## METHODS

The age of the male was compared to the age of the female in pairs where both birds were of known age, using data from 2001, 2002 and 2003. Pairs were included only if they produced eggs, and trios (see introduction) were not considered. For those pairs that were observed in more than one of these years (23 pairs were seen in 2 years, and 2 pairs in 3 years), a single entry was selected at random. A total of 101 different pairs, with both the male and female of known age, were observed in one of these 3 years. As neither male age nor female age was normally distributed, values were $\log_{10}$ transformed before testing by parametric correlation. The difference between male and female age in pairs was also non-normally distributed, and non-parametric tests were used with this variable.

## RESULTS

In 61% of known age pairs the female was older than her mate, in 25% the male was older, and in 14% the two partners were of the same age (table 2.1). With the exception of one pair, all the partnerships in which the members were of the same age contained birds under 8 years old. Overall, the age of the male and female in pairs was correlated (after $\log_{10}$ transformation, $r = 0.33$, $n = 101$, $p = 0.001$, figure 2.1). There was a small decline in the strength of this relationship when the youngest (less than 4 year old) individuals were excluded (after $\log_{10}$ transformation, $r = 0.25$, $n = 81$, $p = 0.028$). The relationship between male and female age was best described by a quadratic regression (table 2.2). However, there was no significant correlation between male and female age among females over 9 years of age ($\log_{10}$(age), $p > 0.05$). The difference in age between members of a pair increased with female age ($r_s = 0.64$, $n = 101$, $p < 0.001$, figure 2.2), even when pairs with members of the same age (mostly young breeders) were excluded ($r_s = 0.62$, $n = 87$, $p < 0.001$).

## DISCUSSION

Although among young shags age within pairs is highly correlated, the relationship breaks down by the age of 10 years. The most parsimonious explanation for the correlation among young shags is that it is governed by differences in arrival time among birds of different age, with young birds arriving later in the season at the breeding colony, by which time older individuals are already paired and unavailable as partners. As the reproductive performance of young shags, independently of laying date, is lower than that of older individuals (Daunt *et al.* 1999), all birds should preferentially pair with an older bird, but differences in arrival may prevent this. Alternatively, older shags may reject young individuals when they have a choice of partners. A breakdown in the relationship between the ages of pair members is expected if birds re-pair after the death of their partner. More surprising is the difference observed between the sexes in the relative age of their mate, and the positive relationship of the age gap within pairs to female age.

The age correlation in pairs may be maintained by the costs of changing mates or nest site tenacity. Potential costs of mate change are time spent searching for, and soliciting to, a new mate, costs of fighting rivals, the risk of ending up with a poorer mate and initial inefficiency or poor co-ordination with a novel partner (review Ens, Choudhury and Black, 1996). Shags of both sexes on the Isle of May show strong fidelity (over 95%) to the area in which they have previously bred (Aebischer, 1995). On average, 56% of surviving males nest on the same site in consecutive years, although site fidelity is lower for females (Aebischer, Potts and Coulson, 1995). Perhaps the female's attachment is to the male rather than the site, with only 6% of females breeding on their former site in the absence of their former mate. Of those females whose mate moved, 48% joined him on the new site (Aebischer, Potts and Coulson, 1995). The renewal of the pair-bond thus depends on whether both birds return to the breeding colony and whether previous partners can locate each other, which is a function of how far they move from last year's site. I saw several cases where an old female returned to her previous nest site, and remained unpaired at that site for a prolonged period or did not breed at all, after her established partner failed to return. Cases of mate loss like this may contribute to the 25% of shags that skip breeding at least once in their lifetime after recruitment (Aebischer and Wanless, 1992).

The weakening of the age correlation in pairs from about 10 years of age might result from higher mortality in old birds, and so a greater chance of non-return by one member of the original pair. On the Farne Islands and the Isle of May 46% of separations were due to non-return of the partner (Aebischer, Potts and Coulson, 1995), but whether this proportion changes with respect to the ages of the pair members was not examined. A significant decline in survival has been shown for shags older than 13 years of age (Harris *et al.* 1994*b*). The surviving pair member will have a limited choice of replacement partner, and there will be an overall larger number of young birds available. In addition, young birds may be more likely to change mates than old birds. The overall rate of divorce among two-year-old males is more than twice that of older males due to the lower site fidelity of the youngest males (Aebischer, Potts and Coulson, 1995). Males are also more likely to move sites after a failed breeding attempt than after successful breeding, which may explain this difference in site fidelity among age groups. The lack of a correlation between the ages of pair members in old shags contrasts to the scenario among wandering albatrosses (*Diomedea exulans*), which show active selection of mates of similar age. In this albatross, experienced birds whose mates had died predominantly re-paired with other widowed birds, which were on average also old (Jouventin, Lequette and Dobson, 1999).

In 61% of the known age pairs in this study the female was older than the male, which is possibly an effect of the younger age of recruitment to the breeding colony of males (see introduction). However, the difference in recruitment age between the sexes is only one year. The difference in age between members of a pair also increases with female age, and the oldest females mate with the youngest males. In our data there was no indication that old males were similarly pairing with very young females, although the sample of males did not extend to such advanced ages as in females (the oldest male was 18, the oldest female 21 years old). Moreover, it appears that this pattern cannot be explained by sex differences in adult mortality. In those studies that report differential mortality of the sexes, the tendency overall is for adult females to show poorer survival than males. If this were true in old shags, it would suggest that old males are more likely than females to be widowed, and thus be forced to find a replacement mate. However, the pattern of mortality with regard to sex may not be constant across age groups, as the most common causes of

mortality may change with age. For example, senescence may have a greater impact upon the survival of one sex. Alternatively, if senescence affects the condition or reproductive performance of males and females differently, the worse affected sex may find itself more often divorced and constrained in the quality of mate it is able to acquire. To explain the observed pattern of the oldest females paired with very young males by this means, senescence would be expected to take a greater toll on females. A skew towards more males than females among young birds seeking breeding partners, resulting from earlier first breeding by males, could partly explain why old widowed females are more likely to re-pair with a young bird than are old widowers. This pattern might be exacerbated if females show lower post-fledging survival to breeding age than males (though sex-specific estimates of survival at this stage have never been calculated in shags).

European shags display assortative pairing with respect to crest size (Daunt *et al.* 2003). Both sexes in this species grow a crest of feathers on the head before pair formation which is retained until incubation (Snow, 1963). There is considerable variation in the size of this crest, which relates to laying date and subsequent reproductive performance. However, this character was not found to be affected by age, and therefore may represent an age-independent signal of condition.

Although shags on the Isle of May are generally monogamous (albeit with a few trios), some covert polygyny does occur. Potts (1968) reported that most cases of repeated egg breakages at the nest were the result of interference by females in simultaneous polygynous relationships, and this occurs on the Farne Islands in 3.5% of shags. Promiscuity by male shags on the Isle of May has also been shown directly by time-lapse photography (Harris, 1982). Observations suggest that on the Isle of May approximately 14% of copulations by males are not with the female with which they rear young, and DNA fingerprinting revealed 18% of chicks have extra-pair paternity (Graves *et al.* 1992).

The pattern of relative age in pairs described here complicates investigation of age-specific reproductive performance because it means, with the exception of young birds, that the age of one member of a pair cannot be used as a proxy for the age of its mate. Therefore, in pairs with an old and a young bird, low breeding performance could be a consequence of senescence-related changes in the old individual, age-related constraints, restraint, or a lack of experience in the young bird, or the fact that

the pair is newly formed. To demonstrate senescence-related change in old birds, it may be necessary to rule out re-pairing with an unfamiliar or young mate as the cause of declining reproductive success. This was recognised by Black and Owen (1995), who showed that falling breeding success in old barnacle geese *Branta leucopsis* was not due to loss of the established mate or re-pairing with a younger, less proficient bird. Rather the declining success of very old female geese was due to their partnership with very old males. In contrast, in the shag old birds especially may differ greatly in age from their mate, and this information where possible should be included in analyses of reproductive performance.

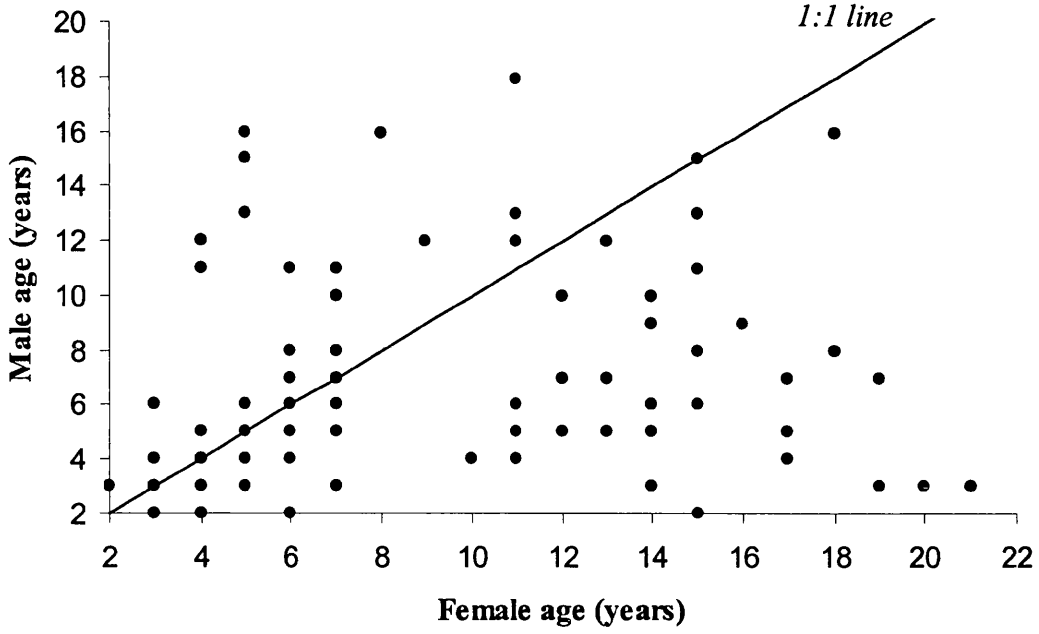
## FIGURES

| female age (yrs) | male age (yrs) | | | | | | | | | | | | | | | Total | Mean male age ± 1 S.E.[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 15 | 16 | 18 | | |
| 2 | | 1 | | | | | | | | | | | | | | 1 | 3 |
| 3 | 2 | 3 | 1 | | 1 | | | | | | | | | | | 7 | 3 ± 0.5 |
| 4 | 2 | 2 | 4 | 2 | | | | | | 1 | 1 | | | | | 12 | 5 ± 0.9 |
| 5 | | 1 | 4 | 3 | 2 | | | | | | | 1 | 1 | 1 | | 13 | 7 ± 1.3 |
| 6 | 1 | | 2 | 2 | 2 | 3 | 1 | | | 1 | | | | | | 12 | 6 ± 0.7 |
| 7 | | 1 | | 4 | 3 | 1 | 1 | | 1 | 1 | | | | | | 12 | 6 ± 0.7 |
| 8 | | | | | | | | | | | | | | 1 | | 1 | 16 |
| 9 | | | | | | | | | | | 1 | | | | | 1 | 12 |
| 10 | | | 2 | | | | | | | | | | | | | 2 | 4 |
| 11 | | | 1 | 2 | 1 | | | | | | 2 | 1 | | | 1 | 8 | 9 ± 1.8 |
| 12 | | | | 3 | | 1 | | | 1 | | | | | | | 5 | 6 ± 1.0 |
| 13 | | | | 1 | | 1 | | | | | 1 | | | | | 3 | 8 ± 2.1 |
| 14 | | 1 | | 1 | 2 | | | 1 | 1 | | | | | | | 6 | 7 ± 1.1 |
| 15 | 1 | | | | 2 | | 2 | | | 1 | | 1 | 1 | | | 8 | 9 ± 1.5 |
| 16 | | | | | | | | 1 | | | | | | | | 1 | 10 |
| 17 | | | 1 | 1 | | 1 | | | | | | | | | | 3 | 5 ± 0.9 |
| 18 | | | | | | | 1 | | | | | | | 1 | | 2 | 12 ± 4.0 |
| 19 | | 1 | | | | 1 | | | | | | | | | | 2 | 5 ± 2.0 |
| 20 | | 1 | | | | | | | | | | | | | | 1 | 3 |
| 21 | | 1 | | | | | | | | | | | | | | 1 | 3 |
| Total | 6 | 12 | 15 | 19 | 13 | 8 | 5 | 2 | 3 | 4 | 5 | 3 | 2 | 3 | 1 | 101 | |

[a] when a sample of one occurred, the male age in that pair is shown.

**Table 2.1** The frequency of age combinations in known age pairs in 2001, 2002 and 2003 (no pairs repeated). Ages not present in the data are not shown in the table (e.g. there were no males of age 14 years).

*(a)* $n$ = 101 pairs of known age




*(b)*

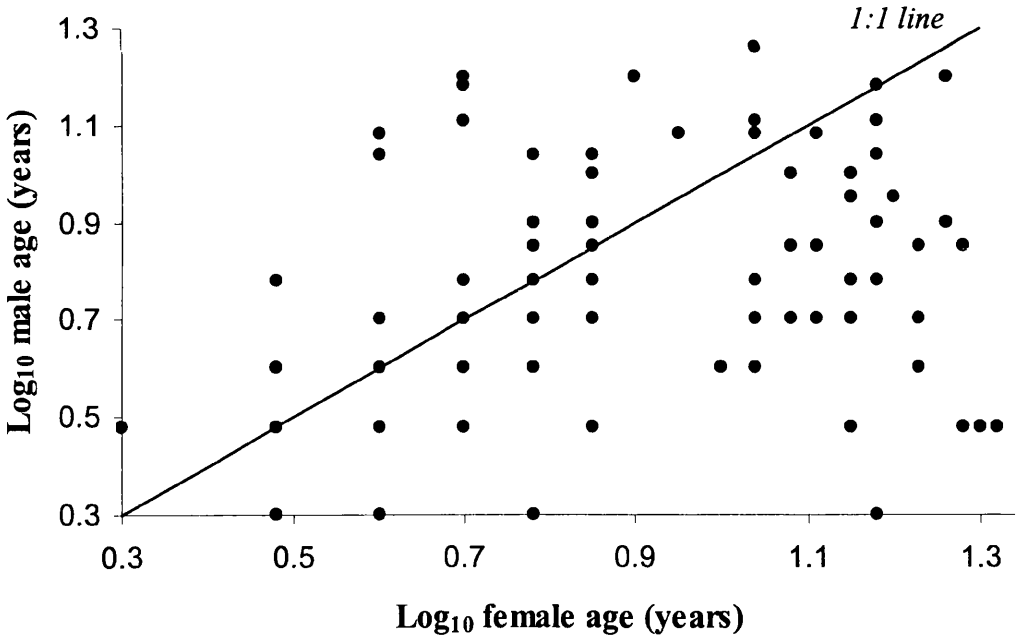



**Figure 2.1** The relationship between *(a)* the age of the male and the age of the female, and *(b)* these values $\log_{10}$ transformed, in pairs in which both birds are of known age for the years 2001, 2002 and 2003 (each pair appearing only once in the dataset). The 1:1 lines are shown for comparison.

| Regression | $F$ | $p$ | adj. $R^2$ |
|---|---|---|---|
| Linear | $F_{1,99} = 11.79$ | < 0.001 | 0.10 |
| Logistic | $F_{1,99} = 12.83$ | 0.0005 | 0.11 |
| **Quadratic** | $\mathbf{F_{2,98} = 11.54}$ | **< 0.0001** | **0.17** |

**Table 2.2** The relationship between the ages of pair members was best described by the quadratic regression (shown in bold), suggesting that the positive correlation between male and female age among young breeders broke down in middle age. These analyses were carried out on $\log_{10}$ (age).


**Figure 2.2** There was a positive correlation between the difference in age of members of a pair and the age of the female. Thus, the oldest females were paired with males of much younger ages, with the age gap extending to over 15 years.

# Egg volume in relation to female age, size and measures of maternal state

## ABSTRACT

Resource allocation to eggs can have consequences for the fitness of both offspring and parents. Individual females may adjust, or be constrained, in their level of egg investment in relation to their current state, and state can be age-related. In this chapter egg volume in the European shag is examined with respect to female age and several potential state variables, with a particular emphasis on changes in old age. Female size and the time in the season at which eggs are laid are also considered as possible explanatory variables. Evidence for a decline in egg size in old females is presented. This decline is observed at both the population and individual level, possibly constituting a senescence effect. Laying date also explained part of the variation in egg size, with early laying females producing larger eggs. In contrast female size, blood parameters and cell-mediated immune response were unrelated to egg volume. A negative relationship between female body condition at chick rearing and clutch volume highlighted the need to consider the time at which adult state variables are measured. A range of mechanisms that might be responsible for the decline in egg size in old age are discussed.

## INTRODUCTION

Individuals vary in the size, quality and number of eggs they lay (Price 1998, Christians 2002). These traits are important for two main reasons. Firstly, egg attributes directly affect the offspring that hatch from those eggs. Secondly, investment in egg production can affect the condition of the laying female, which in turn influences subsequent reproductive performance and survival.

As egg characteristics may depend on the current state of the laying female, in this chapter clutch and egg size in the European shag are investigated in relation to female age, size, and other physical traits related to condition in birds. In particular I have looked for changes in these variables in old shags, as a potential indicator that there are constraints on reproduction imposed by senescence. Analyses of egg size changes within individuals with age are carried out in order to test whether patterns at the population level also occur in individuals, and are not solely due to selection. Longitudinal analysis also controls for differences among females, such as inherent quality. Throughout the introduction and discussion, the term egg size is used for measures of volume or mass, which are highly correlated (e.g. Reid and Boersma 1990, Arnold 1992, Meathrel *et al.* 1993, Smith, Ottoson and Ohlsson 1993, Viñuela 1997).

### *What are the predictions for egg differences among females of different age, size, or condition?*

#### *1. Age of the laying female*

Maternal age can affect egg attributes, and hence offspring. Increasing clutch or egg size between young, often first-time, breeders and older individuals is a common pattern in birds, and is one component of a general tendency for early improvement in reproductive success (e.g. common tern *Sterna hirundo*: Nisbet, Winchell and Heise 1984, glaucous-winged gull *Larus glaucescens*: Reid 1988, great skua *Catharacta skua*: Hamer and Furness 1991, Ratcliffe, Furness and Hamer 1998, kittiwake *Rissa tridactyla*: Thomas 1983, lesser snow goose *Anser caerulescens caerulescens*: Rockwell *et al.* 1993, wandering albatross *Diomedea exulans*: Croxall, Rothery and Crisp 1992, Weimerskirch 1992, see chapter 1). This has also been previously documented in shags (Snow 1960, Coulson, Potts and Horobin 1969,

Potts, Coulson and Deans 1980, Daunt *et al.* 1999). However, whether egg characteristics change in old age as a consequence of senescence has received much less attention.

There are several alternative hypotheses regarding the effects of old age on reproductive output (Clutton-Brock 1984, Martin 1995). Breeding performance could stabilise, increase, or decrease in old age. A levelling-off in the productivity of old individuals would occur if there is no impact of senescence on reproduction, and early improvements lead to optimisation of breeding and foraging ability in middle-age. Continued improvement in these skills throughout life would, in contrast, lead to a pattern of increase in old age. In birds that form stable, long-term pair bonds, the increasing experience of and with a mate may also be important (Cézilly and Nager, 1996). Increasing reproductive output in old age could alternatively relate to greater reproductive effort by the oldest individuals, due to falling residual reproductive value with the diminishing number of future opportunities to breed (Fisher 1930, Williams 1966*a*, *b*, Pianka and Parker 1975, Charlesworth 1980). In contrast, declines in reproductive performance and egg investment in old birds may be imposed by ageing processes, perhaps constraining the amount of effort old birds can invest in reproduction, or masking increased effort. The other possibility is that declining reproductive performance represents a strategy to reduce effort and prolong life.

There are difficulties in assessing senescence in the wild (Nisbet 2001, chapter 1). One problem is that old individuals could become reproductively inactive, which may mean they are not present at the breeding colony or, if present, may fail to breed. This would reduce their chances of being sampled in most studies. Nevertheless, there is some evidence for declining egg size in the oldest birds, which supports the idea that reproductive senescence can affect these parameters (e.g. Antarctic blue-eyed shag *Phalacrocorax atriceps*: Shaw 1986, common tern: Nisbet, Winchell and Heise 1984, but see Nisbet, Apanius and Friar 2002, glaucous-winged gull: Reid 1988, great skua: Hamer and Furness 1991, Hawaiian *geese Branta sandvicensis*: Woog 2002, herring gull *Larus argentatus*: Davis 1975, red-billed gull *Larus novaehollandiae scopulinus*: Mills 1979, wandering albatross: Weimerskirch 1992, yellow-eyed penguin *Megadyptes antipodes*: Richdale 1955, review Newton 1989). Declining clutch size in old age has also been demonstrated (e.g. peregrine

falcon *Falco peregrinus*: Clum, 1995). However, very few studies have confirmed that patterns seen at the population level also occur within individuals as they age. Longitudinal comparisons that do demonstrate senescence (in clutch size) have been made in the white-tailed ptarmigan *Lagopus leucurus* (Wiebe and Martin, 1998), Eurasian sparrowhawk *Accipiter nisus* (Newton and Rothery, 2002) and red-billed chough *Pyrrhocorax pyrrhocorax* (Reid *et al.* 2003).

There is also some evidence for continued increases in reproductive performance among the oldest birds (Pugesek 1981, Nisbet, Apanius and Friar, 2000). In fact, there is no reason why different mechanisms cannot act in different species or populations. These are likely to depend on the particular life-history characteristics of the group. An example concerning age-specific reproduction in early life comes from the extremely long-lived wandering albatross, in which the average age of first breeding is 11-12 years (Weimerskirch and Jouventin, 1987). In these birds, there is no improvement in foraging performance with age among early breeders, and neither is adult survival influenced by reproduction at early ages (Lequette and Weimerskirch 1990, Croxall 1991, Weimerskirch 1992, Berrow, Humpidge and Croxall 2000). So, in contrast to shorter-lived species, breeding is delayed until individuals are fully competent foragers and the risk of increased mortality at first breeding has disappeared. Even within a population, in practice more than one mechanism could act simultaneously on reproductive output, for instance there may be ongoing weak selection for increased effort, but some constraints imposed by the ageing processes. Various components of breeding may be affected differently, for example old white-tailed ptarmigans show reproductive senescence for laying date and clutch size, but still have the highest overall production of any age class, suggesting that parental chick rearing ability compensates for the reduced egg production (Wiebe and Martin, 1998). Generally, senescence-related changes in birds have more commonly been seen in the laying and egg production stages of reproduction, and are less often evident in the rearing and production of young (Newton, 1989).

### *2. Size of the laying female*

Size can refer to either the structural (body) size, or the mass, of an organism. These two measures are different (though likely to be correlated) because they reflect food

intake over two different time scales, different types of growth, and only mass reflects the level of stored reserves that can be mobilised when needed. While structural size in most birds becomes fixed at maturity or in early adulthood, and thus is a function only of juvenile growth and development, mass can vary greatly within and among years throughout life (e.g. Ankney 1982, Croxall 1984). Hence mass, or mass in relation to structural size, is a measure of current state, and is discussed in the following section on female condition (but see Merilä, Kruuk and Sheldon, 2001). Here I consider only how egg size might relate to differences in the structural size of females.

Body size is often correlated with important life history traits such as survival (e.g. Boag and Grant, 1981) and fecundity (e.g. Petrie 1983, Alisauskas 1987). Intra-specifically, body size differences may be influenced by environmental variation (Larsson and Forslund 1991, Sedinger, Flint and Lindberg 1995, Merila 1997). Large body size may enable females to store more reserves (Sedinger, Flint and Lindberg, 1995), which are then available to invest in egg production. A positive relationship between body size and egg volume has been demonstrated in the following birds: dunlin *Calidris alpina* (Ricklefs, 1984), black brant *Branta bernicla* (Sedinger, Flint and Lindberg 1995), Cape petrel *Daption capense* (Weidinger, 1996), snow petrel *Pagodroma nivea* (Barbraud *et al.* 1999), and American oystercatcher *Haematopus palliatus* (Nol, Baker and Cadman, 1984, but not the Eurasian species *Haematopus ostralegus,* Jager, Hulscher and Kersten 2000), as well as in other animals (review Roff 1992). In some cases, this may relate to constraints imposed by the size of the pelvic aperture (Congdon and Gibbons, 1987). In an analysis that simultaneously tested for effects of inter-annual environmental differences, female breeding experience, and female body size on the size of eggs in the northern fulmar *Fulmarus glacialis*, female body size was found to explain most variation (Michel *et al.* 2003).

### *3. Condition of the laying female*

Adult body condition is likely to have a large influence on reproductive decisions (e.g. Drent and Daan 1980, Winkler and Wilkinson 1988, de Laet and Dhondt 1989), including egg allocation. However, it has been difficult to find adequate measures of condition, because of uncertainty surrounding their physiological basis or the time-

scale over which they are affected (Drent and Daan 1980, chapter 5). Frequently used indices include body composition measures related to nutritional status, such as mass, mass controlled for structural size, or subcutaneous fat scores. Other measures frequently used are blood parameters, such as haematocrit or white cell counts, which are assumed to relate to health (but see Dawson and Bortolotti 1997), and measures of immune response, such as reaction to the antigen phytohaemagglutinin (review Brown, 1996). The time at which condition is measured in relation to the breeding cycle may be particularly important in determining cause and effect: parental condition may influence reproductive decisions, but it will also be affected by them, and these two interpretations should be separated. Unfortunately, in wild birds it may only be possible to measure individuals at particular times or stages in the breeding cycle. For instance, the shag is most amenable to capture during chick rearing. It may also be difficult and undesirable (because of disturbance) to measure individuals repeatedly.

In some studies, egg size relates positively to female condition (mass-based measures: Murphy 1986, Hepp *et al.* 1987, Wiggins 1990, Smith, Ottosson and Ohlsson 1993, Wiebe and Bortolotti 1995, Hanssen, Engebretsen and Erikstad 2002, Parker 2002, fat stores: Smith and Moore 2003, blood parameters: Moreno *et al.* 2002). Adult condition can be experimentally increased by supplemental feeding, often with positive effects on clutch size, egg size and egg quality (review Price, 1998).

Senescence is expected to entail a fall in condition, and declines in survival with increasing age are often associated with reduced physical condition, at least in mammals (Clutton-Brock, 1988). Lower body condition (lower weight) in old age has been observed in female wandering albatrosses (Weimerskirch, 1992) and white-tailed ptarmigans (Wiebe and Martin, 1998), which also lay smaller eggs or clutch sizes respectively than younger birds. A condition-dependent morphological trait, the length of the outermost tail feathers, decreases and parasite infestations increase in old barn swallows *Hirundo rustica*, which also exhibit declining reproductive success and an increase in age-specific mortality (Møller and de Lope, 1999). In contrast, immunity levels in common terns appear to be unrelated to age (Apanius and Nisbet, 2003). Overall, a link between declining reproductive performance in old age and declining condition might be predicted (but see chapter 5).

### *Effects of egg production on parental fitness*

The success of the current breeding attempt, future attempts and parental survival can be influenced by changes in parental condition resulting from egg production. There is good evidence that reproduction in general is costly (reviews Nur 1988, Winkler and Wilkinson 1988, Clutton-Brock 1991, Roff 1992, Hochachka 1992, Stearns 1992, also see Drent and Daan 1980, Gustafsson and Sutherland 1988). For instance, female red deer (*Cervus elaphus*) that produce a calf in one breeding season have a reduced chance of surviving to the next season, and if they do survive are less likely to calve that year (Clutton-Brock, Guinness and Albon, 1982). In *Drosophila*, females that are highly fertile early in life have shorter lives (Rose and Charlesworth 1981*a*,*b*, Luckinbill *et al.* 1984), and this has also been observed in a wild population of red-billed choughs (Reid *et al.* 2003). Summer survival rates are lower among reproducing willow (*Parus montanus*) and crested tits (*Parus cristatus*) than non-reproducing individuals, and survival is negatively related to clutch size (Ekman and Askenmo, 1986). Clutch size of older individuals in the collared flycatcher (*Ficedula albicollis*) is negatively affected by their reproductive effort early in life (Gustafsson and Pärt, 1990). Where costs of reproduction have not been observed, this may be a consequence of covariation among life-history traits, for instance with individual state (Partridge and Harvey, 1985, van Noordwijk and de Jong 1986). Alternatively, reproductive costs may only be evident when individuals are stressed or environmental conditions are particularly poor (e.g. Fairburn 1977, Haukioja and Hakala 1978, Tinbergen, van Balen and van Eck 1985, Williams and Christians 2003).

Until recently it was generally assumed that the main cost of reproduction in species with parental care was the cost of rearing offspring (Lack 1947, Lessells 1991, Roff 1992, Stearns 1992). Larger broods are more costly to raise (e.g. Dijkstra *et al.* 1990, Daan, Deerenberg and Dijkstra 1996). However, it has now been demonstrated that costs of egg production *per se* and incubation in birds are not insubstantial (Carey 1996, Williams 1996, Monaghan and Nager 1997, but see Ward 1996, Williams and Vézina 2001). Independently of effects on egg quality or effects of rearing an enlarged brood, laying extra eggs reduces the ability of parents to subsequently provision their offspring (Monaghan, Nager and Houston, 1998). There is also a negative effect on egg quality when extra eggs are laid (Monaghan,

Bolton and Houston 1995). This results from greater depletion of protein reserves, mostly from flight muscle (Houston *et al.* 1995), as more eggs are produced (Carey 1996, Bolton, Monaghan and Houston 1993), although the extent to which different females are affected may be state-dependent (Heaney and Monaghan 1996, McNamara and Houston 1996, Monaghan, Nager and Houston 1998).

Incubating extra eggs can also be costly (review Reid, Monaghan and Nager, 2002): the chicks of common terns given an extra egg to incubate (removed before hatching, so that the increased cost was solely due to incubation) experienced poor growth compared to a control group (Heaney and Monaghan 1996). In barnacle geese *Branta leucopsis*, a prolonged incubation period leads to lower body condition of the incubating female at hatching (Tombre and Erikstad, 1996). The energy costs of incubation increase with larger clutch sizes (Haftorn and Reinertsen, 1985). In blue tits *Parus caeruleus* where both sexes feed the offspring but only the female incubates, large clutch sizes result in weight loss and greater mortality of the female, but not the male, parent (Nur, 1984).

So, current reproductive investment trades-off against future survival and reproductive performance (Williams 1966*a*, *b*, Lindén and Møller 1989, Roff 1992, Stearns 1992). Parental condition influences the reproductive decisions of iteroparous breeders (Clutton-Brock 1991, Monaghan, Uttley and Burns 1992, Weimerskirch *et al.* 1997), and these variables will have a bearing on the performance of old birds.

### *Paternal effects on egg traits*

As well as effects of the mother's phenotype, some of which are mediated in birds by egg differences, offspring can also be influenced by the father's phenotype (review Bernardo 1996). In fact these paternal effects can also act via eggs, where fathers make a nutritional investment as in the case of courtship feeding (Nisbet, 1973) or, in insects, transmission of a nutrient-rich spermatophore (Butlin, Woodhatch and Hewitt, 1987). In addition, characteristics of the male partner can influence the reproductive effort of females (e.g. Burley 1988, de Lope and Møller 1993), including their investment in eggs. Female peahens (*Pave cristatus*) lay more eggs when they have mated with a preferred male (Petrie and Williams, 1993), as do female zebra finches *Taeniopygia guttata* (Balzer and Williams, 1998), which also

invest different amounts of testosterone in their eggs in relation to male attractiveness (Gil *et al.* 1999). In mallards (*Anas platyrhynchos*), females lay larger eggs after mating with a preferred male and smaller eggs after mating with a less preferred male (Cunningham and Russell, 2000). In species where feeding territories are held by males, the male's phenotype can indirectly affect the egg production of his mate via differences in territory quality and thus food available to the laying female (e.g. Potti, 1993). These examples all suggest that characteristics of the male parent can have consequences for egg attributes. Although paternal effects on eggs in the shag are not investigated here, choosing instead to concentrate on effects of the mother's phenotype, paternal characteristics should be borne in mind as a potential explanation for some of the residual variation in egg size.

### *Studies of European shag eggs*

A number of studies have reported the egg characteristics of European shags (Snow 1960, Potts 1966, Coulson, Potts and Horobin 1969, Potts, Coulson and Deans 1980, Aebischer 1985, Barrett, Strann and Vader 1986, Stokland and Amundsen 1988, Barrett 1989, Amundsen and Stokland 1990, Grau 1996, Daunt *et al.* 1999, 2001, Daunt 2000). In summary, these have shown the following:

1. No difference in clutch size with parental age, with more than 70% of birds laying three eggs.
2. A slight decrease in clutch size across the season (Snow 1960, but none found by Potts, Coulson and Deans 1980).
3. A distinct pattern of intra-clutch egg size variation, with the second-laid egg the largest in clutches of three, and very little difference in the volume of the first- and last-laid egg. The variation in egg size is greater among than within clutches.
4. Young females lay smaller (both length and breadth) and lighter eggs than older females. However, the overall lower breeding success of the youngest breeders was found to be unrelated to differences in egg quality or hatching success (Daunt *et al.* 1999, 2001, Daunt 2000).
5. A seasonal decrease in egg breadth and volume.
6. The interval between nesting and laying correlates with the size of eggs laid.

7. No indication of a change in the shape index of eggs in any clutch size; differences in egg breadth are usually accompanied by changes in length.
8. The size of eggs laid by different females may be in proportion to their own body weight (Snow, 1960), though this trend was derived from a sample of only 7 individuals (all weighed after eggs were laid). Re-analysis of this data showed the relationship was close to significance ($r = 0.74$, $n = 7$, $p = 0.055$).
9. Replacement eggs laid after loss or removal are smaller than their respective first clutches. However, egg production in the shag was calculated to impose only a small additional food need (Grau 1996; also gives details of egg composition).
10. Double brooding is a very rare event in this species, occurring among pairs that breed early in the season, in years when the breeding season begins early. Few second clutches result in fledged chicks (Wanless and Harris, 1997).
11. There has also been some work on the occurrence of pollutants in shag eggs (Potts 1968, Allen and Thompson 1996). These have found considerable variation in the levels of polychlorinated biphenyls and organochlorine pesticides in different eggs collected at the same location. In one case the level of dieldrin correlated with total clutch or brood failure (Potts, 1968), but the concentration of this toxin in eggs was not related to maternal age (Robinson *et al.* 1967).
12. There is no difference in the volume of eggs from which male and female chicks hatch (Daunt *et al.* 1999).

There is also evidence from these studies that egg size in the shag has consequences for offspring. There is a significant correlation between egg volume and the mass of chicks within 5 hours of hatching, although the volume difference of the first and second eggs is generally counteracted in the chicks by hatching asynchrony. There is also a growth advantage to chicks hatching from large eggs, which is not solely explained by differences in parental quality (Amundsen and Stokland 1990, but also see Daunt *et al.* 2001). As is evident from this summary, the eggs of old birds have not previously been specifically studied in the shag.

# METHODS

### *Measuring egg dimensions in the field*

Egg dimensions were measured and used to calculate volume, rather than weighing eggs. As egg mass changes during incubation, it should be measured when the eggs are freshly laid. For large samples this is logistically difficult in shags, as many nests must be monitored for precise laying times, and this can cause disturbance-shags are most vulnerable to disturbance during laying and early in incubation. The linear dimensions of the eggs in contrast do not change during incubation and so, provided the eggs are not lost, can be measured at a time when disturbance is minimal. Volume and mass have been shown in previous studies to be highly correlated (see introduction).

In 2001, 2002 and 2003 the length and breadth of eggs was measured (to 0.1 mm) at the nest using callipers. Eggs from a total of 106 clutches were measured in 2001. Of these clutches, 8 were excluded from analysis for one of the following reasons. Firstly, cases were excluded if single eggs were laid and lost before a full clutch was established, when these eggs were not measured. Secondly, cases were excluded if it was likely that two females laid at one site, either because they were both seen occupying that site, or because unusually large clutch sizes appeared and eggs were found off the nest. Thirdly, egg measurements from replacement clutches, laid after a female had already lost a clutch either at the same site or nearby, were removed from the data before analysis. In 2001 this left 98 clutches. These reasons for exclusion were also applied to data from other years: in 2002 this left a sample of 84 clutches. In 2003, 25 clutches of three eggs only were measured, and no cases were excluded.

In 2001 and 2002, the order in which eggs were laid was recorded by making regular nest checks and marking eggs at the nest with indelible markers. Egg volumes in some clutches for which the laying sequence was not fully known were also recorded. The 'A' egg refers to the first laid egg in a clutch, 'B' the second, and 'C' the third. In 2003 laying sequences were not monitored. Laying date refers to the day on which the first egg in a clutch was laid. This was observed either directly by daily nest checks, derived by counting back through the laying sequence (eggs are

normally laid every third day, Snow 1960), or calculated from the day on which the first egg hatched, using the mean laying-to-hatching period of 35 days

### *Egg volume and relative egg mass calculations*

Egg volume was calculated from the linear dimensions of eggs by the formula: Volume (cc) = 0.51 (length * breadth$^2$) (Stonehouse 1966, Preston 1974, Hoyt 1979). Hoyt (1979) estimated that the volume of most bird eggs can be determined to within 2% using this formula and volume coefficient. To calculate egg mass relative to the mass of the laying female, our volume measurements were converted using an average value of 1.05 g cm$^{-2}$ for specific gravity and the formula: weight = 0.51 × (length × breadth$^2$) × specific gravity (Bergtold 1929, Stonehouse 1966). It was necessary to derive an estimate of egg mass from volume for the comparison of egg mass to the mass of the laying female, because fresh egg mass was not measured. Weights calculated for each egg in a clutch were summed to give clutch weight, which was then divided by female weight. Egg composition was not measured, as this generally requires destructive sampling.

Patterns of clutch size, relative egg mass and intra-clutch egg size differences were investigated in 2001 and 2002. The proportion of the variation in egg size among females was compared to intra-clutch egg size variation. For certain analyses, including those in relation to female age, 2002 data were excluded. Measurements in 2002 were biased because work that year focussed on a manipulation in which clutches of three were exchanged between females of different age, matched for laying date (see appendix).

### *Comparisons of egg volume in relation to laying date, maternal age, size and potential state variables*

#### *1. Cross-sectional comparisons*

Egg size was compared among females of different age, and in relation to laying date by a cross-sectional analysis of 2001 data. In line with the prediction that senescence may cause declining reproductive performance in old age, egg volume relationships were tested by quadratic regression with respect to age, compared to linear and logistic models. The quadratic relationship is reported where it provided

the best explanation of the data. Further confirmation of declining performance in old age was made by testing for a negative correlation between egg volume and age beyond the peak in volume- this allows a decline to be distinguished from a levelling-off in egg volume at old age. Clutch size was controlled in egg volume analyses by restricting these to clutches of the most common size, three eggs.

Laying date, age and interaction effects on egg volume were simultaneously assessed in a General Linear Model (GLM) that included female age, female age$^2$ and laying date as covariates. For last-laid eggs, the influence of laying date was also examined by plotting, for early- and late-layers, the standardised residuals from the quadratic regression on female age.

As well as considering egg attributes in relation to female age, relationships with linear size measures (head and bill, wing and tarsus length), mass, mass controlled for structural size, the blood parameters haematocrit and mean corpuscular volume (MCV), and a test of cell-mediated immunity (PHA test) in females were also investigated. Details of how these were measured in the field and laboratory are given in chapter 5, where it is also shown that they bear no relationship to adult age. Although not confounded with age, these physical traits could explain part of the residual variation in egg volume. A principal components analysis (PCA) of the three linear size measures was carried out for females, and the first factor from this analysis compared to egg and clutch volumes in 2001. The first factor explained approximately 67% of the variance in these three size measures that year. It is important to bear in mind the time at which the physical traits of females were measured- after egg production and incubation, when the birds were rearing medium to large chicks.

### *2. Longitudinal analysis*

The longitudinal dataset comprised egg volume measurements I made in 2001, 2002 and 2003, plus the egg volumes of any of these females also measured in 1997, 1998 or 1999 (by Francis Daunt). Analysis was restricted to individuals for which egg number and volumes were known in more than one year. Variability in the clutch sizes laid by individuals among years was assessed in relation to age. For females that consistently laid three eggs, the proportion of the variance in the A, B and C egg volume of individuals among years was calculated and compared to that among

females. I tested for differences in the average volume of three egg clutches among years and between measurers. For females of known age that laid three eggs in each year, the change in the volume of consecutive clutches was compared in relation to female age in the first year. A paired *t* test was used to compare clutch volume at age $x$ to that at age $x + 1$. In order to examine whether the magnitude of change varied with age, the clutch volume changes of known age females that laid three eggs in both 2001 and 2002 were also plotted in relation to their ages when first measured.

Year, and the number of years between measures were included in a GLM for clutch volume change with female age (in this analysis comparisons were not restricted to consecutive years). Standardised clutch volume residuals, accounting for year and the number of years between measures, were plotted for different age categories of females.

A residual maximum likelihood (REML) mixed model was used to test for the quadratic relationship of female age to clutch or C egg volume in the longitudinal data, which incorporated differences among years and among females as random effects. Female identity was nested within year as a random effect in this analysis. To confirm the decline in old age, these analyses (without the quadratic for female age) were repeated for only those females aged 10 years or older. The model was also repeated incorporating laying date as an additional covariate.

Repeatabilities of egg size were calculated using the methods described by Lessells and Boag (1987), and Becker (1984). All analyses except the REML mixed models were carried out in SPSS (release 10.0, copyright 1989-1999, SPSS Inc.). REML models were tested in SAS (release 8.2, copyright 1999-2001, SAS Institute Inc.), using the Satterthwaite method for calculating degrees of freedom (to account for repeated measures of the same individuals). Means and slope estimates are shown ± 1 S.E.

# RESULTS

## *General patterns of egg number and size*

In total, 87% of clutches measured in 2001 consisted of three eggs, while only 9% of females laid two eggs and 4% laid four eggs. In both 2001 and 2002 clutches of three ($n$ = 54, $n$ = 73 respectively) weighed approximately 9% of mean female body mass (1628g in 2001, 1680g in 2002). The relative weight of two egg clutches was 5- 6% (2001 $n$ = 8, 2002 $n$ = 6), and of four egg clutches 11-12% (2001 $n$ = 4, 2002 $n$ = 2).

The volumes of the A, B, and C eggs in clutches of three are significantly different, and this pattern of intra-clutch variation did not differ between 2001 and 2002 (repeated measures ANOVA, volumes of A, B and C eggs: $F_{2,174} = 43.92$, $p < 0.001$, year: $F_{1,87} = 0.39$, $p = 0.54$, interaction term: $F_{2,174} = 0.21$, $p = 0.81$, figure 3.1). The B egg was on average the largest egg within clutches of three, with the C egg approximately 1.4% and the A egg 4.0% smaller than the B egg. The B egg is of both greater length and greater maximum breadth than the other eggs in the clutch (table 3.1). Despite this, not all clutches display the average intra-clutch egg size pattern: the B egg was the largest egg in 56% of three egg clutches in 2001. Of the clutches in which the B egg was not the largest ($n$ = 24), there were 7 clutches in which the A egg was largest, 16 clutches in which the C egg was largest, and 1 clutch in which the A and C egg were of equal volume.

The average dimensions of eggs in clutches of two, three and four for which laying order was known in 2001 are shown in table 3.2. In 8 out of 9 clutches of two the B egg was larger than the A egg, and in 2 of 4 clutches of four the B was the largest egg in 2001. The volume of A and B eggs did not differ between clutches of two ($n$ = 8) and clutches of three ($n$ = 54, Mann-Whitney test for A eggs $z = 0.12$, $p > 0.05$, for B eggs $z = 0.10$, $p > 0.05$). Egg size differences were greater among females than within individual clutches: in 2001 67% of the variance in egg volumes occurred among females (S.E. = 0.05, 95% C.I. = 0.56, 0.76, $F_{84, 170} = 6.97$, $p < 0.001$).

## Cross-sectional comparisons of egg number and size

### 1. With female age

There was no significant difference in the ages of females that laid two, three or four eggs (Kruskal-Wallis test $\chi^2_2 = 1.75$, $n = 77$, $p = 0.42$). In 2001, in clutches of three for which laying order was known, egg volume was correlated within clutches (A and B egg: $r = 0.76$, A and C egg: $r = 0.67$, B and C egg: $r = 0.82$, in all cases $n = 54$, $p < 0.001$, figure 3.2). The volume of the A egg was not related to female age (linear, logistic and quadratic regressions $p > 0.05$). However, both B and C egg volumes showed significant quadratic relationships with female age (quadratic regressions B eggs: $F_{2,41} = 7.65$, $p = 0.002$, *adj.* $R^2 = 0.24$, C eggs: $F_{2,47} = 8.69$, $p < 0.001$, *adj.* $R^2 = 0.24$, figure 3.3). The linear and logistic regressions were not significant ($p > 0.05$). The peak in B egg volume and that in C egg volume occurred between 10 and 11 years of age (figure 3.3). For females older than 11 years, there is a significant negative correlation between female age and B or C egg volume (B eggs: $r = -0.64$, $n = 13$, $p = 0.02$, C eggs: $r = -0.61$, $n = 14$, $p = 0.02$). Consequently, the quadratic regression of clutch volume on female age, for three egg clutches in 2001, was also significant ($F_{2,63} = 4.83$, $p = 0.011$, *adj.* $R^2 = 0.11$, figure 3.4). There was no significant relationship between female age and the volume of the smallest egg (A) / volume of the largest egg (B) ($r_s = -0.14$, $n = 44$, $p = 0.38$).

### 2. With laying date

Laying date varied with female age in 2001 (for egg volume dataset, $F_{1,55} = 7.60$, $p = 0.008$, *adj.* $R^2 = 0.11$), such that young females laid later than old females (figure 3.5, also see chapter 4). A plot of clutch size against laying date did not indicate that the number of eggs laid differed across the season in either 2001 or 2002, as clutches of two and four tended to occur close to the median laying date in both years (figure 3.6).

In 2001, laying date had no effect on the volume of A or B eggs in clutches of three (A egg volume: $F_{1,58} = 0.34$, $p = 0.56$, B egg volume: $F_{1,46} = 1.93$, $p = 0.17$). However, there was a significant, though weak, negative relationship between laying date and the volume of the C egg ($F_{1,53} = 7.12$, $p = 0.010$, *adj.* $R^2 = 0.10$, figure 3.7*a*). When the length and breadth of the C egg were considered separately, only breadth

showed a significant negative relationship ($r$ = -0.37, $n$ = 55, $p$ = 0.006). This was not explained by females of different size laying at different times in the season, as there was no correlation between female body size and laying date in 2001 ($r$ = 0.27, $n$ = 21, $p$ = 0.24). The overall volume of three egg clutches also declined significantly as laying date advanced ($F_{1,70}$ = 4.33, $p$ = 0.04, figure 3.7*b*), although laying date alone explained only a very small amount of the overall variation (*adj.* $R^2$ = 0.05).

When laying date was included in a GLM for C egg volume in 2001 with female age and female age$^2$, laying date was not significant (laying date: $F_{1,40}$ = 2.58, $p$ = 0.12, female age$^2$: $F_{1,40}$ = 11.13, $p$ = 0.002), and there was only a very small increase in the amount of variation explained by the model (*adj.* $R^2$ from 0.24 for the quadratic regression on female age to 0.26 when laying date was included). The interaction between the quadratic term for female age and laying date was also not significant ($F_{1,38}$ = 0.05, $p$ = 0.82). Residuals from the quadratic regression of C egg volume on female age were compared between females that laid early (on or before the median laying date) and females that laid late (after the median laying date) in 2001. After controlling for age there was a tendency, though non-significant ($t_{42}$ = 1.46, $p$ = 0.15), for late-laying females to lay smaller C eggs (figure 3.8). Figure 3.9 shows the relationship between C egg volume, female age and laying date relative to the median laying date in 2001.

### 3. *With female size, mass, and other physical traits*

The frequencies of two-, three- and four-egg clutches in light (< median mass) and heavy (≥ median mass) females in 2001 did not differ from expected frequencies for the null hypothesis of an equal clutch size distribution in each female weight class ($\chi^2_5$ = 2.69, $n$ = 44, $p$ > 0.05). This was also the case when females were classed according to their body condition (mass/ head and bill length, $n$ = 42), or size (factor 1 scores from the PCA of linear size measures, $n$ = 42). Clutch volume (in three egg clutches) was negatively correlated to female body condition (mass corrected for size) during chick rearing in 2001 ($r_s$ = -0.60, $n$ = 17, $p$ = 0.010, figure 3.10). However, when included as a covariate with female age and female age$^2$ in a GLM on clutch volume, female condition (mass/PCA score for size) at chick rearing was not significant (female condition: $F_{1,13}$ = 0.44, $p$ = 0.518, female age$^2$: $F_{1,14}$ = 6.25, $p$

= 0.025). No additional variation in clutch volume was explained by inclusion of female condition as a variable with female age (*Adj.* $R^2$ for female age$^2$ = 0.23, also including female condition = 0.20). Neither female mass nor female size alone correlated with clutch volume ($p > 0.05$). There was no relationship between clutch volume and any other measured physical characteristic of females (in all cases $p > 0.05$).

### *Comparisons of egg number and size within individual females (Longitudinal analysis)*

There were a total of 61 females for which egg volumes were measured in more than one year. Most females (79%) consistently laid three eggs. Among females of known age, 12/57 did not lay three eggs in every year. Of these, 3 individuals consistently laid a clutch of two, while the remaining 9 females had varying clutch sizes each year. There were no individuals in our data that consistently laid four eggs. There was a significant difference between the mean age (calculated using age at first observation) of females that consistently laid two eggs, consistently laid three eggs and those for which clutch size varied between years ($\chi^2_2 = 6.44$, $n = 57$, $p = 0.040$). Females with varying clutch sizes across years were of greater mean age at the first measure than either females that consistently laid three eggs or females that consistently laid two eggs, which were of youngest average age (figure 3.11*a*). Figure 3.11*b* shows, in relation to female age, the frequency distribution of females in each of these categories of clutch size consistency.

For females that laid three eggs in each year, the proportion of the variance in clutch volume among birds was greater than the yearly differences of individuals ($F_{53,69} = 6.95$, $p < 0.001$, $r$ (95% *C.I.*) = 0.72 (0.58, 0.83)). Yearly variation in the clutch volume of individual females thus contributed 17 - 42% of the variance. When the repeatabilities of A, B and C egg volumes in three egg clutches were calculated separately, the proportion of the variance attributed to differences among years for individuals was greatest for C eggs and lowest for the A egg (C eggs: $F_{34,44} = 3.26$, $p < 0.001$, $r$ (95% *C.I.*) = 0.50 (0.24, 0.70), B eggs: $F_{34,44} = 5.64$, $p < 0.001$, $r$ (95% *C.I.*) = 0.67 (0.47, 0.81), A eggs: $F_{34,44} = 7.51$, $p < 0.001$, $r$ (95% *C.I.*) = 0.74 (0.57, 0.86)). Overall, there was no difference in the mean volume of three egg

clutches among the years 2001, 2002 and 2003 ($F_{2,134} = 0.065$, $p = 0.94$, mean for all three years (95% *C.I.*) = 138.14 (136.37, 139.90) $cm^3$).

Before looking at egg volume change within individual females as they aged, I tested for observer effects; in 1997, 1998 and 1999 eggs were measured by a different person than in 2001, 2002 and 2003. However, there was no difference between measurers in the mean volume of three egg clutches recorded ($t_{122} = 0.19$, $p = 0.85$). Table 3.3 shows the sample sizes of known age females that laid three eggs in each year, in terms of the year in which the first and last measurements were taken. From these data, 42 pairs of clutch volume measurements taken in consecutive years for individual females were extracted. Table 3.4 shows the number of comparisons available for each combination of age $x$ and $x + 1$. Across all ages in this dataset, there is a correlation between clutch volume at age $x$ and that at age $x + 1$ ($r = 0.89$, $n = 27$, $p < 0.001$). The difference between consecutive years is not significant (paired $t$ test $t_{26} = 1.87$, $p = 0.072$). There is no clear pattern with age in the direction of clutch volume change in consecutive years, although this may be a consequence of the small sample sizes for many of these comparisons, or to confounding effects of year *per se*. It is also perhaps unlikely that a large change in egg volume would occur in the space of only one year. In order to rule out effects of year, clutch volume change between 2001 and 2002 (the pair of consecutive years for which the sample size was greatest) was plotted in relation to female age in the first year, for known age females that laid three eggs in both these years. There was no clear relationship between clutch volume change from 2001 to 2002 and female age (figure 3.12, $r = -0.24$, $n = 34$, $p = 0.17$).

In a GLM analysis for the change in clutch volume, testing the effect of covariates female age at the first observation ($\log_{10}$ transformed) and the number of years between measures, and of the random effect year, the following terms were significant: the interaction between female age and year ($F_{3,39} = 3.35$, $p = 0.029$), the interaction between female age and the number of years between measurements ($F_{1,39} = 12.32$, $p = 0.001$), and all three main effects (female age: $F_{1,39} = 8.37$, $p = 0.006$, time between measures: $F_{1,39} = 11.32$, $p = 0.002$, year: $F_{3,39} = 3.12$, $p = 0.037$). The change in clutch volume after controlling for year and the number of years between measures is shown in relation to female age (figure 3.13).

In a REML mixed model the clutch volume of females that laid three eggs in more than one year was tested in relation to female age in the first year, with the random effects year and female nested within year. This analysis differs from the cross-sectional regressions of egg volume on female age, as it incorporates data on more than one clutch for individual females, thus testing for changes with age within those individuals. There was a significant quadratic relationship between clutch volume and female age ($F_{1,109} = 5.21$, $p = 0.024$, clutch volume = 128.92 + 2.23 (female age) –0.12 (female age$^2$)). The random effect female identity nested within year was also significant ($p < 0.0001$). The linear relationship between female age and clutch volume was not significant ($F_{1,111} = 1.11$, $p = 0.293$). This pattern was also apparent for the volume of the C egg in three egg clutches (quadratic relationship with female age: $F_{1, 74.5} = 5.78$, $p = 0.019$, C egg volume = 40.89 +1.02 (female age) – 0.05 (female age$^2$), random effect year (female): $p = 0.0003$, linear relationship with female age: $F_{1,71.8} = 0.10$, $p = 0.758$). In females aged 10 years or older, for both clutch and C egg volume the tendency was for a reduction with age, but only for C eggs was this decline significant (clutch volume $b = -0.75 \pm 0.50$, $t_{43.8} = 1.50$, $p = 0.142$, C egg volume $b = -0.43 \pm 0.19$, $t_{28.8} = 2.21$, $p = 0.035$). The influence of laying date on clutch volume in the longitudinal data was also tested in a REML analysis for three egg clutches, specifying the same random effects as before. When included as a covariate with female age and female age$^2$, the time of laying in the season had a significant effect on clutch volume, in addition to age (female age$^2$: $F_{1,95.9} = 7.11$, $p = 0.009$, laying date: $F_{1,30.6} = 8.67$, $p = 0.006$, all interactions $p > 0.05$).

## DISCUSSION

This work confirms a number of previous observations regarding the eggs of the shag, such as the intra-clutch variation in egg size, but also extends these studies by showing that the oldest females lay smaller eggs than middle-aged birds. Evidence that this population-level pattern also occurs within individuals, as they grow older, is provided. In addition, laying date influenced clutch volume: regardless of age, females laying late in the season laid smaller eggs than females that laid early. In contrast, no relationship between the size of eggs laid and female size was found, nor was egg size related to most measures of female state. The exception among these was the mass of females, after correcting for size, where it appears that females in lower condition during chick rearing previously laid larger eggs. In the following section, I discuss possible interpretations of this relationship, and the probable causes and effects of declining egg size in old age.

Most shags on the Isle of May laid a clutch of three eggs. Clutch size did not differ among females with regard to age, and there was also no obvious pattern of clutch size change across the season, as the few clutches of two or four that were laid occurred close to the median laying date in each year. Longitudinal analysis of individuals also showed that most females consistently lay three eggs. In females that did not lay clutches of three in every year, the majority varied their egg number rather than consistently laying a clutch of two or four. Thus, clutch size variation appears to reflect current condition or differences in environmental circumstances among years, rather than variation in the inherent quality of females. All females consistently laying two eggs were young (all under 6 years of age). A peak in the frequency of females that consistently laid three eggs occurred at age 5-6 years (refer to figure 3.11*b*). Hence, among old females a greater proportion displayed clutch size variation from year to year.

The volume of eggs in clutches of different size did not differ; shags with smaller clutch sizes are not laying larger eggs. Thus, clutch size reduction in this species corresponds with reduced overall egg allocation. The mass of a clutch of three eggs equates to approximately 9% of female body mass, while two- and four-egg clutches are proportionately smaller, or larger, respectively. The intra-clutch pattern of egg size was the same in 2001 and 2002, with the second-laid egg on average the largest. However, this pattern did not occur in every clutch, and in 30%

of clutches it was the C egg, rather than the B egg, that was largest. Intra-clutch differences in egg size did not vary in relation to female age, and are less than the egg size variation observed among females.

Differences in egg volume resulted from changes in both length and breadth. For instance, within clutches of three, the second-laid egg was on average of both greater length and greater maximum breadth than either other egg. As the last-laid egg is generally of smaller breadth than the second-laid egg, this difference cannot be attributed to constraints imposed on the breadth of the first egg by a small cloacal diameter at the start of laying. In small-bodied species of freshwater turtle the size of the pelvic aperture constrains egg width (Congdon and Gibbons, 1987), but in the shag there is clearly some flexibility in the width of egg that can be produced. This suggests shags are not laying eggs of the maximum size physically possible, but instead that egg size in this species is either constrained or adjusted in relation to other factors. The decline in C egg volume with laying date was due to a change in the breadth of these eggs. However, there was no evidence that females of different size laid at different times in the season.

Cross-sectional analysis showed that as well as initially increasing with age among younger females, clutch volume declines in old shags. This bell-shaped pattern of clutch volume with age is due to differences in the volume of the second- and third-laid eggs. The peak in clutch volume occurs between 9 and 11 years of age. The average volume of three egg clutches of the oldest females sampled (17 year olds) was not as small as that of first-time breeders (3 year olds), but was similar to the clutch volumes laid by 4 year olds. Of the age range measured in 2001, the youngest females (3 year olds) laid clutches 15% smaller, and the oldest females clutches 8% smaller than mean peak clutch volume. So, early improvement in egg volume in the shag is greater than the subsequent decline in old age, although it may be that clutch volume continues to decline in females older than those measured here. Females up to the age of 22 years have been observed to lay eggs on the Isle of May, though these very old individuals are scarce.

The population level pattern of egg size variation with age could result from either changes in egg volume within individuals, or selective mortality, whereby females that lay smaller eggs live longer than females who invest more in egg production and lay larger eggs. In fact, both scenarios could be operating at once, as

has been observed in a wild population of red-billed choughs (Reid *et al.* 2003). Here, a number of different approaches to longitudinal analysis were taken.

There proved insufficient clutch volume data for known age females that laid three eggs in each year to fully examine changes in consecutive years with respect to age. The complicating factor of measurements being taken in different years also hampered analysis by that means. When non-consecutive year comparisons were included, there was an effect of female age, year *per se* and the number of years between measurements on clutch volume change in females. As shown by the residual plot (figure 3.13), young females were more likely, irrespective of year or the time between clutch volume measures, to show an increase in their clutch volume, while old females were more likely to show a decline. In order to confirm this pattern, a mixed model that incorporated year and female as random effects was used. Both clutch and C egg volume changes within individual females showed significant quadratic relationships to age in this model. The regression coefficients for female age and female age$^2$ in the longitudinal analysis are similar to those shown by the cross-sectional quadratic regression (cross-sectional analysis *b* (female age) = +3.89 ± 1.25, *b* (female age$^2$) = -0.19 ± 0.06, longitudinal analysis *b* (female age) = + 2.23 ± 1.08, *b* (female age$^2$) = -0.12 ± 0.05). The age of peak clutch volume predicted by the longitudinal analysis was 9 - 10 years, which is similar to that shown in the cross-sectional regression. This result, together with the residual plot of clutch volume change among different female age classes, strongly suggests the cross-sectional pattern also occurs at an individual level. From year to year, the volume of the last laid egg in clutches of three varied the most within individuals.

Selective mortality on the basis of clutch volume cannot be completely ruled out, as the survival of birds with differing levels of investment was not investigated. However, in long-lived organisms it is less likely that differential selection explains age-specific reproductive performance, because low annual mortality can only cause small changes in the proportion of phenotypes among age classes (Forslund and Pärt, 1995). It is possible that both senescence and changes in the phenotypic composition of the breeding population with age are causing the pattern of egg volume change observed. On a cautionary note, Coulson and Fairweather (2001) identified a depressive effect on performance at the last breeding attempt prior to death in kittiwakes of all ages, which they suggest could be the result of terminal

illness-induced falls in condition, but might be confused with age-related senescence. This cannot currently be excluded in the shag, but no indications of reduced health were observed in the old birds in this study (see chapter 5), and in addition several old females did return and breed in the following years.

Part of the difference in egg volume with age may relate to the time in the season at which the eggs were laid, as laying date negatively correlates with the volume of the C egg, and also varies with female age. However, there was no indication that old females laid later than middle-aged birds. When laying date was included in the longitudinal analysis of clutch volume, there was no interaction with female age. Thus, the effect of time of laying on egg size is consistent at all ages. Although laying date added only a very small amount to the explanatory power of the quadratic regression of C egg volume on female age in the cross-sectional analysis, it was a significant effect in the longitudinal comparison. In terms of senescence, even though late laying females are more likely to lay smaller eggs than early laying females at all ages, this is most relevant to birds younger than 10 years of age, because the majority of late-layers occur in that age group.

A reduction in clutch volume in old age suggests that females may become constrained in their allocation of resources to egg production, as a result of senescence. This could occur despite the lack of obvious signs of physical ageing, and may mask effects of increased reproductive effort. Senescence has been suggested as the reason for smaller eggs being laid by old individuals in other bird species (e.g. glaucous-winged gulls Reid 1988, wandering albatrosses Weimerskirch 1992). However, there are several other possible explanations that should be considered. Firstly, given that male traits as well as those of the female (see introduction) can influence egg size and quality, differences in the eggs produced during old age could result from a greater incidence of mate change at that time. Old birds are more likely to have lost their long-term mate through mortality. Cézilly and Nager (1996) suggested that observed declines in reproductive performance in old age could partly result from over-representation of new pairs in older age classes. New pairs were hypothesised to be less successful because of the reduced experience of the new partner, or reduced experience of co-ordinating the breeding attempt. The disruption of a change in breeding partner could also affect egg production, if females are forced, for instance due to delays in nesting, or choose, perhaps in

relation to male quality or attractiveness, to adjust their investment in the clutch. In support of this idea, there is some evidence that the otherwise tight correlation between the ages of pair members breaks down in old shags, so older cohorts will contain more newly formed pairs (see chapter 2). There have been very few tests of the effects of pair-bond duration on breeding performance in old age, but one study in the barnacle goose found no link between declining reproductive success in old birds and mate retention (Black and Owen, 1995).

A second alternative to constraints imposed by senescence is that although old females are capable of laying large eggs, they may refrain from doing so, perhaps to guard their own, aged and hence vulnerable, condition. Self-maintenance is pronounced in long-lived species, which may defer breeding when conditions are poor (Hamer, Schreiber and Burger, 2002), or maintain their own body condition at the expense of the condition of their current brood. For instance, increased flight costs during breeding produced by artificially reducing wingspan in Leach's storm petrel *Oceanodroma leucorhoa* led to no change in the nutritional condition of parents but a reduction in that of offspring, which gained mass more slowly than controls (Mauck and Grubb, 1995). However this may only be the case when levels of extrinsic mortality are low, such that there is a high probability of survival to the next breeding attempt (Williams, 1966*a*, *b*). Survival of shags declines in old age (Harris *et al.* 1994*b*). Thus, this idea is contrary to standard restraint hypotheses, which state that old individuals will show less reproductive restraint as the number of future opportunities to breed decrease. The reduction in egg size in old birds could be adaptive for other reasons; for instance laying small eggs allows birds to lay earlier (Birkhead and Nettleship, 1982). Old females may be better able to compensate for small eggs at subsequent stages of reproduction. Indeed, that has been found in relation to smaller clutch sizes in old ptarmigans (Wiebe and Martin, 1998, see introduction), and discussed in relation to the decrease in clutch volume in great skuas older than 15 years (Hamer and Furness, 1991).

In this study, because egg composition was not measured, it is not known how egg volume changes relate to differences in particular egg components, such as the proportion of yolk. It is difficult to estimate the additional costs of producing a larger egg when it is not known specifically which egg components occur in greater quantities. Grau (1996) measured the composition of shag eggs and estimated the

nutrient needs required for egg formation, although not in relation to egg size. It was concluded that egg production does not impose a major food need in these birds. However, the costs of egg production may depend on individual state, and there is recent evidence that in general these costs have been underestimated (e.g. Monaghan and Nager, 1997, see introduction).

In the longitudinal data there was greater variation in clutch volume among females than within individuals among years. While some of the inter-individual variation is due to age, these differences could also relate to variation in female quality or condition. While female size, mass, blood parameters and cell-mediated immune response measured during chick rearing are unrelated to female age (see chapter 5), it was hypothesized they may reflect differences in female quality, current state or the level of previous reproductive investment, of which egg number and size are components. However, there was no difference between the clutch size laid by females of low or high mass, or among females of different size. Neither size, blood measures, nor immune response were related to the clutch volume laid by females. The variation in these adult traits in our sample may reflect only a small amount of the variation in the population as a whole, because the sample included only birds that had already successfully reared chicks to medium size. This may have reduced the chance of observing differences in these traits in relation to egg size.

There was a significant negative correlation between clutch volume and the mass of females, after correcting for their structural size, when this was measured during chick rearing. This suggests either that laying clutches of large volume lowers the mass of the laying female, or that females of lower mass tend to lay larger eggs. Although our data cannot distinguish between these two alternatives, and ideally female mass would be measured both pre- and post-egg production in order to do so, the former explanation seems intuitively more likely. As described in the introduction, it has been demonstrated in other birds that laying more eggs depletes female body reserves (Bolton, Monaghan and Houston 1993, Carey 1996). The same may be true of laying larger eggs in shags, although it is surprising that the comparatively small increase in egg size observed in this case could have this effect. The pattern observed here contrasts with Snow's (1960) findings, which suggested females lay eggs in proportion to their own body mass, although in that case body

size was not taken into account. It also contradicts Grau's suggestion that the nutritional requirements for egg production in the shag are small. Clearly the relationship between female mass, body condition and egg investment requires further investigation in this species, and it would be particularly useful (though difficult) to have adult measures taken at different stages in the breeding cycle.

A positive effect of greater egg size for chicks in the period shortly after hatching is generally observed in birds (Roff 1992, Williams 1994). Egg size often correlates positively with offspring mass and size within the first week of hatching, and to early nestling growth (e.g. Williams 1980, Birkhead and Nettleship 1982, Furness 1983). These effects have also been found to be independent of female quality in a number of egg exchange studies (e.g. Amundsen and Stokland 1990). As hatchling mass can influence post-hatching survival (Parsons, 1970), egg size may also have a positive impact on the survival of nestlings (e.g. Nisbet 1973, 1978, Lundberg and Väisänen 1979, Moss *et al.* 1981, Bolton 1991). Whether the differences in egg size reported here, or other differences in egg quality such as composition, among females of different age ultimately influence their breeding success is addressed in the next chapter (also see appendix).

## FIGURES

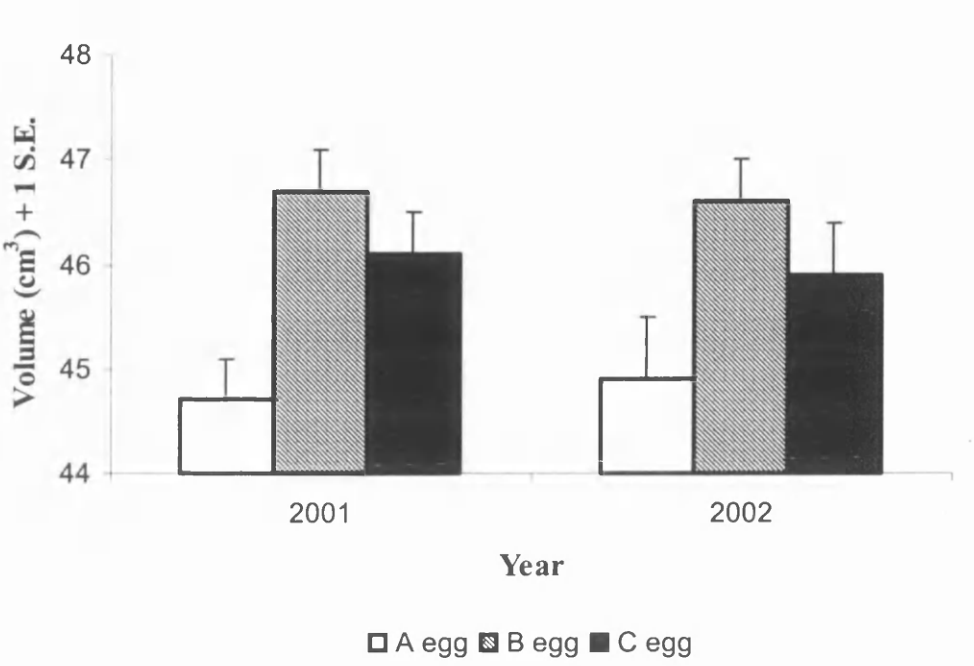



**Figure 3.1** The intra-clutch pattern of A, B and C egg volumes in clutches of three in 2001 and 2002. Volumes of A, B and C eggs within a clutch differ consistently.

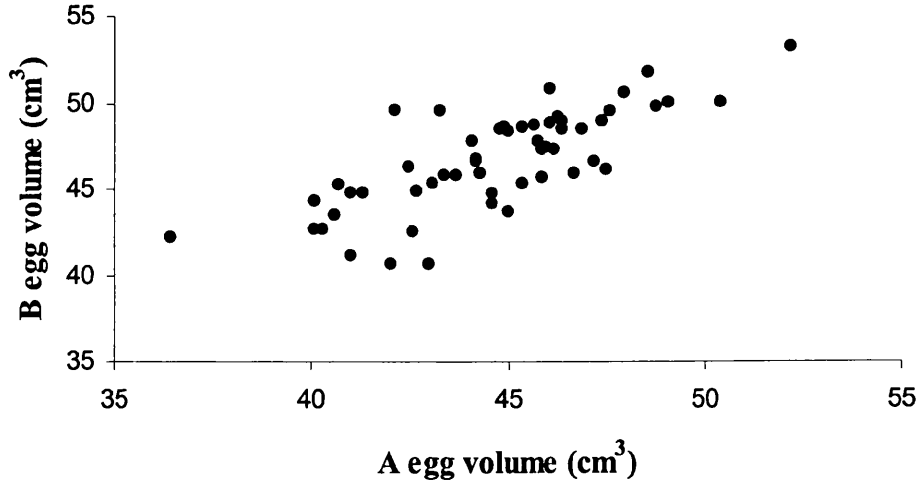
| Year | Egg | Mean length (mm) +/- 1 S.E. | Mean breadth (mm) +/- 1 S.E. | Mean volume ($cm^3$) +/- 1 S.E. | Sample size (*n*) |
|---|---|---|---|---|---|
| 2001 | A | 61.1 +/- 0.3 | 37.9 +/- 0.1 | 44.7 +/- 0.4 | 54 |
| | B | 62.0 +/- 0.3 | 38.5 +/- 0.1 | 46.7 +/- 0.4 | 54 |
| | C | 61.8 +/- 0.3 | 38.2 +/- 0.1 | 46.1 +/- 0.4 | 54 |
| 2002 | A | 60.7 +/- 0.3 | 38.0 +/- 0.2 | 44.9 +/- 0.6 | 73 |
| | B | 62.0 +/- 0.3 | 38.4 +/- 0.1 | 46.6 +/- 0.4 | 73 |
| | C | 61.6 +/- 0.3 | 38.2 +/- 0.1 | 45.9 +/- 0.5 | 73 |

**Table 3.1** Average lengths, breadths and volumes of A, B, and C eggs in clutches of three in 2001 and 2002.

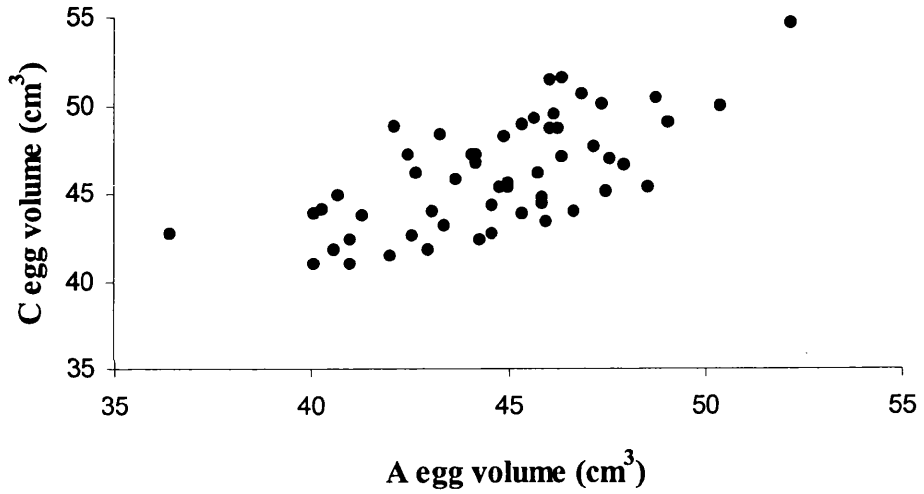
| Egg | Clutch size | Mean length (mm) +/- 1 S.E. | Mean breadth (mm) +/- 1 S.E. | Mean volume ($cm^3$) +/- 1 S.E. | Sample size (*n*) |
|---|---|---|---|---|---|
| A | 2 | 61.0 +/- 1.4 | 37.9 +/- 0.6 | 44.8 +/- 2.2 | 8 |
| | 3 | 61.1 +/- 0.3 | 37.9 +/- 0.1 | 44.7 +/- 0.4 | 54 |
| | 4 | 63.6 | 36.3 | 42.6 | 1 |
| B | 2 | 62.5 +/- 1.5 | 38.3 +/- 0.4 | 46.8 +/- 1.9 | 8 |
| | 3 | 62.0 +/- 0.3 | 38.5 +/- 0.1 | 46.7 +/- 0.4 | 54 |
| | 4 | 57.7 | 38.9 | 44.5 | 1 |
| C | 3 | 61.8 +/- 0.3 | 38.2 +/- 0.1 | 46.1 +/- 0.4 | 54 |
| | 4 | 69.0 | 36.2 | 46.1 | 1 |
| D | 4 | 58.6 | 38.7 | 44.8 | 1 |

**Table 3.2** The average length, breadth and volume of each egg in the laying sequence in clutches of 2, 3 or 4 in 2001.

*(a)*




*(b)*




*(c)*

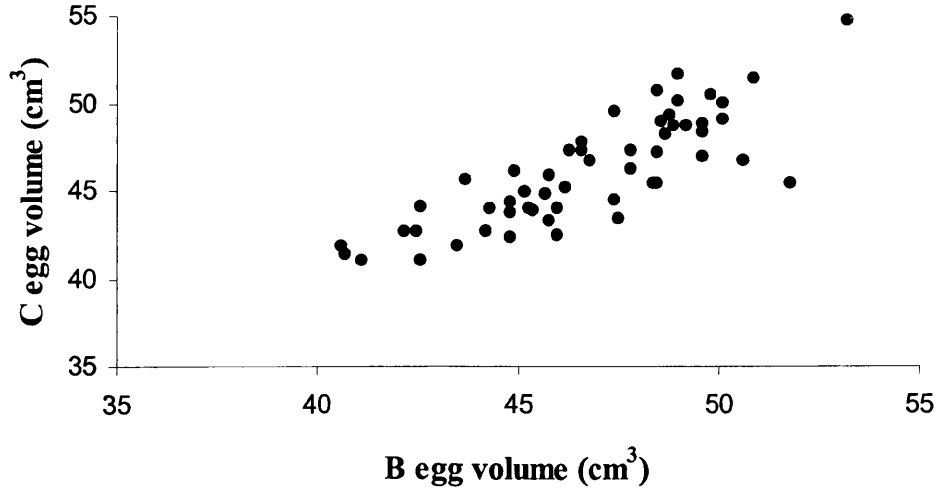



**Figure 3.2** Egg size is correlated within a clutch. For clutches of three laid in 2001 ($n$ = 54) are shown the volume of the *(a)* A egg in relation to B, *(b)* A egg in relation to C, and *(c)* B egg in relation to the C egg.

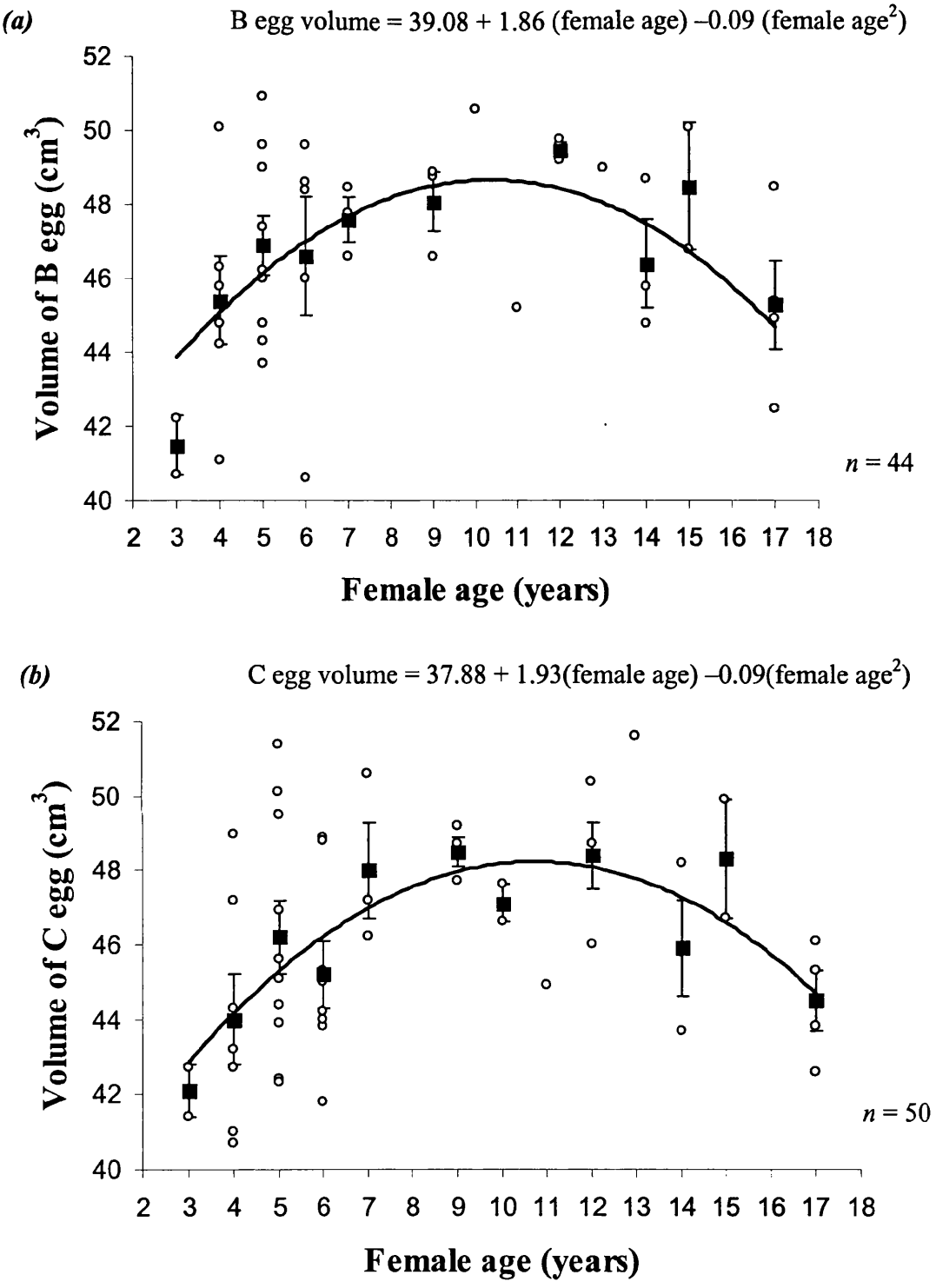



**Figure 3.3** The volume of *(a)* B and *(b)* C eggs in clutches of three in 2001 plotted against female age. Circles represent individual measurements and squares are the means for each age, ± 1 S.E. The curve is the quadratic regression, fitted to individual measurements (equation given in each chart). Female age explained 24% of the variance in both cases.

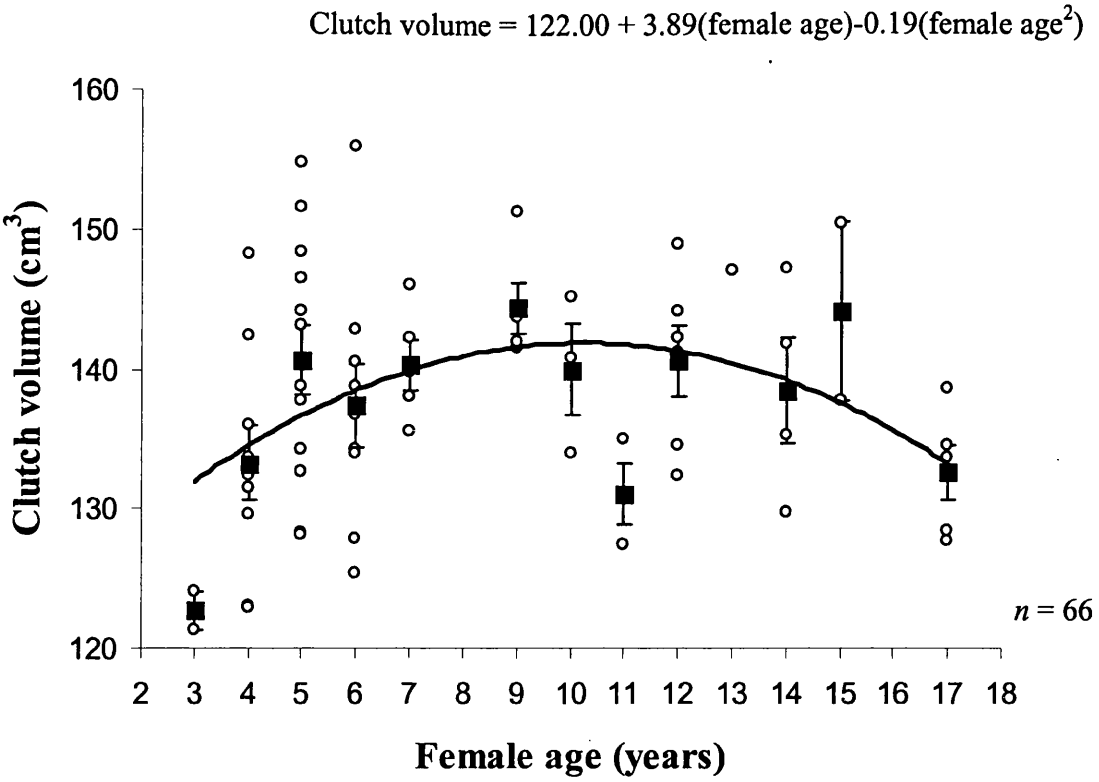



**Figure 3.4** Clutch volume in three egg clutches in 2001 plotted against female age, showing the quadratic regression. Individual measurements are shown as circles, and means for each age ± 1 S.E. as squares. The regression was performed on the individual observations.

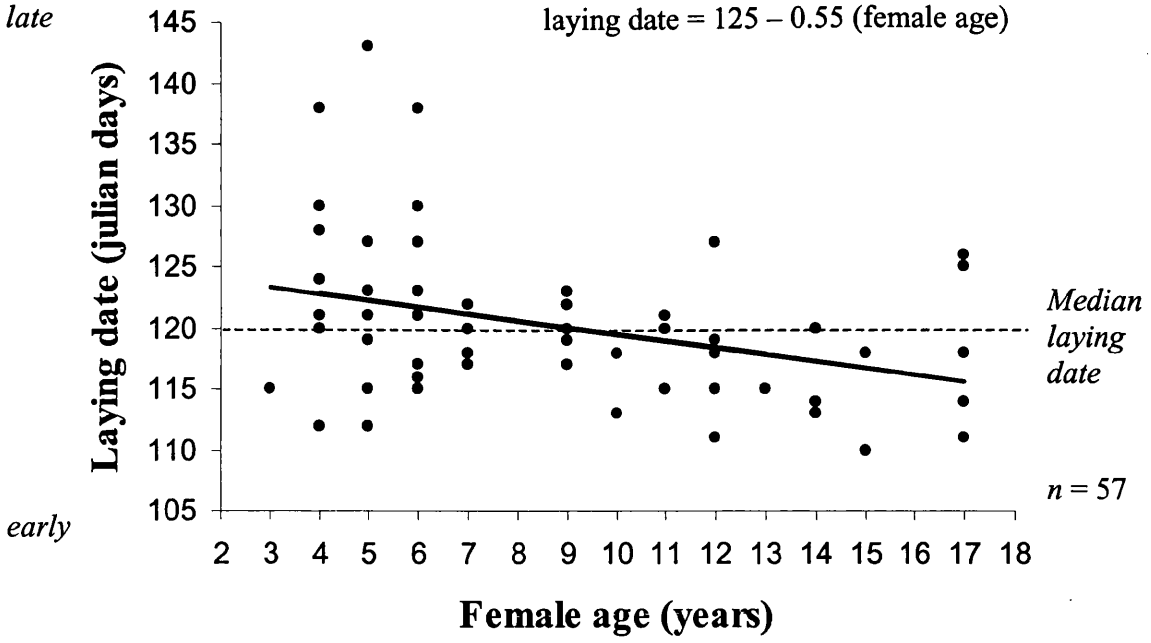



**Figure 3.5** In 2001 there was a significant negative relationship between female age and laying date. The equation for the linear regression is given on the chart, and the regression line shown as solid and bold. The dashed line indicates the median laying date.

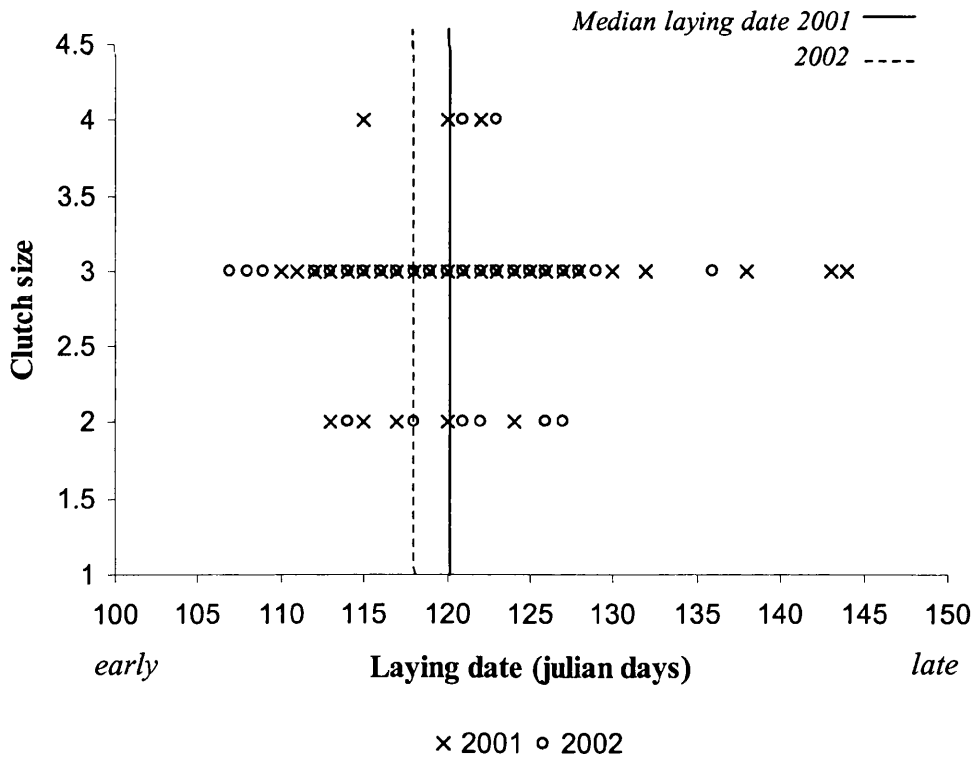
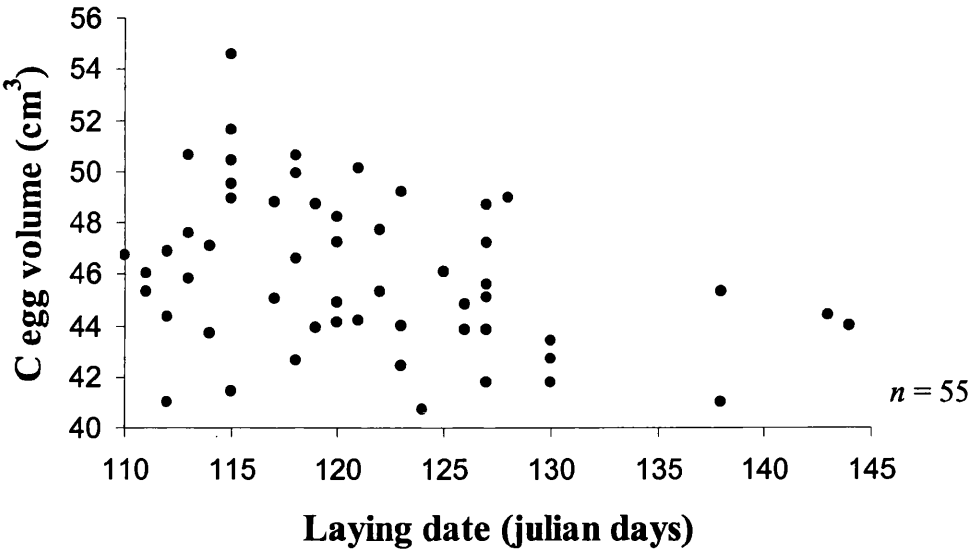



**Figure 3.6** There was no indication that clutches of four or clutches of two were laid exceptionally early or laid in the season, in either 2001 or 2002.

*(a)*




*(b)*

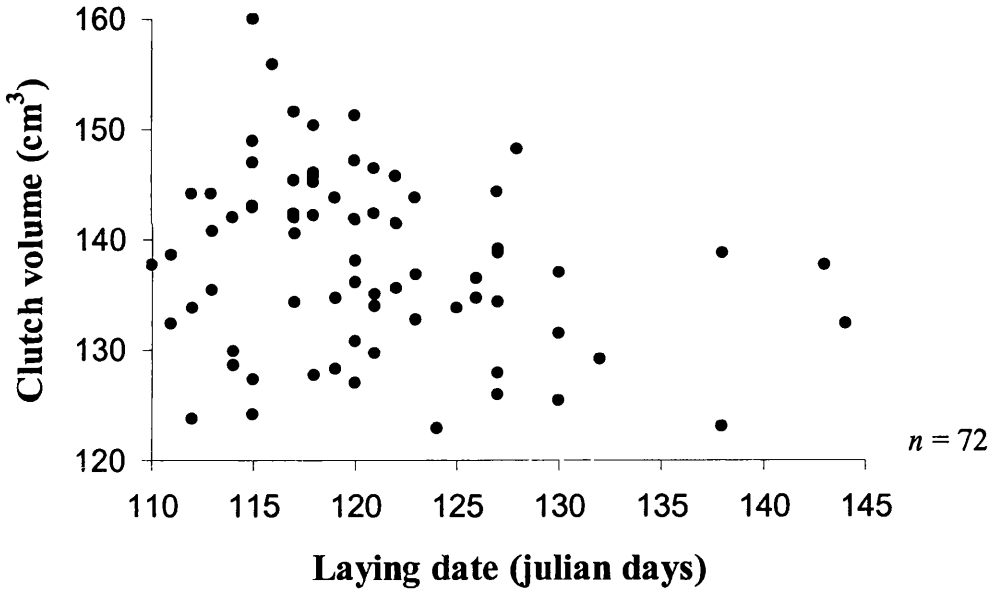



**Figure 3.7** *(a)* There was a significant negative relationship between laying date and the volume of the C egg in clutches of three in 2001 *(b)* Clutch volume also showed a negative relationship to laying date. Julian day 110 corresponds to April 20th and day 144 is May 24th. The median laying date of females for which egg volumes were measured in 2001 was April 30th (julian day 120).

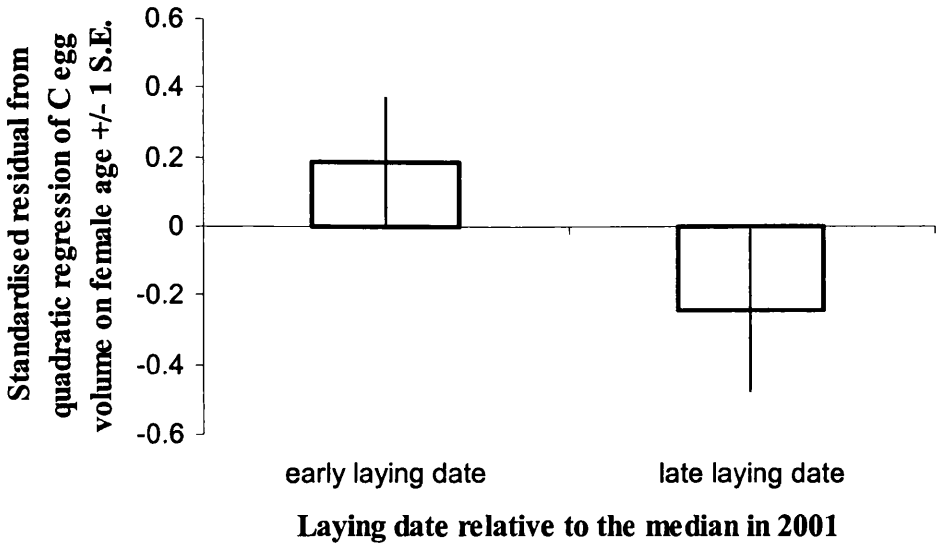



**Figure 3.8** Residuals from the quadratic regression of C egg volume on female age, in relation to relative laying date in 2001.

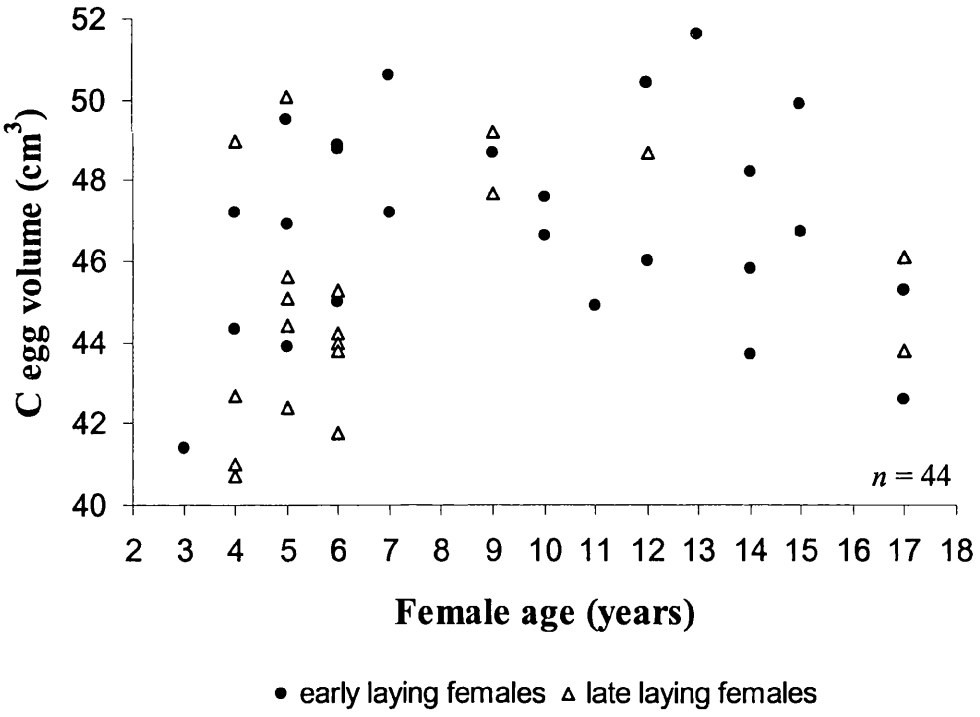



**Figure 3.9** The pattern of C egg volume for three egg clutches with female age in 2001, with relative laying dates indicated by different symbols. Early laying females were those that laid on or before the median laying date in 2001 (which was April 30th), while late laying females were defined as those laying after the median laying date.

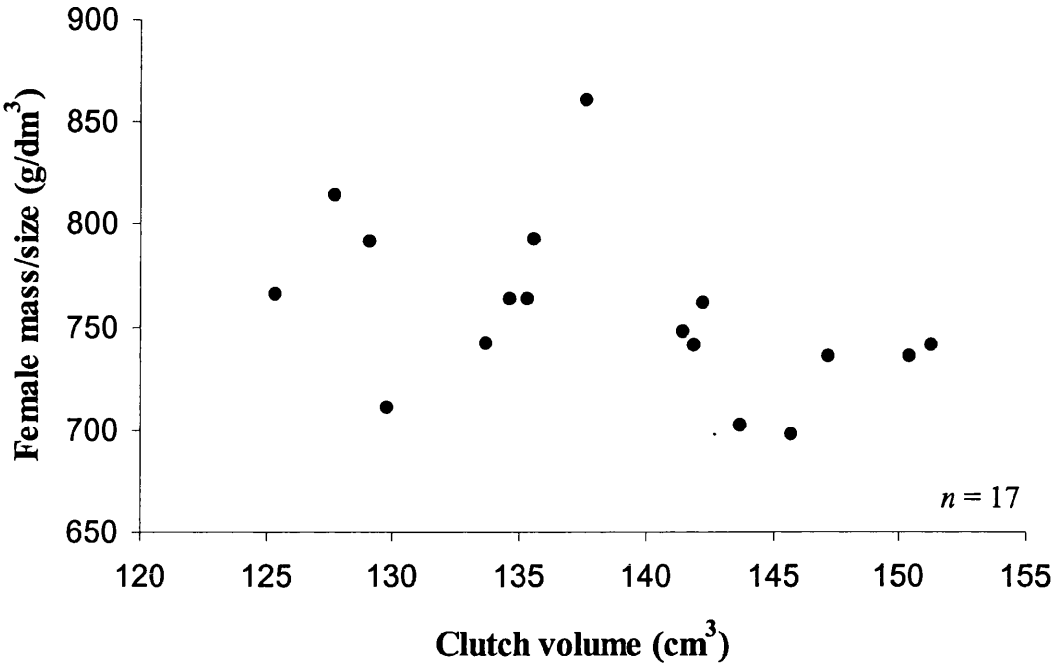



**Figure 3.10** In 2001, females that laid large volume clutches of three were of poorer body condition (mass corrected for size) during chick rearing than females that laid three egg clutches of smaller volume.

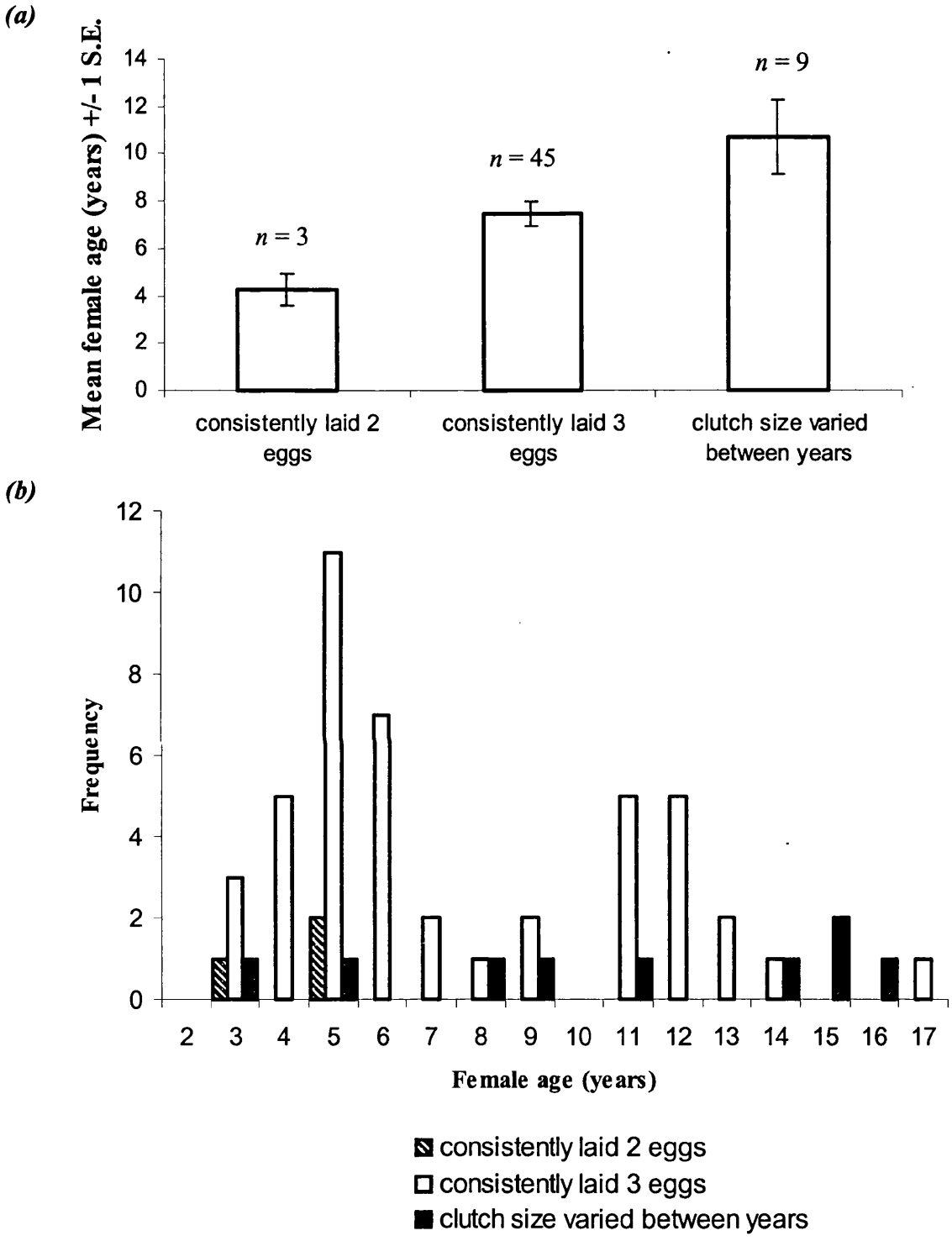



**Figure 3.11.** The *(a)* mean age (at first observation) and *(b)* frequency of females that consistently laid two eggs, consistently laid three eggs or laid clutches of varying size among years.

| First year in which egg volumes were measured | Last year in which egg volumes were measured | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1997 | | 1998 | | 1999 | | 2001 | | 2002 | | 2003 | | |
| | Years between first and last egg measurements | Number of females | Years between first and last egg measurements | Number of females | Years between first and last egg measurements | Number of females | Years between first and last egg measurements | Number of females | Years between first and last egg measurements | Number of females | Years between first and last egg measurements | Number of females | Total |
| 1997 | ___ | _ | 1 | _ | 2 | _ | 4 | **4** | 5 | **4** | 6 | **2** | **10** |
| 1998 | ___ | _ | ___ | _ | 1 | _ | 3 | **2** | 4 | **2** | 5 | _ | **4** |
| 1999 | ___ | _ | ___ | _ | ___ | _ | 2 | **2** | 3 | **3** | 4 | _ | **5** |
| 2001 | ___ | _ | ___ | _ | ___ | _ | ___ | _ | 1 | **26** | 2 | 4 | **30** |
| 2002 | ___ | _ | ___ | _ | ___ | _ | ___ | _ | ___ | _ | 1 | 1 | **1** |
| Total | ___ | 0 | ___ | 0 | ___ | 0 | ___ | **8** | ___ | **35** | ___ | **7** | **50** |

**Table 3.3** Sample sizes, and years in which egg volumes were measured in the longitudinal dataset, for females of known age that laid a clutch of three eggs in each year.

| Age $x$ | Age $x + 1$ | Number of comparisons of clutch volume, for clutches of three laid by known age females | Mean clutch volume at age $x$ ($cm^3$, ± 1 S.E.) | Mean clutch volume at age $x + 1$ ($cm^3$, ± 1 S.E.) | Clutch volume at age $x + 1$ – clutch volume at age $x$ [a] ($cm^3$) (± 1 S.E.) |
|---|---|---|---|---|---|
| 2 | 3 | 0 | — | — | — |
| 3 | 4 | 1 | 124.1 | 129.25 | +5.15 |
| 4 | 5 | 5 | 135.82 (4.48) | 135.35 (3.84) | -0.47 (1.58) |
| 5 | 6 | 10 | 138.77 (2.68) | 136.98 (3.03) | -1.79 (1.25) |
| 6 | 7 | 7 | 139.57 (3.24) | 136.85 (3.64) | -2.72 (1.84) |
| 7 | 8 | 1 | 135.6 | 140.1 | +4.50 |
| 8 | 9 | 0 | — | — | — |
| 9 | 10 | 1 | 151.3 | 143.86 | -7.44 |
| 10 | 11 | 2 | 143 (2.21) | 141.89 (0.16) | -1.11 (2.05) |
| 11 | 12 | 3 | 131.75 (1.68) | 125.76 (4.44) | -5.99 (3.56) |
| 12 | 13 | 6 | 142.78 (3.77) | 138.41 (3.77) | -4.37 (2.46) |
| 13 | 14 | 4 | 135.93 (4.72) | 134.13 (2.63) | -1.8 (2.60) |
| 14 | 15 | 1 | 141.8 | 139.65 | -2.15 |
| 15 | 16 | 0 | — | — | — |
| 16 | 17 | 0 | — | — | — |
| 17 | 18 | 1 | 127.7 | 128.77 | +1.07 |
| **Total:** | | **42** | **Means: 137.34** | **135.92** | **-1.42** |

[a] this difference in clutch volume is the mean when the number of comparisons at that age is greater than 1.

**Table 3.4** The change in clutch volume for three egg clutches laid by individual females at age $x$ and $x + 1$.

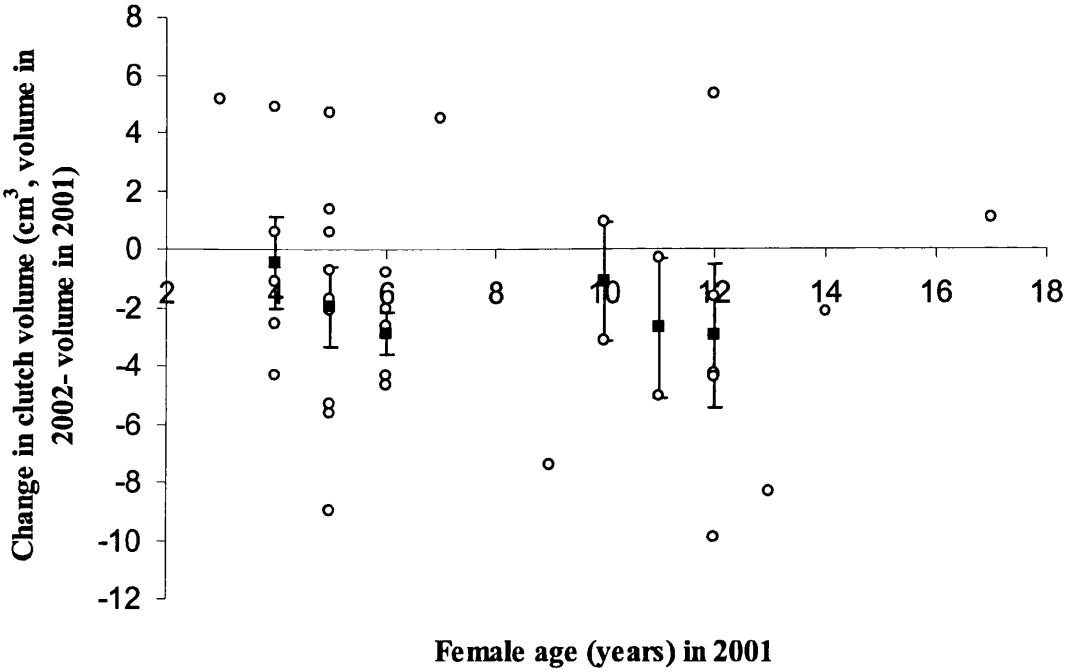



**Figure 3.12** The change in clutch volume within females that laid a clutch of three in both 2001 and 2002. Clutch volume changes for single females are shown as circles, and the mean ± 1 S.E. for each age as squares.

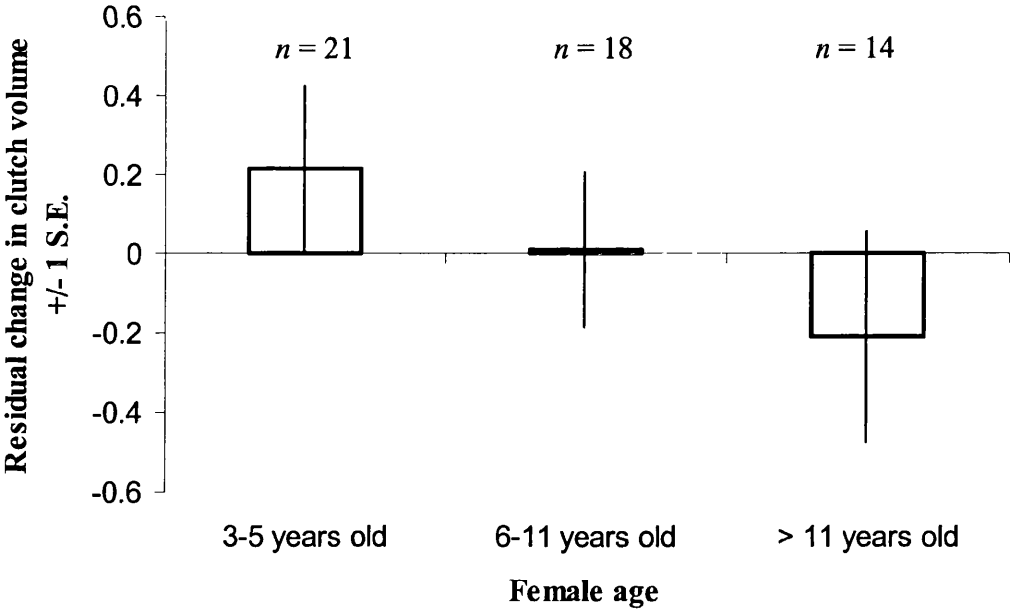



**Figure 3.13** Mean change in clutch volume for three egg clutches, after controlling for year and time over which the change was measured, compared among female age classes. Females > 11 years for which minimum age (but not exact age) was known were included in the age group of old birds. The age range in the oldest age group is 12 to at least 18 years.

# Effects of old age on reproductive performance in European shags


## ABSTRACT

A deterioration in reproductive performance in old age, or even the complete cessation of breeding, occurs in many mammals, and has been reported in captive birds. Reproductive senescence has also been observed in some wild bird populations, but these data are more difficult to obtain. Reproductive performance in relation to old age was examined in European shags breeding on the Isle of May. The nest sites occupied by middle-aged and old breeders were also compared, as any differences in nest site quality could influence reproductive success. No difference in the breeding sites of these two age groups was found. The date on which the first egg was laid was earlier in older breeders than in young birds. With the exception of the oldest female, who did lay comparatively late (though this may reflect her young partner), there was no indication of a senescence-related change in the laying dates of old birds. Hatching success, fledging success and the number of chicks fledged did not differ sigificantly between middle-aged and old shags. However, complete failure either pre-hatching or during chick rearing was associated with a large age difference between breeding partners. This may reflect the lower reproductive performance of newly-formed pairs, the old age of the female in these pairs, or the young age of the male. These factors are confounded, and their negative effects on breeding success may be cumulative. There was no difference in the proportion of male chicks produced at laying by old compared to middle-aged females. The lack of clear signs of reproductive senescence in these birds is discussed with respect to the problems of measuring senescence in the wild, environmental conditions on the Isle of May in the year of monitoring, and the methods of analysis used.

## INTRODUCTION

Age-related variation in reproductive performance is a common observation in birds, although differences are often most pronounced, and have been most often studied, in young breeders (see general introduction). However, there has recently been increasing interest in reproductive senescence (e.g. symposium *Reproductive ageing in avian species*, 21st International Ornithology Congress, Vienna, Austria 1994, and 2nd symposium of organisms with slow ageing, California 2003). In captivity, the fertility of many mammals and birds declines from a peak at young ages, and furthermore individuals live beyond the maximum age at which breeding occurs (Ricklefs, Scheuerlein and Cohen, 2003). It has been suggested that few individuals will survive to such advanced ages in natural populations, due to the high levels of extrinsic mortality (Packer, Tatar and Collins, 1998). Despite predictions that reproductive senescence will be rare or absent in birds, particularly in the wild (Nice 1943, Slobodkin 1966, Comfort 1979, Charlesworth 1980, Williams 1992), reports of declining breeding success in old wild birds have been accumulating (reviews Martin 1995, Holmes and Austad 1995*a*, Bennett and Owens 2002). For instance, the average productivity of the oldest age class is lower than that of middle-aged birds in the kittiwake *Rissa tridactyla* (Thomas and Coulson, 1988), glaucous-winged gull *Larus glaucescens* (Reid, 1988), short-tailed shearwater *Puffinus tenuirostris* (Wooller *et al.* 1990), wandering albatross *Diomedea exulans* (Weimerskirch, 1992), and red-billed chough *Pyrrhocorax pyrrhocorax* (Reid *et al.* 2003, see chapter 1 for further examples). Decreased egg production (smaller clutch or egg sizes), poor responses to cues that normally induce ovulation and laying, reduced viability of the eggs, and low hatching or fledging success have all been documented in old birds (Holmes *et al.* 2003). Old parental ages have also been linked to reduced fitness of the fledged offspring (Saino, 2002). Old female shags on the Isle of May lay smaller B- and C-eggs, and have a lower clutch volume than middle-aged females (chapter 3). Whether these differences in egg size are matched by poorer performance in subsequent breeding stages is investigated here. The relationship of laying date to age, and the effect of the age difference within pairs on reproductive performance are also considered.

### *Why might breeding performance decline in old age?*

Explanations for age-related declines in breeding performance can be addressed on two levels: proximate mechanisms that cause lower performance, and the ultimate, evolutionary explanations for senescence. The focus of this thesis has been identifying firstly whether, and then at what stage, senescence operates in shags, and does not extend to testing hypotheses for how ageing evolves (though the main theories are outlined briefly in chapter 1). Only potential proximate causes are considered here. As introduced in chapter 1 and discussed in chapter 3, a decline in the reproductive performance of old birds may be the result of imposed constraints, or adaptive restraint.

Falling reproductive success may result from physical deterioration or changes in physiology, such as hormonal levels, in old birds. Male dunnocks (*Prunella modularis*) over the age of six years display less competitive behaviour than younger males, occupying small territories between those of breeders, and remaining unpaired throughout the breeding season (Davies, 1992). The lower survival of European shags after 13 years of age (Harris *et al.* 1994*b*) suggests that these old birds are more vulnerable than younger individuals, and so may be in poorer condition (though this survival pattern could also occur if old birds are less likely to return to, or remain at, the breeding colony). Another type of constraint that may act in old age is a forced change of mate, when the former partner dies. The oldest breeding female shags on the Isle of May are paired with young males (chapter 2). In species with long-term pair bonds, reproductive success improves as experience with the breeding partner increases, and so is lower in newly formed pairs (e.g. Bradley, Wooller and Skira 1995, Clum 1995). If one of the partners is young, newly formed pairs may also be less successful because of the poorer performance of young, inexperienced birds. A large number of new pairs in old age classes therefore might be responsible for an overall pattern of reproductive decline in old age (Fowler 1995, Cézilly and Nager 1996). Late-laying by old birds, due either to lower responses to reproductive cues, or delays caused by waiting for a non-returning partner, finding a new mate, or being newly paired with a young male, could constrain breeding performance, as laying late in the season is associated with poor success (young male shags breed late and have lower breeding success than older males: Potts, Coulson and Deans 1980, Aebischer 1993, Aebischer, Potts and Coulson 1995, Daunt *et al.* 1999).

Restraint theories traditionally predict that reproductive effort will increase with age, as residual reproductive value falls (Williams 1966*b*, Gadgil and Bossert 1970, Stearns 1976), although there have been very few demonstrations of this in old birds (one of the few examples of greater effort in old birds comes from California gulls *Larus californicus*: Pugesek, 1981). A decline in reproductive success in old age might be observed irrespective of greater effort, if this effort does not translate into positive results, i.e. old birds may be working hard but still failing. Alternatively, the decline in survival probability of old birds at the breeding colony may not represent a large reduction in residual reproductive value, and therefore old individuals will still maintain their own condition for future breeding attempts (self-maintenance, at the cost of the current reproductive attempt when conditions are harsh, is a characteristic of the life-history of long-lived birds, e.g. Hamer, Schreiber and Burger 2002). Old birds may adjust their allocation to reproduction in line with their own state, perhaps falling as a result of the ageing process.

On a population level, poor breeding success in the oldest age group could reflect selective mortality, whereby highly productive individuals die at a younger age. The selection hypothesis has also been used as an explanation for early improvements in young breeders, in which case poor reproductive performers die sooner, or disappear from the study population due to a higher tendency to disperse (review Forslund and Pärt 1995). Which of these two processes is paramount will depend on the balance between the costs of reproduction and the covariation between survival and reproductive performance mediated by individual quality. There is substantial evidence for the costly nature of reproduction and for greater mortality rates in highly fertile individuals (see chapter 3 introduction). Selective processes operating at the level of the population must be kept in mind when assessing differences in reproductive performance across birds of differing age. Other population level effects that should also be considered are variation in environmental conditions over time, and so in relation to different cohorts of the population. There is evidence that in some species both within-individual reductions in reproductive performance, and differential mortality, with more productive birds dying at a younger age, operate to produce an overall pattern of low performance in old birds (peregrine falcons *Falco peregrinus*: Clum, 1995, red-billed choughs: Reid *et al.* 2003).

### *Nest site quality*

Nest site quality may influence breeding success, either independently of parental age or as one aspect of age-related differences. Snow (1960) first described in general terms the nest sites occupied by shags (on Lundy Island), for instance recognising the need to avoid sites that become drenched by heavy spray in stormy weather, which can destroy eggs and chicks. On the Farne Islands, Potts (1966) ranked nest sites according to their access to the sea, protection from heavy seas, exposure to attack and their size. He found that the earliest nesting birds each spring occupied the best sites, while two-year-old birds, which arrived and began nest building later, occupied inferior sites. The quality of the nest site also partly accounts for variation in the number of young fledged (Potts, Coulson and Deans, 1980). On the Isle of May, Aebischer (1985) found access to the sea to be unimportant, but the size of the ledge on which the nest was built, dampness of the site after rain, exposure to attack, and wave reach all significantly influenced mean chick production by shags. Aebischer also confirmed the pattern of poorer quality nests being occupied by younger birds, as a consequence of age-related differences in the timing of breeding. However, this finding was not replicated by Daunt (2000), who found no significant difference between the nest site quality of pairs containing a two-year-old male and those in which the male was at least three years old, on the Isle of May.

A recent study by Velando and Freire (2003) has confirmed at another location (Islas Cíes, Galicia, northwest Iberian peninsula) the link between the timing of breeding, characteristics of the nest site, and reproductive success in the European shag. As on the Isle of May and Farne islands, site characteristics had a significant effect on breeding success. Laying date was earlier at better sites, but even after controlling statistically for laying date, the effect of nest site was significant. This suggests that there is an effect of site quality on breeding success that is independent of variation in parental quality, though this requires experimental confirmation. Differences in nest site characteristics were not investigated in relation to age in this case.

If the nest sites occupied by old shags differ in quality from those occupied by younger birds, this factor may contribute to age-related variation in reproductive performance, including any differences that occur in old age. Whether the nest sites

occupied by very old shags differ from those of middle-aged birds has not previously been studied. The age of a bird's mate as well as its own age may influence the nest site it obtains. There is a positive relationship between the age difference within pairs and female age (chapter 2), as a result of the oldest females pairing with very young males. Differences in the nest site characteristics of old females therefore could reflect the young male to which they are paired, or retention of the site by the female. For this reason, the nest site scores of old females should be assessed with respect to the age of their male partner. A difference in the quality of the nest sites occupied by old individuals might be expected if competitive ability varies with respect to age, for example if very old individuals are less able to fight off intruders at their site. As the focus of this study is senescence and its effects on reproductive performance, differences in the nest sites of old individuals are explored only in relation to middle-aged birds. The nest site quality of young shags, which has been previously investigated, is not examined again here.

### *The sex of offspring*

Adult European shags are sexually dimorphic, with males on average larger than females (Cramp and Simmons 1977, Daunt *et al.* 2003, chapter 5). For this reason, growth, condition and fledging success may differ between male and female offspring. In a cross-fostering experiment that controlled for the timing of breeding and egg quality, male chicks raised by young shags grew more slowly and attained lower peak mass than those raised by older pairs, though there was no difference for female chicks (Daunt 2000, Daunt *et al.* 2001). Therefore, chick sex should be included in analyses of these factors with respect to parental age. Individuals may also adjust the sex ratio of their offspring in relation to their own condition, which might be influenced by age. For instance, condition-related sex ratio skews have been previously found in lesser black-backed gulls *Larus fuscus* (Nager *et al.* 1999), and great skuas *Catharacta skua* (Kalmbach *et al.* 2001). In the European shag, a change in the brood sex ratio with laying date has been reported (Velando, Graves and Ortega-Ruano, 2002), with early broods biased towards males and late broods towards females. This change in the sex ratio with laying date was attributed to manipulation of sex in the first laid egg. However, as chicks in that study were only

sexed at between 20 and 30 days of age, this pattern may reflect sex-biased mortality rather than a sex ratio bias at laying.

It is possible to sex chicks from their DNA, which can be extracted from blood cells (avian red blood cells, as well as white blood cells, are nucleated). In 2001 and 2002 shag chicks were sexed in this way. Chick sex was taken into account in analyses of offspring growth and condition, and the brood sex ratio was also explored in relation to maternal age.

## METHODS

The breeding success of 115 pairs was monitored from egg laying through to fledging on the Isle of May in 2001. Both partners were of known age in 55 pairs (48%), only the female was of known age in 34 pairs (30%) and only the male was of known age in 18 pairs (16%). In the remaining eight pairs the birds were either of minimum known age (i.e. ringed as adults) or were of unknown age (not ringed). Minimum age birds were targeted if they were old, in order to increase the sample size of this age group. Three nests were excluded from analysis because two females were breeding with one male at those sites. A small number of polygynous trios are regularly observed on the Isle of May and the Farne Islands (Potts 1966, Potts, Coulson and Deans 1980, Aebischer 1985). Study nests in 2001 were distributed in six areas of the island (figure 4.1), the choice of study nests governed by the distribution of known age adults, and the ease of access to nests (with minimum disturbance).

### *Nest site quality*

Occupied breeding sites on the Isle of May were scored for nest site quality in 2001 and 2002. Nest site quality scores were based on four factors that have previously been shown to influence the breeding success of shags (on the Farne islands: Potts, Coulson and Deans 1980, on the Isle of May: Aebischer 1985). These factors are ledge size, dampness of the site after rain, exposure to level attack from conspecifics, and sea or wave reach to the nest (table 4.1). Of 119 nests assessed for these factors in 2001, 106 (89%) had previously been allocated their score, and I scored the remainder of the sites. In 2002, 171 nests were newly allocated site quality scores, by an independent observer (Judith Hamilton) to prevent bias. The average score for each nest site characteristic was compared between old (13+ years) and middle-aged (5-9 year old) males and females, separately in each year. As the nest site scores are ordinal, non-parametric tests were used. Grouping individuals into age classes maximised the number of old birds in the dataset, as birds of minimum age 13 years (i.e. old individuals ringed as adults) could be included in the old age group. Table 4.2 shows the sample sizes in each year of males and females in these age classes for which nest sites were scored. The average overall rating of nest site quality was also

compared between age groups. This rating was calculated using the following equation (derived by Aebischer, 1985):

nest site rating = 0.259 (ledge size) + 0.166 (risk of flooding after rain)
– 0.0042 (risk of level attack by other shags) + 0.230 (wave reach)

To test for effects of male partner age, spearman rank correlations between the nest site scores of old females and the age of the male with which they were paired were carried out.

### *Laying date*

Laying dates were recorded for 82 known age females, 66 known age males, and 50 pairs in which both birds were known age. The date of laying was determined by one of three means. At 22% of nests laying date was determined by daily nest checks. Alternatively, laying date was derived by counting back through the laying sequence (21% of nests, eggs are laid every third day, Snow 1960), or was calculated from the day on which the first egg hatched, by subtracting the mean laying-to-hatching period of 35 days (57% nests). The sample analysed here is larger and includes older females than that tested in chapter 3, where only females for which egg volumes were also measured were included.

The relationship between laying date and female or male age was explored by regression analysis. Where plots suggested the relationship was not linear, quadratic or logarithmic regressions were also performed and compared to the linear fit. In all cases, the quadratic function was accepted only when it showed a stronger relationship to age than the linear regression. Additionally, in order to confirm quadratic relationships, correlations between the dependent and independent variable after the peak were tested. Given the small numbers of very old birds present in the sample, and the potential of these to strongly influence correlations, these were removed from analyses to check whether relationships remained significant.

In known age pairs, the effect of male and female age on laying date were tested together in a multiple regression, which included the interaction between the age of breeding partners. To separate the influence of male age from that of female age on the time of laying, known age pairs in which the male was of the same age were selected. Among those pairs only, laying date was then tested in relation to the variation in female age.

### *Reproductive success*

A number of measures of breeding performance were recorded. In addition to overall breeding success (number of chicks fledged), these were clutch size, egg size, hatching success (proportion of eggs laid that hatched), offspring growth and condition (see following section), and fledging success (proportion of chicks hatched that fledged). Clutch and egg size were examined in relation to female age in chapter 3. In analyses of hatching success, there is no attempt to discriminate between different causes of hatching failure, as in most cases the precise cause is not known. Poor hatching success therefore refers to failure of eggs to hatch, or egg breakage by the parents, other shags, or during predation by gulls. The first-hatched chick in a brood is referred to as the A chick, the second hatched the B chick, and the third-hatched chick as the C chick. Fledging success corresponds to chick survival over the rearing period, a distinct measure from breeding success (number of fledged chicks per nest), which is affected by failures at any stage of breeding and by clutch size.

When testing for the effects of parental age or laying date on the dependent variables hatching success, fledging success or fledging number either non-parametric tests or generalised linear models with appropriate error distributions and link functions were used. In parametric tests of hatching success and fledging success, a binomial error distribution and logit link function were specified. For the analysis of the number of chicks fledged a poisson distribution and log link were used. Non-significant terms were removed in turn from the full model. In known age pairs, breeding performance was also explored in relation to the age difference between pair members.

To test specifically for a difference in performance between middle-aged and old birds, individuals were grouped according to age. This also has the advantage of increasing the sample size of old birds, as individuals of minimum known age (ringed as adults) that fall into the old age category are included in addition to individuals whose exact age is known. The age range defined as old was 13+ years, because from this age a significant fall in survival has been reported (Harris *et al.* 1994*b*). In the fledging success analysis and that of chick condition (following section) in relation to male age this cut-off point for old birds led to prohibitively small sample sizes. Therefore for these analyses the old age category was extended to also include 12-year-old birds. The middle-aged category was set at 5 - 9 years, in

order to exclude first-time and inexperienced breeders, as well as avoiding inclusion of aged individuals.

### *Chick growth and condition*

Chick growth rates during the linear phase of growth (chick age 8-30 days) and the body condition of 114 chicks from 49 broods were measured in 2001. Mass, head and bill length, wing length and tarsus length were recorded three times over this period. Head and bill length was measured to the nearest millimetre with a 220mm measure, wing length to the nearest mm with a wing rule, and tarsus length to 0.1mm with callipers. Mass measurements were made using a 1kg Salter spring balance (to the nearest 5g) and thereafter with a 2.5kg Pesola spring balance (to the nearest 20g), at approximately five-day intervals between the ages of 13 and 27 days. In order to reduce the error in weight measurements associated with differences among chicks in the time since their last feed, chicks were weighed early in the morning (before 6 am), when it was assumed they had not yet been fed. The gradient of the line through the mass measurements of each chick provided its linear growth rate. Two chicks died during the period over which mass measurements were taken, and therefore growth rate could not be calculated for those chicks. Asymptotic mass was not recorded due to difficulties in handling shag chicks close to fledging, at which time they are highly mobile. The body condition of individual chicks was calculated as their mass divided by the product of the three linear size measures, at the time of the first measurement (chick age 13-19 days, mean age 15 ± 0.09 days), and at the last measurement (chick age 23-27 days, mean age 24 ± 0.09 days). The magnitude of the condition change in individual chicks between these two measurement points was also calculated and compared in relation to the age of their parents.

Before the effects of parental age on chick growth rates or condition could be tested, it was necessary to take into account a number of other variables likely to have an influence on these measures. These were brood size, laying date, chick hatching order, and chick sex. The growth rate of chicks that subsequently died was compared to that of chicks that survived to fledge in order to assess whether these chicks should be included in the analyses. As more than one chick was sampled per brood, chicks are not independent data points. Different approaches to the analysis that can account for this include taking the average value per brood, the value of one chick per brood

selected at random, or using all chick measurements and incorporating a random factor for brood identity. The last approach was preferred, as it uses all the data. In order to include a random factor for brood identity, and because this dataset was unbalanced, residual maximum likelihood (REML) mixed model analyses were used. Degrees of freedom were calculated by the Satterthwaite method, to account for non-independence of chicks from the same nest, and the consequent smaller sample size of nests than chicks. Chick growth and condition were tested in relation to female age, male age, the interaction between the age of the male and the female, and the age difference within known age pairs. Significant interactions were investigated by plotting separately for each level of one factor the mean response in relation to the second factor.

### *Chick sex*

Blood samples were collected (under Home Office licence) from chicks shortly after hatching (up to a few days old) in 2001 and 2002, for subsequent sexing from DNA using a polymerase chain reaction (PCR)-based method (Griffiths, Daan and Dijkstra 1996, Griffiths *et al.* 1998). A few drops of blood were drawn from the tarsus using a fine needle (25G or 29G), and transferred to an approximately equal volume of 100% ethanol before freezing. When necessary in order to obtain sufficient DNA for sexing, a second blood sample was taken from surviving chicks at a later date. It was possible to take more blood for these second-round samples, as the chicks were by that time larger. In the laboratory, DNA was extracted from the blood samples (protocol from Sambrook, Fritsch and Maniatis, 1989) and the sex of each chick determined by PCR (see references above). Chick sex was incorporated in the analyses of chick growth and condition in relation to parental age.

For broods of three in which the sex of all the chicks was known, the proportion of male offspring in the brood was compared among females of differing age. Only broods where all three chicks were sexed were used in this analysis. The brood sex ratio of three chick broods was recorded for 27 known age females in 2001, and in addition 5 old females of minimum known age (12+ years). In 2002, these data were collected from 50 known age females, and additionally 2 old females of minimum known age (both 13+ years). As 10 females occurred in both the 2001 and 2002 datasets, for these individuals their brood sex ratio in 2002 only was selected before

analysis, which meant the sex ratio at the older age in each case was used. The combined data from 2001 and 2002 after selecting one case per female therefore consisted of 74 females, ranging in age from 3 to 18 years. The number of male chicks in each brood was compared across known age females by entering female age as a continuous variable in a generalised linear model with poisson error distribution and log link function. The brood sex ratios laid by middle-aged females were compared directly to old females by entering age class as a fixed factor. The laying date of each female was also entered as a covariate in the latter analysis. In both cases, year was included as a random factor.

Throughout, all means are reported ± 1 S.E. Analysis by generalised linear models (binomial or poisson error distributions) and mixed models with normal error distribution were carried out in SAS (release 8.2, copyright 1999-2001, SAS Institute Inc.), using the GLIMMIX and PROC MIXED procedures respectively (see Littell *et al.* 1996 for further details). All other analyses were carried out in SPSS (release 10.0, copyright 1989-1999, SPSS Inc).

# RESULTS

## *Nest site quality*

Average nest site quality differed among scorers ($F_{2,287} = 45.0$, $p < 0.001$), and so between years ($t_{288} = 7.64$, $p < 0.001$). Therefore each year was analysed separately. In neither 2001 nor 2002, did old birds differ significantly from middle-aged birds in the quality of nest sites they occupied. The scores for ledge size, dampness of the nest after rain, exposure of the site to level attack and wave reach to the nest did not differ significantly between these age classes in either year (Mann-Whitney tests, in all cases $p > 0.05$, figure 4.2). The same results were obtained using Aebischer's (1985) formula to calculate a single rating for each nest: there was no significant difference between middle-aged (5-9 year old) and old (≥ 13 year old) males or females in either year ($p > 0.05$, table 4.3). Among old females, there was also no correlation between male partner age and the quality of the nest site occupied by the pair in 2001 ($n = 15$, male ages 2-15 years), or 2002 ($n = 21$, male ages 2- 16 years, in both years for all four nest site features $p > 0.05$).

## *Laying date*

Younger females laid later in the season than older birds (figure 4.3). However, the relationship of laying date to female age was best described by a quadratic regression (table 4.4*a*), suggesting that the oldest females are not the earliest to lay. In order to confirm the trend towards later laying in the oldest females, the correlation between laying date and female age in only birds aged 13 years or older was tested. However, the correlation was not significant in that age range (for females aged 13-21 years, $r = 0.28$, $n = 18$, $p = 0.27$). This suggests that the significant quadratic relationship overall may largely reflect a levelling-off of laying date among old birds, although the 21-year-old (and oldest) female did lay comparatively late. After removal of the oldest female from the data, quadratic regression no longer gives the best fit, and instead a logarithmic regression of laying date on female age explains the most variation (table 4.4*b*, figure 4.4). Logarithmic regression also allows for the greater change in laying date with age among young breeders, and more gradual differences in relation to age later in life.

Late laying by the 21-year-old female might reflect the age of her mate, a three-year-old male in 2001. The best description of the relationship between laying date

and the age of the male was provided by logarithmic regression (figure 4.5, table 4.5). There was insufficient data in known age pairs to separate the effects on laying date of female age from those of male age. For pairs in which the age of both sexes was known ($n$ = 50, table 4.6), neither male age nor female age had a significant effect on laying date (table 4.7*a*). The logarithmic regression of laying date on male age was the closest to significance ($p$ = 0.06). There was no significant interaction between the ages of pair members when both were included in a multiple regression against laying date (table 4.7*b*). Holding male age constant, and selecting male ages with the largest sample sizes (three and five year old males, table 4.6), the effect of female age on laying date was not significant ($p > 0.05$).

## *Reproductive success*

### *1. Hatching success*

In 2001 study nests (after excluding trios, $n$ = 103), clutch size ranged from two to four eggs, with a mean clutch size of 3 ± 0.04. Up to three eggs hatched per nest (mean 2.35 ± 0.09). Hatching success did not differ significantly among clutches of two ($n$ = 8), three ($n$ = 85), or four ($n$ = 10) eggs (Kruskal-Wallis test $\chi^2_2 = 3.47$, $n$ = 103, $p$ = 0.18, figure 4.6). Overall in 2001, mean hatching success in monogamous pairs was 78 ± 3%. Hatching success in trios (polygyny, with one male and two females at the same nest site) was much lower (0- 29%). There was no significant difference in the hatching success of clutches laid early in the season (on or before the median laying date of April 29$^{th}$) and late-laid (after the median laying date) clutches ($F_{1,95} = 2.22$, $p$ = 0.14, figure 4.7).

In 60% of three-egg clutches (51/85) all the eggs hatched successfully. When there was failure at the egg stage, the most common pattern was for a single egg to fail to reach hatching (21/34 clutches with egg failure). Complete hatching failure occurred in only 5% of three-egg clutches (4/85). Of the 21 cases in which one egg failed to hatch from a clutch of three, the laying order of the eggs was known at 12 nests. In 7/12 clutches it was the C egg, in 2/12 cases the B egg, and 3/12 cases the A egg that failed to reach hatching. This does not differ significantly from the null hypothesis that there is an equal chance of pre-hatching failure for all eggs in the clutch ($\chi^2_2 = 3.5$, $p > 0.05$).

The mean clutch volume of three egg clutches in which all the eggs hatched was compared to that of clutches in which at least one egg did not reach hatching. There was no significant difference in the average clutch volume of these two groups (average clutch volume: pre-hatching failure group 137.9 ± 1.6 $cm^3$, all eggs hatched group 139.1 ± 1.3 $cm^3$, $t_{62} = 0.58$, $p = 0.57$). The intra-clutch variation (measured as the volume of the smallest egg (A) / volume of the largest egg (B)) also did not differ between these two groups ($t_{38} = 0.48$, $p = 0.64$). The sample size for testing intra-clutch variation is lower than that for clutch volume because the former requires that the laying order of the eggs is known.

Hatching success in relation to parental age is shown in figure 4.8. The sample sizes for hatching success in known age females, known age males and known age pairs are given in these figures and table 4.8. The two oldest females in the dataset (a 20- and 21-year-old) both failed to hatch any eggs. However, there is no indication of a decline in hatching success prior to these extreme ages. No decline at all in hatching success at old age is seen in males, and the oldest males (three 17-year-olds and one 19-year-old) had 100% hatching success. The trend is for an increase in hatching success with male age. Figure 4.8 *(c)* shows hatching success in relation to the age difference between the parents, for pairs in which the age of both members is known. From this plot, it is apparent that pairs in which the female is much older than the male have poor hatching success.

For three-egg clutches, the hatching success of 64 females of known age, 53 known age males, and 39 pairs in which both members were of known age was recorded in 2001. Although the data is scant for complete hatching failure (because this is rare), in all four cases where complete failure occurred the female was aged 10 years or older, including the oldest (21-year-old) female in the dataset. The male was of known age for only two of these cases, but in both he was young (3 or 4 years old). The age difference between members of a pair was significantly larger in pairs that failed to hatch any of the eggs in their three-egg clutch ($F_{3,35} = 2.93$, $p = 0.047$, figure 4.9). Female age also differed significantly among pairs in which none, one, two or all three eggs hatched from clutches of three ($\chi^2_3 = 8.92$, $n = 64$, $p = 0.030$, figure 4.10). However, if the clutches in which no eggs hatched are excluded, female age does not differ significantly in relation to hatching number ($\chi^2_2 = 5.74$, $n = 60$, $p = 0.057$). Hatching success in three-egg clutches did not differ significantly in

relation to male age ($\chi^2_3 = 3.22$, $n = 53$, $p = 0.36$). Across clutches of all sizes, in a generalised linear model with a fixed factor describing laying date (early or late laying), hatching success did not differ with regard to male ($F_{1,42} = 0.23$, $p = 0.63$) or female age ($F_{1,43} = 0.93$, $p = 0.34$). The interactions between male and female age, and between parental age and laying date also were not significant (in all cases $p > 0.10$).

### *2. Fledging and breeding success*

Between zero and three chicks fledged from each study nest in 2001 (mean number of chicks fledged per nest 2.08 ± 0.10, $n = 103$ nests). For those nests at which at least one chick hatched, 78% of pairs reared all their chicks to fledging. Clutches from which no chicks hatched are excluded from the analysis of fledging success (proportion of hatched chicks that fledged), but are included in that of breeding success (total number of chicks fledged). There was no significant difference in the fledging or breeding success of clutches laid early (on or before the median laying date) compared to those laid late (after the median laying date) (fledging success: $F_{1,92} = 0.63$, $p = 0.43$, breeding success: $F_{1,95} = 2.15$, $p = 0.15$), although in both cases the average value was higher for early-laid clutches (figure 4.11).

Fledging and breeding success in relation to the age of the female parent, male parent, and the age difference in known age pairs is shown in figures 4.12 and 4.13. There is no decline in these breeding parameters in old females or old males. When fledging success was compared between middle-aged (5 - 9 year old) and old (12+ years, actual range 12-21 year old) females, there was no difference between these two groups ($F_{1,61} = 0.27$, $p = 0.61$). There was also no difference in the fledging success of middle-aged (5 - 9 year old) and old (12+years, actual range 12-19 year old) males ($F_{1,35} = 2.12$, $p = 0.15$). Neither were female or male age class significant when tested together (female age class: $F_{1,18} = 0.77$, $p = 0.39$, male age class $F_{1,18} = 0.27$, $p = 0.61$). Figure 4.12*c* shows no clear pattern in fledging success with regard to the age difference in known age pairs, although the three pairs at which there was complete failure during rearing did all have a comparatively large gap in age between breeding partners (age difference −11, +8 and +11 years). The difference in the absolute age gap in pairs (i.e. age difference irrespective of which sex is older) that failed completely during chick rearing and pairs that fledged at least one chick is

significantly different (age difference in pairs that failed during rearing: 10 ± 1 years, in pairs that fledged at least one chick: 2.9 ± 0.5 years, $z = 2.75$, $n = 43$, $p = 0.006$).

No chicks were fledged by the two oldest females in the dataset, but this is due to complete hatching failure rather than poor rearing performance. There was no difference overall in the number of chicks fledged by old compared to middle-aged females ($F_{1,65} = 0.02$, $p = 0.90$), or by old males in comparison to middle-aged males ($F_{1,37} = 1.64$, $p = 0.21$, age classes as before). Female age class and male age class also did not significantly influence breeding success when tested together (female age class: $F_{1,19} = 0.82$, $p = 0.38$, male age class: $F_{1,19} = 0.09$, $p = 0.77$). Although female age – male age in pairs does not differ significantly among known age pairs that fledged 0, 1, 2 or 3 chicks ($\chi^2_3 = 7.53$, $n = 47$, $p = 0.057$), the absolute age difference (i.e. disregarding which member of the pair is older) is significantly different among these groups ($\chi^2_3 = 7.92$, $n = 47$, $p = 0.048$). There was a greater age difference in pairs that fledged no chicks than in pairs that fledged one, two or three (figure 4.14).

### *Chick growth*

Overall, the average growth rate of chicks during the linear phase was 58.9 ± 0.8 g day$^{-1}$, and ranged from 33.3 to 74.4 g day$^{-1}$ ($n = 112$ chicks, 49 broods). Four chicks (from three broods) for which the growth rate is known died before reaching fledging. There is a significant difference in the growth rate of chicks that died and chicks that fledged (within-brood comparison, mean growth rate chicks that died: 40.3 ± 1.0 g day$^{-1}$, mean growth rate chicks that fledged: 53.0 ± 0.6 g day$^{-1}$, paired $t$ test: $t_1 = 28.1$, $p = 0.023$). In the following analyses, broods in which the chicks died have been excluded. There was a significant effect of brood identity on the growth rates of individual chicks ($F_{48,63} = 3.44$, $p < 0.001$).

The age range of females in this dataset is 3 – 17 years old, and of males 3- 19 years old. There was no relationship between the average brood growth rate and the age of the female or male parent (female age: $F_{1,24} = 0.15$, $p = 0.70$, male age: $F_{1,29} = 1.40$, $p = 0.25$, figure 4.15), when laying date, brood size and quadratic terms for parental age were included. The interaction between male and female age also was not significant (in known age pairs, $n = 19$ broods, $p > 0.05$). Neither the age of the female parent ($r = 0.32$, $n = 12$, $p = 0.32$), nor the age of the male parent ($r = 0.04$, $n$

= 15, $p$ = 0.89) correlated with intra-brood growth rate variation (greatest rate of growth in the brood/ lowest rate of growth in the brood) in broods of three. However, there was a significant effect of the difference in age between pair members (female age- age of her partner) on the mean growth rate of broods, when brood size was taken into account (age difference of parents: $F_{1,15} = 7.60$, $p = 0.015$, brood size: $F_{2,15} = 13.15$, $p = 0.001$, interaction: $F_{2,13} = 0.32$, $p = 0.73$, figure 4.16). Broods raised by pairs in which the female was substantially older than the male had lower average growth rates than broods of the same size raised by pairs in which both birds were of similar age.

In a mixed model analysis of the linear growth rate of individual chicks raised by known age pairs, incorporating brood size, laying date, hatching order, chick sex and the random factor brood identity, female age had a significant effect on chick growth (table 4.10). Although male age was not a significant main effect, the interaction between male age and hatching order was significant. Male age influences the growth of A, B and C chicks differently (see figure 4.17). The growth of individuals is also influenced by the size of brood in which they are raised, their hatching order and their sex. Overall, male chicks showed higher rates of linear growth than females (one chick per nest selected at random males: 63.8 ± 1.6 g day$^{-1}$, females: 59 ± 1.4 g day$^{-1}$, $t_{43} = 2.24$, $p = 0.030$). Laying date was the only non-significant variable, and was removed from the model. The average growth rate of broods also did not correlate significantly with laying date (laying date $\log_{10}$ transformed, all brood sizes: $r = -0.25$, $n = 45$, $p = 0.10$, broods of three only: $r = -0.20$, $n = 21$, $p = 0.39$).

If brood size, hatching order, and chick sex are included as random factors with brood identity in a mixed model testing the growth rate of chicks in relation to the age of their parents (with quadratic terms for parental age), neither quadratic term is significant (in both cases $p > 0.10$). There is also no significant interaction between male and female age ($F_{1,15.1} = 0.56$, $p = 0.47$). Female age significantly influenced chick growth (male age: $F_{1,16.9} = 3.04$, $p = 0.099$, female age: $F_{1,19.1} = 5.76$, $p = 0.027$), but only when male age was taken into account (i.e. if male age is removed from the model, female age: $F_{1,\ 17.5} = 2.60$, $p = 0.12$). Male age was not significant when tested alone ($F_{1,15.3} = 0.18$, $p = 0.68$). The parameter estimate for female age is negative (-0.79 ± 0.33), while that for male age is positive (+0.70 ± 0.40). This is

consistent with the negative relationship found between the age difference in pairs (female age - male age) and average brood growth rate, as chicks raised by an old female and a young male will have the lowest rate of growth (figure 4.18).

### *Chick condition*

Chick condition (mass, g/linear size$^3$, dm$^3$) at the first (mean chick age 15 days) and last measurement (mean chick age 24 days), after excluding broods in which one or more chicks subsequently died, was known for 98 and 101 chicks respectively, from 45 broods. Sample sizes differ between the two measurement periods due to a small number of missing values for mass or size. Overall, chick condition scores decrease between the ages of 15 and 24 days (mean condition score at first measure: $2.75 \pm 0.04 \times 10^3$ g dm$^{-3}$, at last measure: $1.41 \pm 0.02 \times 10^3$ g dm$^{-3}$, paired $t$ test: $t_{97} = 37.38$, $p < 0.001$). This negative change reflects the structural growth of chicks over time. Change in this condition score does not correlate with linear growth rate according to mass ($r = 0.08$, $n = 98$, $p = 0.45$).

Both parents were of known age in 20 broods (41 chicks), with female age ranging from 3 to 17 years, and male age from 3 to 15 years. For known age pairs, the effect of parent age on chick condition was tested in a mixed model that incorporated brood size, laying date, hatching order, chick sex, and the random factor brood identity. Quadratic terms for male and female age were also tested. Female age did not have a significant effect on chick condition at 15 days, 24 days, or on the change in condition (table 4.11). However, the interaction between male age and chick hatching order was significant for all three measures (see figure 4.19). Hatching order *per se* also had a significant effect on chick condition at both chick ages and on condition change. Within broods of three for which the condition of all the chicks was recorded (18 broods), the condition of C chicks differed significantly from that of either the A or B chick in the same brood at 24 days of age (mean condition A chicks: $1.36 \pm 0.03 \times 10^3$ g dm$^{-3}$, B chicks: $1.35 \pm 0.02 \times 10^3$ g dm$^{-3}$, C chicks: $1.52 \pm 0.04 \times 10^3$ g dm$^{-3}$, repeated measures ANOVA $F_{2,34} = 11.74$, $p < 0.001$), presumably because C chicks are structurally smaller than their siblings. Chick sex had a significant influence at 15 days of age and 24 days of age, but did not emerge as a significant factor for condition change. Brood size was only significant in the analysis of body condition at chick age 24 days. Neither the

interaction between male and female age, nor the quadratic term for the age of either parent was significant. The age difference between pair members (female age- male age) was also tested for an effect on chick condition score. However, this variable (which ranged from –1 to 12 years in this dataset) was not significant ($p > 0.10$).

The age range of males in known age pairs for which chick condition was recorded included young, middle-aged and old birds. In order to test whether the significant effect of male age on chick condition included a difference in old males (rather than only a difference between young and middle-aged birds), males were grouped into middle-aged (5-9 year olds) and old (12+ year olds, actual age range 12-19 years) age classes for all broods in which the male was of known age. There were 13 broods (and 26 chicks) with a middle-aged father and 9 broods (and 23 chicks) with an old father. In a mixed model including the random factors brood identity, brood size, chick sex and hatching order (shown to influence chick condition in the previous analysis) chick condition at 15 days of age, 24 days of age and the change in condition was compared between chicks raised by middle-aged and old males. Male age group had a significant effect on chick condition score at 15 days of age (with a middle-aged father, overall mean chick condition: 2.95 ± 0.10, with an old father: 2.69 ± 0.06, $F_{1,15.7} = 4.66$, $p = 0.047$), but was not significant for chick condition at 24 days ($F_{1,18} = 1.87$, $p = 0.188$), or for the change in condition ($F_{1,16.3} = 3.19$, $p = 0.093$).

### *Brood sex ratio*

The sample sizes at each female age in the combined dataset for 2001 and 2002 brood sex ratios (only three chick broods in which all the offspring were sexed) are shown in table 4.12. There was no significant effect of female age (across all ages) on the proportion of the brood that was male ($F_{1,65} = 0.08$, $p = 0.78$, figure 4.20). The number of male chicks in broods of three produced by old females (13+ years) did not differ from the number produced by middle-aged females (5 - 9 years old, laying date included as a covariate, female age class: $F_{1,55} = 0.02$, $p = 0.88$, laying date: $F_{1,55} = 0.48$, $p = 0.49$, interaction: $F_{1,53} = 0.78$, $p = 0.38$, figure 4.21).

## DISCUSSION

There was no difference in the quality of nest site occupied by old and middle-aged shags on the Isle of May. For old females, the quality of the nest site was not related to the age of their partner. Any differences between these age groups in reproductive performance therefore cannot be attributed to differences in the quality of their breeding sites. In addition, this suggests that variation in reproductive success with regard to nest site (Potts, Coulson and Deans 1980, Aebischer 1985, but also see Olsthoorn and Nelson 1990) is independent of differences in the age of the birds at those sites (for middle-aged and older birds).

With the exception of the single oldest female in the dataset (a 21-year-old), laying date in relation to female age was best described by logarithmic regression. This indicates that older females lay earlier than young birds, but that the change with age gradually levels off. Laying date showed the same relationship to male age. As the oldest female was paired with a young male, her comparatively late laying may have been a consequence of the age of her partner. Therefore, there is no change in the overall age-related trend in laying date in old birds, and aside from effects due to new pairings with a young partner, laying is not delayed. There was insufficient data to separate the effects on laying date of male age from those of female age.

Hatching success in monogamous pairs was close to 80%, and when hatching failure did occur in three-egg clutches, the most common pattern was for one egg not to hatch. Although no significant effect of laying order on the chance of each egg hatching was found, this was investigated for only a very small sample of known order eggs. Hatching success was not related to average egg size in clutches, but this was not explored on an individual egg basis. There was no significant difference in the hatching success of early and late-laid clutches. Complete hatching failure was rare, but the two oldest females both failed to hatch any eggs. Female age was significantly greater in pairs with complete hatching failure, but did not differ significantly among pairs that hatched one, two or three eggs from a clutch of three. The two oldest females were paired with young males, and therefore there was also a large age difference between breeding partners in these pairs. There was no indication of a gradual decline in hatching success with age among old birds, and in

males the trend was for a continued increase with age, up to at least 19 years old. For neither female nor male age was there a significant overall relationship to hatching success.

The survival of chicks during the rearing period was high in 2001, and at almost 80% of nests that produced chicks the full brood fledged. Both fledging and breeding success show a similar pattern overall with parental age as that seen in hatching success. Fledging success did not differ between old and middle-aged males or females. The age difference within pairs was significantly greater at nests that failed completely during chick rearing than at nests from which at least one chick fledged. Thus, complete failure at either the egg or chick stage is associated with a large difference in age between pair members. When old parents are compared to middle-aged parents, they do not differ significantly in the total number of chicks fledged. It is not unusual to find no overall difference in the reproductive success of old birds. Despite study over many years, no senescent decline has been observed in the reproductive performance of old common terns (*Sterna hirundo*, cross-sectional comparison, Nisbet, Apanius and Friar 2002), although terns over 12 years old do lay significantly smaller clutches and smaller eggs than younger females (Nisbet, Winchell and Heise, 1984).

Variation in the rate of chick growth was high, with a significant effect of natal brood. Surprisingly, there was no effect of laying date on either the average growth of broods or the growth of individual chicks. Average growth in broods was not affected by the age of either parent alone, but the age difference in pairs did have a significant effect on brood growth, when the size of the brood was taken into account. The average growth rate of the brood was lower in pairs where the female was much older than her partner. Taking into account differences among chicks in brood size, hatching order, chick sex and the age of the male parent, the growth of individual chicks is also significantly affected by female age. The growth rate of chicks is lower among old females, while the trend with male age is in the opposite direction. There is also a significant interaction between male age and hatching order: the greatest difference between young and old males was in the growth rate of C chicks, which increases with male age more steeply than the growth of the A or B chick in broods of three.

There was no significant effect of female age on chick condition. Male age did influence the condition of chicks when brood size, hatching order and chick sex were taken into account. As with chick growth, the effect of male age varied with chick hatching order. The condition score of B and C chicks in three chick broods increased gradually with male age, while the condition of A chicks was lower in broods raised by an old than by a young male. Comparing old males to middle-aged males only, the effect on chick condition was evident at chick age 15 days, when the condition of chicks with an old father was lower than that of chicks with a middle-aged father. Chicks with high condition scores are heavy for their size, which is generally assumed to be advantageous, and reflect good provisioning by the parents. On this assumption, chicks raised by old males do worse than chicks raised by middle-aged males, at least up to chick age 15 days. However, as condition declined during the growth period, this simple interpretation of body condition scores may not hold during growth. A low score may not necessarily mean that chick has been poorly fed, but instead that it has undergone greater structural growth, which may be advantageous if it allows early fledging. To assess the benefits of high or low condition at different chick ages it may be necessary to monitor offspring after fledging; there may be delayed effects of the rate of growth (see Metcalfe and Monaghan, 2001). The age difference within pairs did not affect chick condition.

A large age gap between pair members corresponded with poor breeding performance at hatching, in the growth of offspring, fledging success and the number of chicks fledged, although admittedly these findings are all based on the same small number of pairs, consisting of an old female and a young male. So, old female age is confounded with the age difference within pairs. A large age gap within pairs suggests the pair is comparatively newly formed. For pairs with a three or four-year-old male, the birds cannot have been breeding together for more than two or three years. Newly formed pairs may be less successful than established pairs irrespective of the age of the pair members. For instance, in captive peregrine falcons (*Falco peregrinus*) productivity drops by on average 53% when females experience a change of mate, and then subsequently increases again over the next few years (Clum, 1995). It is well established that young male shags have lower reproductive success than older males (e.g. Daunt *et al.* 1999, Daunt 2000), so this may add to the poor performance of new partnerships. It remains unresolved whether the old age of

the female is also a contributing factor to this poor performance, but old females do lay smaller eggs (chapter 3). The young age of the male, the old age of the female and the fact that the pair is newly formed may all combine to hamper breeding attempts.

There is no difference between middle-aged and old females in the proportion of male chicks produced, and the brood sex ratio was approximately 50% for both age classes. Including laying date as a covariate does not alter this result. Across all ages of females sampled, there is no indication that the brood sex ratio differs with maternal age. For the broods included in this analysis, there was no chick mortality and we can be reasonably certain that no eggs were laid that were not sexed (there is a small chance that extra eggs may have been laid if these were predated or broken shortly after laying). Thus, the sex ratio measured in this analysis is the sex ratio at laying. Only three-egg clutches were included in this analysis. However, three is the most common clutch and brood size in shags on the Isle of May, so it is unlikely that differences in the proportion of male chicks produced in clutches of other size would substantially change this result.

The lack of any significant effect of old age in males or females on hatching, fledging or breeding success may in part be due to the favourable environmental conditions for breeding shags on the Isle of May in the year data were collected (indicated by their high productivity). 2001 was a very successful year for shags at this location. Although breeding commenced later than in 2000, the number of breeding pairs (count of apparently occupied nests) on the island increased by 36% from the previous year. The count of 734 breeding pairs in 2001 was the highest since the population crash that took place in 1993. Mean productivity was at its highest since 1986 when intensive monitoring began, at 1.53 (± 0.16) chicks per incubated nest (averaged across monitoring plots). Of 104 successful pairs, 36% raised three chicks to fledge in 2001 (Wilson, Wanless and Harris 2002, Scottish Natural Heritage unpublished records). Not only may reproductive costs be more apparent in years when conditions are harsh (e.g. Tinbergen, van Balen and van Eck 1985, also see chapter 5), individuals may adjust their reproductive effort in line with environmental conditions. Constraints imposed when times are hard may be masked in good years. Another possible reason why reproductive declines in old age were not evident may be that only old birds in good condition hold a nest site at the

breeding colony. Alternatively, the oldest birds, in which senescence will have the strongest effects, may either not be present at the colony or may not lay any eggs. The challenges of measuring senescence in wild populations of long-lived birds have been reviewed by Nisbet (2001).

Reproductive performance in relation to age was only assessed by cross-sectional analysis in a single year. Given the variability in success among years, and the role of chance events in determining success, this may not be a sufficient sample for detecting reproductive senescence. Longitudinal comparisons, which control for intrinsic differences among individuals, may be more likely to reveal senescence-related change. However, this requires study of the same individuals over at least several years, which is challenging in the wild and for which long-term intensive monitoring is necessary. Even in longitudinal studies, it is difficult to separate in the wild ecological factors that affect reproduction from physiological effects.

# FIGURES

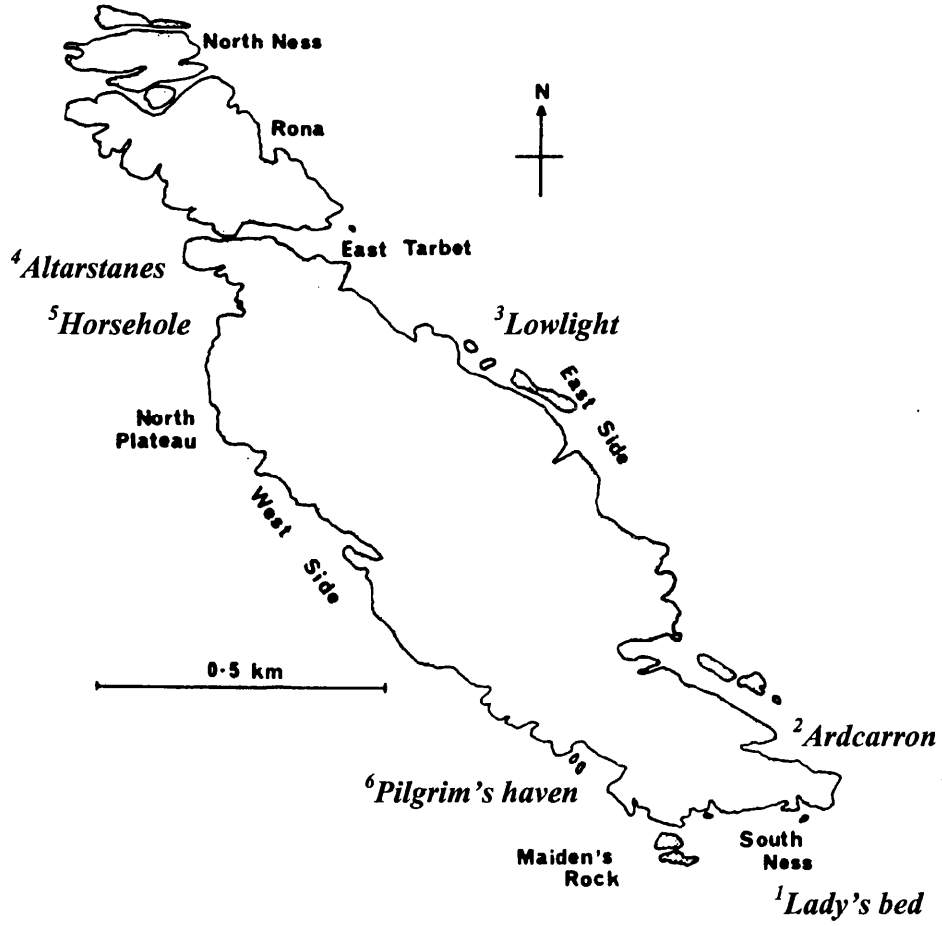



**Figure 4.1** Study nests were located in six main areas of the Isle of May in 2001, numbered 1-6 above. These areas were chosen because there were a large number of nests at each, to which access was possible with minimum disturbance. The number of nests monitored at each site depended on the number of known age pairs located at that site, and ease of access to individual nests.

| Nest site characteristic | | |
|---|---|---|
| **Ledge size** | | |
| Score | Description | |
| 1 | too small to hold 1 large chick and parent | |
| 2 | adequate for 1 large chick and parent | |
| 3 | adequate for 2 large chicks and parent | |
| 4 | adequate for 3 large chicks and parent | |
| 5 | plenty of room for 4 large chicks and both parents | |
| **Dampness after rain** | | |
| 1 | badly waterlogged, no drainage | |
| 2 | wet during rain, poor drainage | |
| 3 | wet during rain, good drainage | |
| 4 | occasionally wetted by rain or run-off | |
| 5 | dry at all times | |
| **Exposure to attack by birds at the same level as the nest** | | |
| 0 | inaccessible from level with the nest | |
| 1 | 0-45° | exposure in the horizontal plane, relative to shelter given by the immediate nest backing |
| 2 | 46-90° | |
| 3 | 91-135° | |
| 4 | 136-180° | |
| 5 | 181-225° | |
| 6 | 226-270° | |
| 7 | 271-315° | |
| 8 | 316-360°, fully exposed | |
| **Sea or wave reach** | | |
| 1 | below the level normally reached by high waves, potentially in danger of being damaged or washed away | |
| 2 | above the level normally reached by high waves, safe from all but the most extreme sea conditions | |

**Table 4.1** The scoring system used for nest site characteristics (after Aebischer, 1985).

| Year | Age class | Sex | |
|---|---|---|---|
| | | male | female |
| 2001 | middle-aged (5-9 years old) | 29 | 31 |
| | old (≥ 13 years old) | 16 | 24 |
| 2002 | middle-aged (5-9 years old) | 60 | 52 |
| | old (≥ 13 years old) | 28 | 38 |

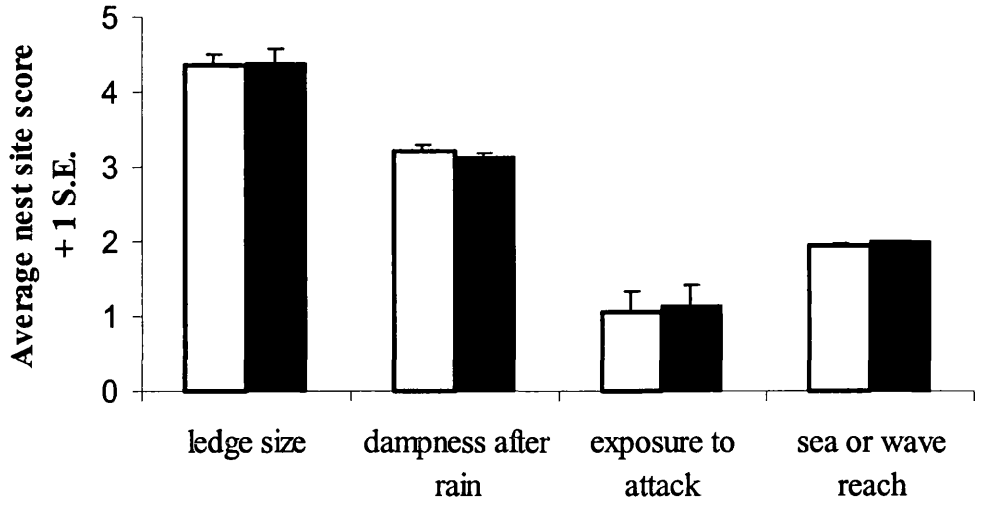
**Table 4.2** The sample size of males and females in each age group for which nest site quality was scored in 2001 and 2002. Some nests, and individuals, appear in both the 2001 and 2002 data. However, years were analysed separately, as the scorer in each year was different.

| Year | Age class | Sex | | | |
|---|---|---|---|---|---|
| | | male | | female | |
| 2001 | middle-aged (5-9 years old) | 2.09 ± 0.05 | *NS* | 2.06 ± 0.04 | *NS* |
| | old (≥ 13 years old) | 2.07 ± 0.05 | | 2.06 ± 0.05 | |
| 2002 | middle-aged (5-9 years old) | 1.83 ± 0.03 | *NS* | 1.86 ± 0.03 | *NS* |
| | old (≥ 13 years old) | 1.83 ± 0.05 | | 1.83 ± 0.04 | |

**Table 4.3** Nest site ratings (after Aebischer, 1985) for old and middle-aged males and females in 2001 and 2002. There was no difference in the quality of the nest site between these age classes in either year. Differences in the average nest site scores between years are likely to be due to different scorers assessing the nests in each year.

*(a)*

*2001*




*2002*

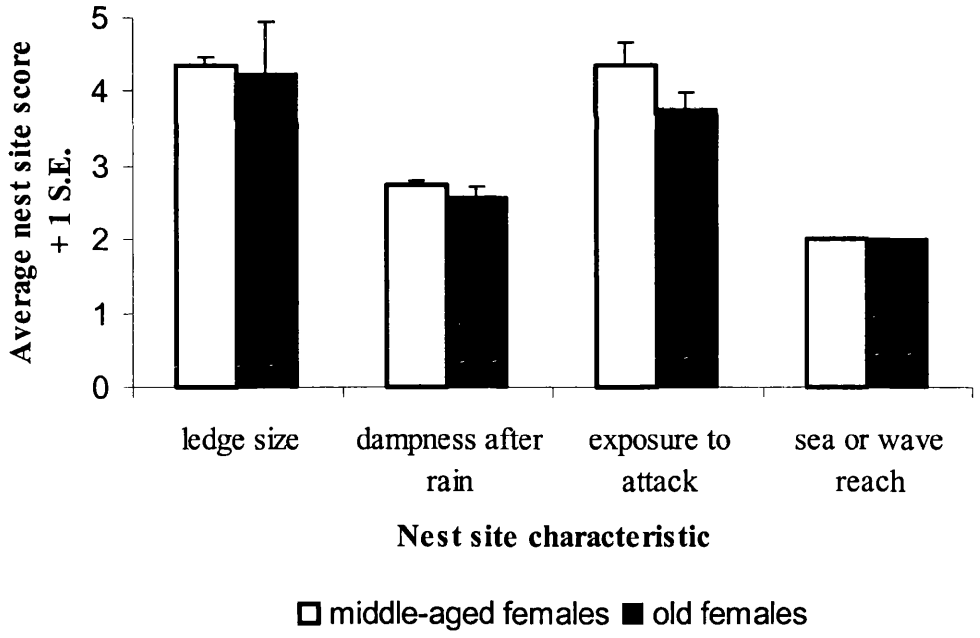
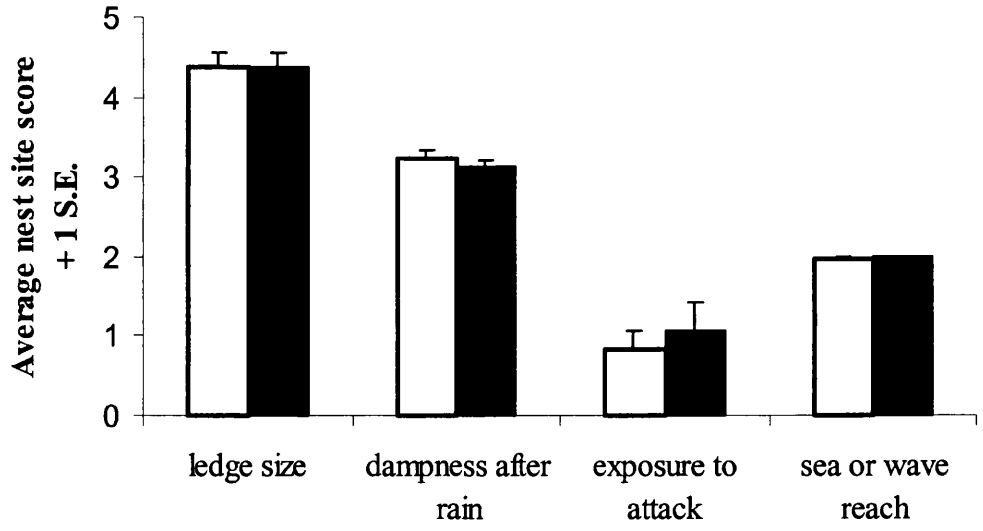



**Figure 4.2** *(a)* There was no difference in any of the four nest site features between sites occupied by old (≥ 13 years) and middle-aged (5-9 year old) females in 2001 or 2002. Comparisons of these nest site characteristics between old and middle-aged males are shown on the following page. The sample sizes of each age category of males and females are provided in table 4.2.

*(b)*

*2001*




*2002*

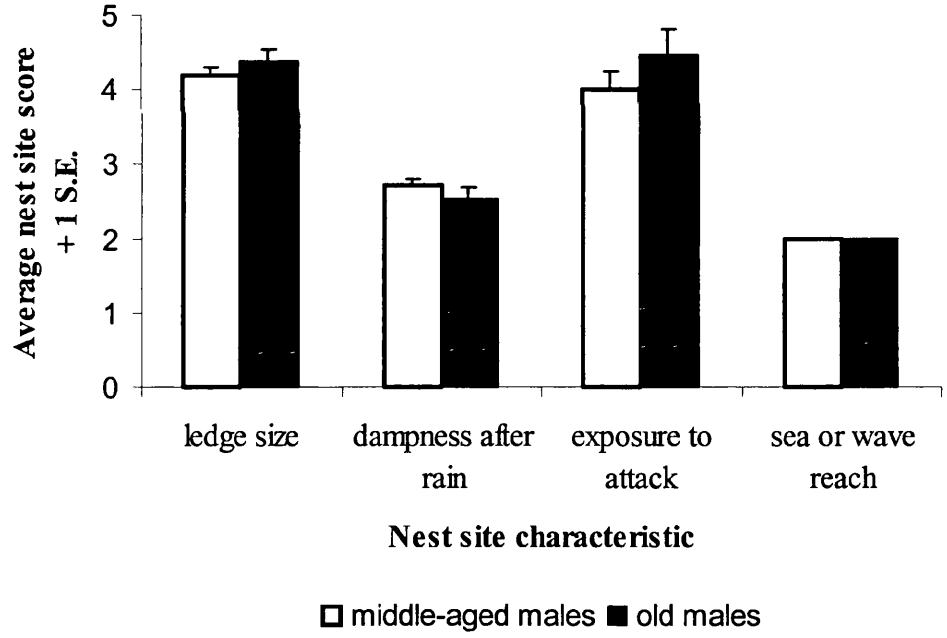



**Figure 4.2** *(b)* The nest sites occupied by old males did not differ from those occupied by middle-aged males in ledge size, dampness of the site after rain, exposure to level attack from other birds or wave reach to the site in either 2001 or 2002.

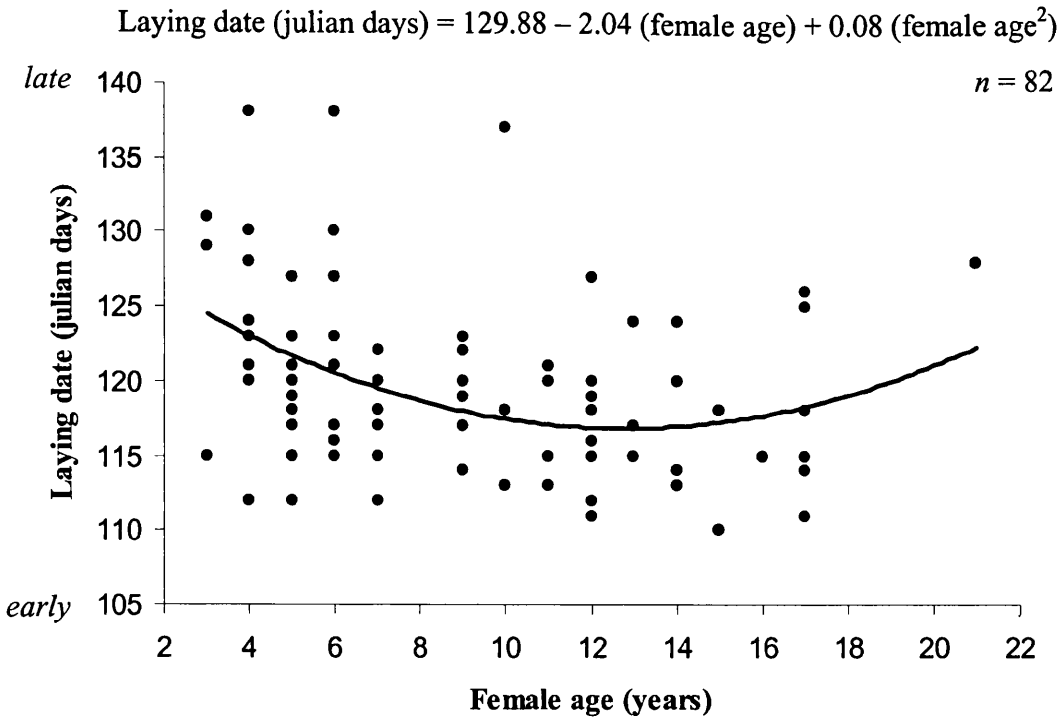



**Figure 4.3** The relationship between laying date and female age in 2001 was best described by a quadratic regression (curve and equation shown in chart). Older females lay earlier in the season than young birds, but this trend does not continue in the very oldest individuals. The median laying date was April 29th (julian day 119).

*(a)*

| Regression | *F* | *P* | *adj.* $R^2$ |
|---|---|---|---|
| Linear | $F_{1,80} = 5.59$ | 0.021 | 0.05 |
| Logarithmic | $F_{1,80} = 7.98$ | 0.006 | 0.08 |
| **Quadratic** | $\mathbf{F_{2,79} = 5.92}$ | **0.004** | **0.11** |

*(b)*

| Regression | *F* | *p* | *adj.* $R^2$ |
|---|---|---|---|
| Linear | $F_{1,79} = 9.02$ | 0.004 | 0.09 |
| **Logarithmic** | $\mathbf{F_{1,79} = 10.69}$ | **0.002** | **0.11** |
| Quadratic | $F_{2,78} = 5.46$ | 0.006 | 0.10 |

**Table 4.4** *(a)* Linear, logarithmic and quadratic regressions of laying date on female age in 2001, and *(b)* when the oldest (21-year-old) female is excluded from the analysis. The regression of best fit is highlighted in each case.

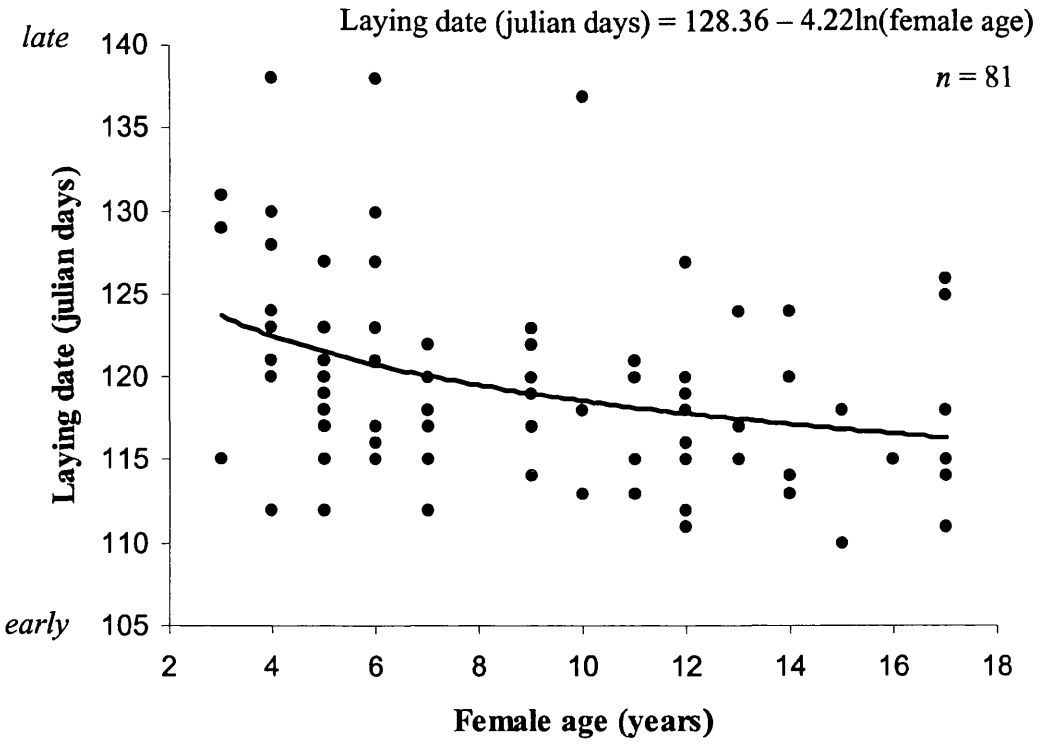



**Figure 4.4** After excluding the oldest female (21-year-old) from the data, the relationship between laying date and female age was best described by a logarithmic regression.

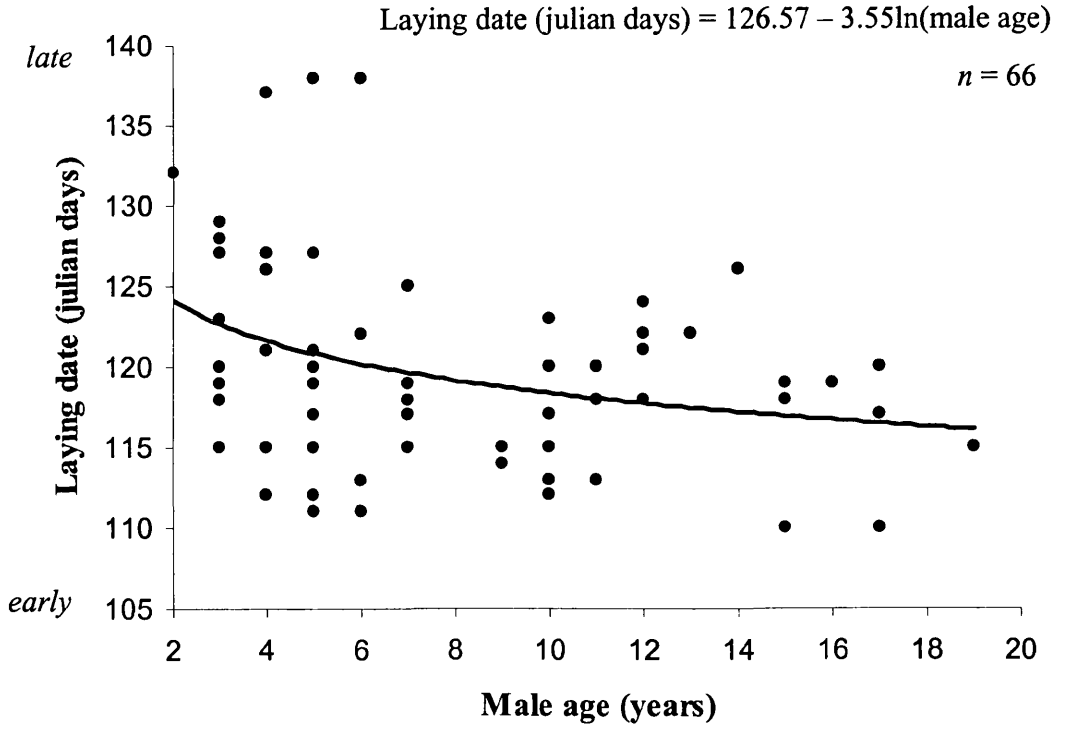



**Figure 4.5** Eggs are laid earlier in the season by pairs with an older male. This relationship is best described by a logarithmic regression.

| Regression | $F$ | $p$ | $adj.\ R^2$ |
|---|---|---|---|
| Linear | $F_{1,64} = 6.23$ | 0.015 | 0.08 |
| **Logarithmic** | $\mathbf{F_{1,64} = 7.78}$ | **0.007** | **0.09** |
| Quadratic | $F_{2,63} = 3.56$ | 0.034 | 0.07 |

**Table 4.5** The relationship of laying date to male age in 2001 was best described by the logarithmic regression.

| Female age (yrs) | Male age (yrs) | | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 | 9 | 10 | 11 | 12 | 15 | 16 | |
| 3 | 2 | | | | | | | | | | | 2 |
| 4 | 2 | 1 | 3 | | | | | | 1 | | | 7 |
| 5 | 3 | 2 | 2 | | | | 1 | | | | 1 | 9 |
| 6 | | | 1 | 1 | 2 | | | | | | | 4 |
| 7 | 1 | | 1 | 1 | | | 1 | 1 | | | | 5 |
| 9 | | | | | | | | | 1 | | | 1 |
| 10 | | 1 | | | | | | 1 | | | | 2 |
| 11 | | 1 | 1 | | | | | | 1 | | | 3 |
| 12 | | | 3 | 1 | | | 1 | 1 | | | | 6 |
| 14 | 1 | | | 1 | | 1 | 1 | | | | | 4 |
| 15 | | | | | | | | | | 2 | | 2 |
| 16 | | | | | | 1 | | | | | | 1 |
| 17 | | 1 | | | 2 | | | | | | | 3 |
| 21 | 1 | | | | | | | | | | | 1 |
| Total | 10 | 6 | 11 | 4 | 4 | 2 | 4 | 3 | 3 | 2 | 1 | 50 |

**Table 4.6** Male and female age in known age pairs for which laying date was recorded in 2001. Ages are only included in the table if they appeared in the dataset (e.g. there were no 8-year-old males in the data so this age is not shown in the table).

*(a)*

| Regression | sex | $F$ | $p$ |
|---|---|---|---|
| Linear | male | $F_{1,48} = 3.31$ | 0.075 |
| | female | $F_{1,48} = 1.01$ | 0.320 |
| Logarithmic | male | $F_{1,48} = 3.59$ | 0.064 |
| | female | $F_{1,48} = 1.66$ | 0.204 |
| Quadratic | male | $F_{2,47} = 1.73$ | 0.188 |
| | female | $F_{2,47} = 2.22$ | 0.120 |

*(b)*

| | Interaction | $F$ | $p$ |
|---|---|---|---|
| *3-way:* | ln(male age) × female age $^2$ | $F_{1,44} = 0.01$ | 0.91 |
| *2–way:* | ln(male age) × female age | $F_{1,45} = 1.55$ | 0.22 |
| | male age × ln(female age) | $F_{1,46} = 1.24$ | 0.27 |
| | ln(male age) × ln(female age) | $F_{1,46} = 1.70$ | 0.20 |
| | male age × female age | $F_{1,46} = 1.57$ | 0.22 |

**Table 4.7** In pairs in which both sexes were of known age, *(a)* there was no significant relationship between laying date and male or female age, and *(b)* the interaction between the ages of pair members did not explain a significant amount of the variation in laying date.

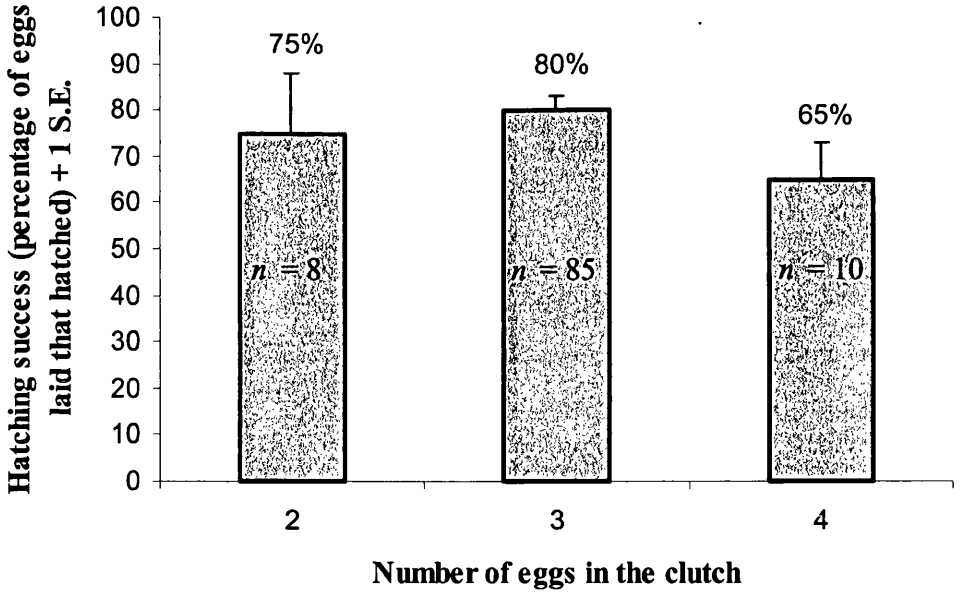



**Figure 4.6** Hatching success did not differ significantly among clutches of two, three or four eggs.

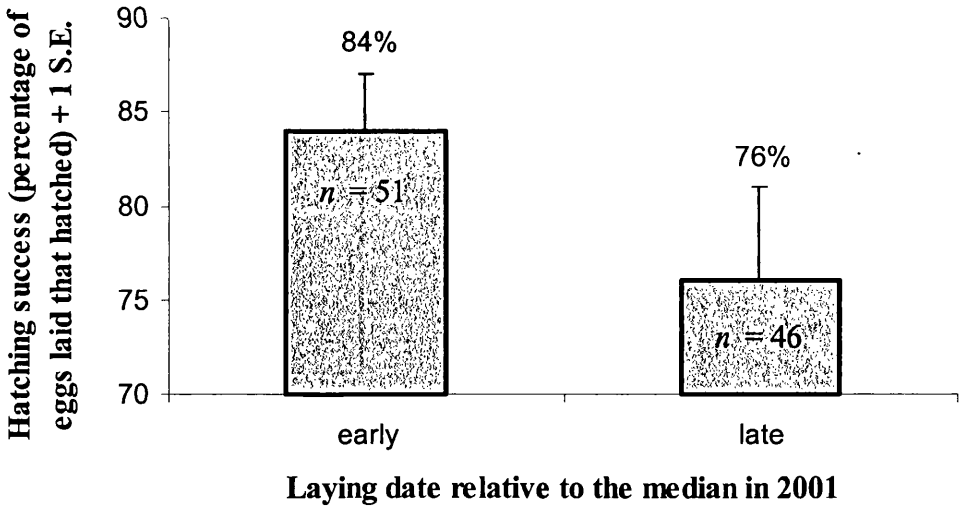



**Figure 4.7** The mean hatching success of early (on or before the median laying date of April 29th) and late-laid (after the median laying date) clutches was not significantly different.

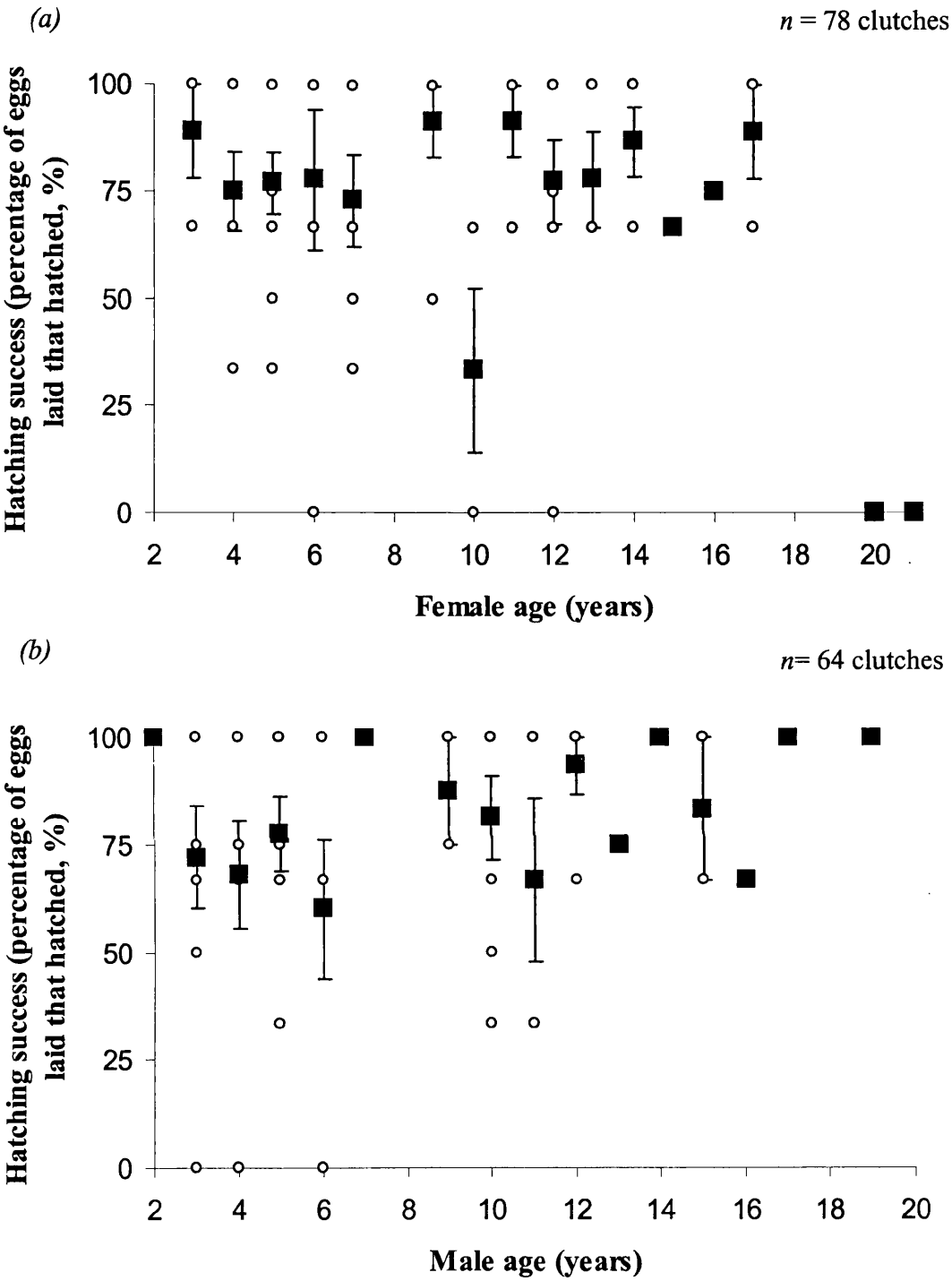



**Figure 4.8** Hatching success in relation to *(a)* the age of the female parent, and *(b)* the age of the male parent. Individual observations are shown as open circles, and the mean hatching success ± 1 S.E. at each age as a closed square. Sample sizes at each age are shown in table 4.8 on the following page. The range of hatching success values observed were: 0% (no eggs hatched), 33% (1/3 hatched), 50% (1/2 or 2/4 hatched), 67% (2/3 hatched), 75% (3/4 hatched), or 100% (all eggs hatched).

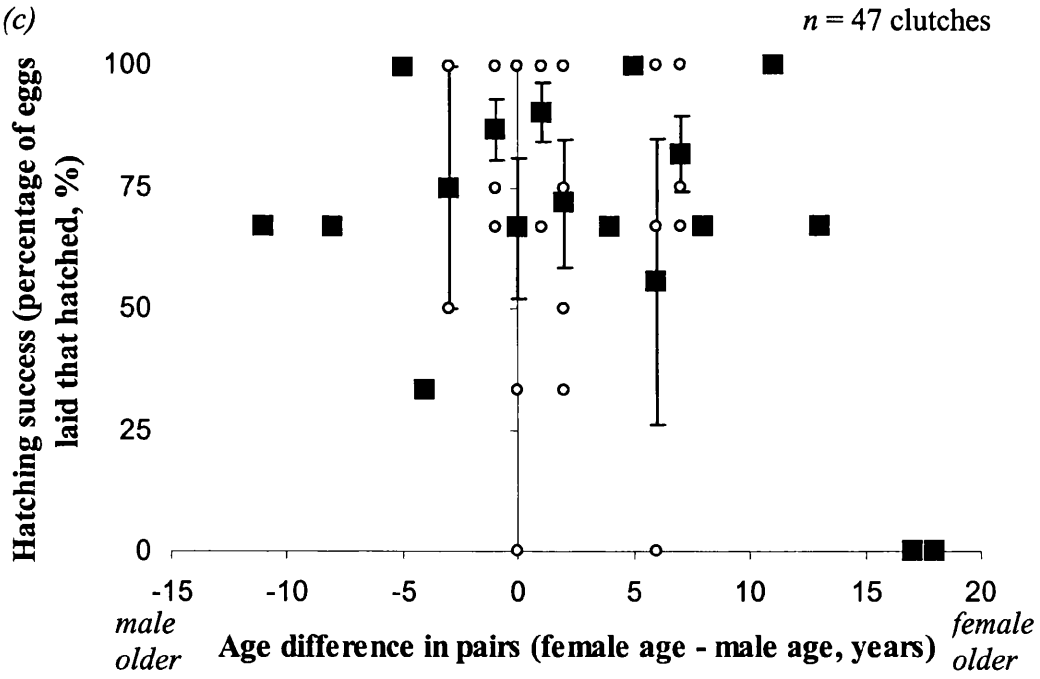



**Figure 4.8** *(c)* Hatching success in relation to the age difference between pair members, for known age pairs in 2001. Open circles show single observations, and the closed squares are mean hatching success ± 1 S.E. for each age gap. Sample sizes of each age difference are shown in table 4.8.

**Table 4.8** The sample sizes of *(a)* each age of female (F) and male (M), and *(b)* each age difference between the members of known age pairs, for which hatching success is known, and as plotted in figure 4.8. In *(b)* only age differences that actually appear in the dataset are shown in the table.

*(a)*

| Sample size at each age (years) | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age | 2 yrs | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| F | - | 3 | 9 | 13 | 6 | 8 | - | 6 | 4 | 4 | 10 | 3 | 5 | 1 | 1 | 3 | - | - | 1 | 1 |
| M | 1 | 11 | 7 | 10 | 5 | 3 | - | 2 | 8 | 3 | 5 | 1 | 1 | 2 | 1 | 3 | - | 1 | - | - |

*(b)*

| Sample size (number of pairs) with each age difference between the sexes (female age- male age, years) | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| gap | -11 yrs | -8 | -5 | -4 | -3 | -1 | 0 | +1 | +2 | +4 | +5 | +6 | +7 | +8 | +11 | +13 | +17 | +18 |
| *n* | 1 | 1 | 1 | 1 | 2 | 7 | 7 | 7 | 5 | 1 | 1 | 3 | 5 | 1 | 1 | 1 | 1 | 1 |

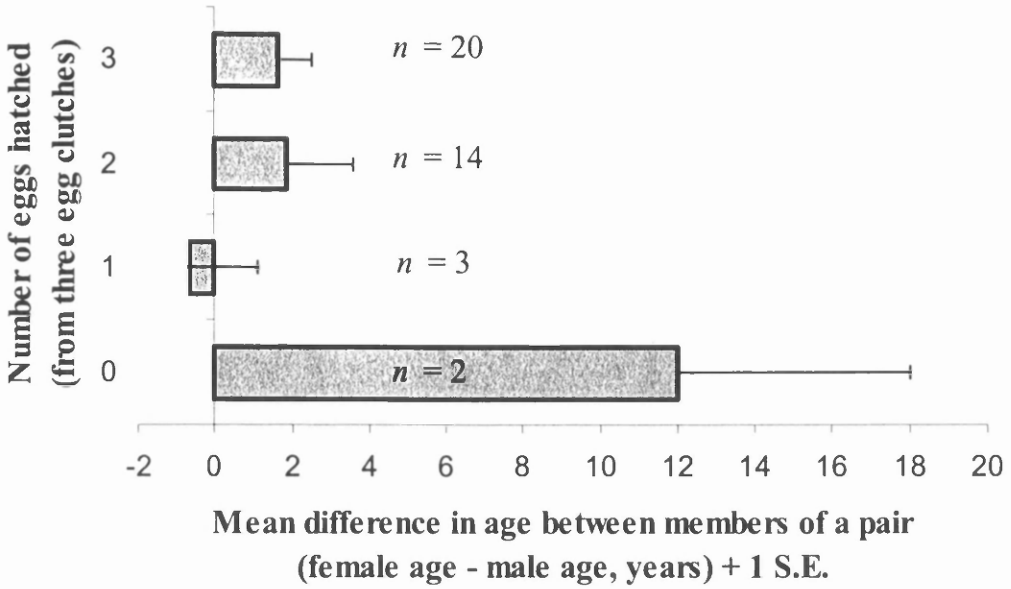



**Figure 4.9** Although the sample size of known age pairs that had complete hatching failure was very small, the age difference in these pairs was significantly greater than in pairs that hatched 1/3 eggs or 3/3 eggs.

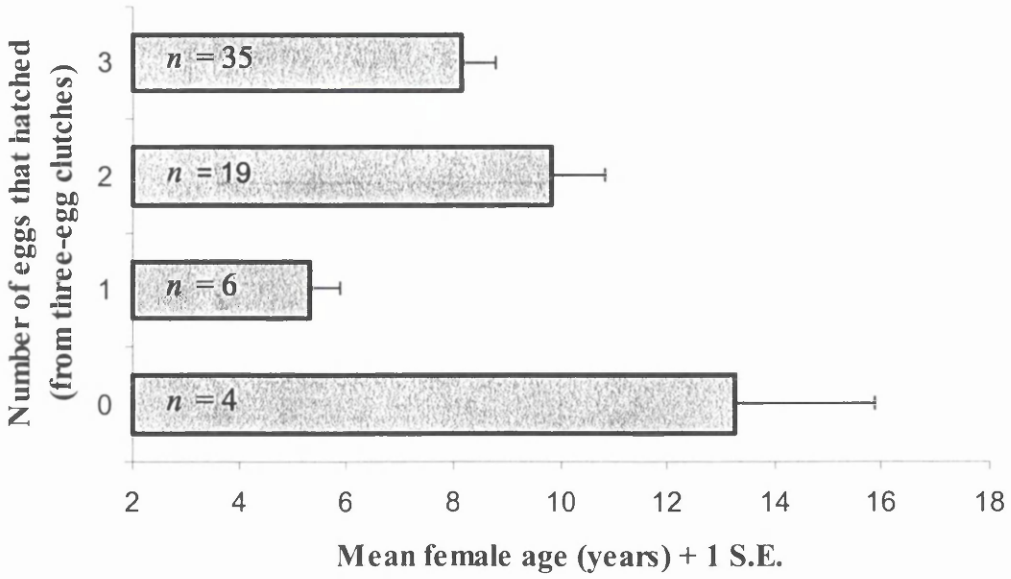
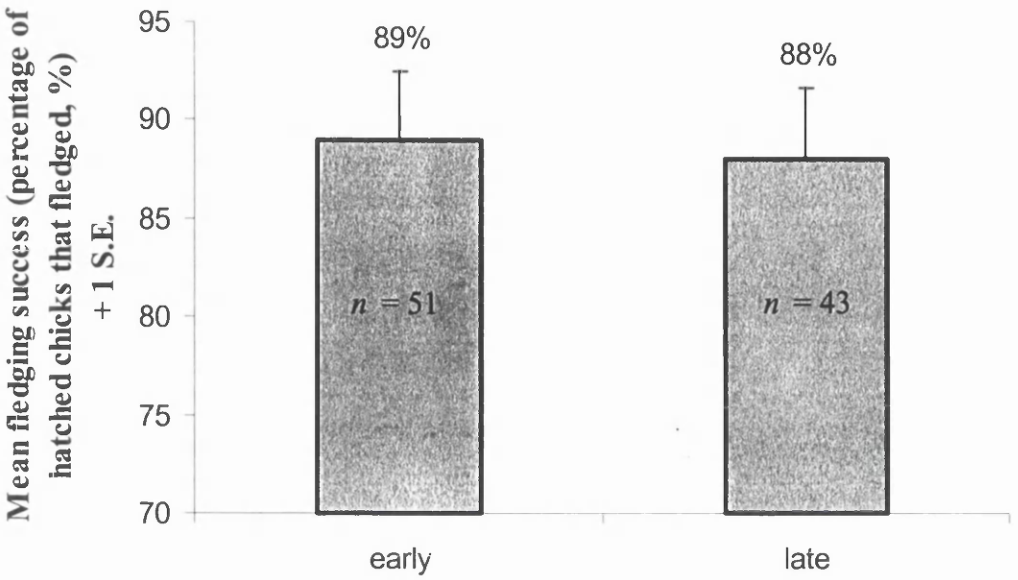



**Figure 4.10** Female age differed significantly among clutches of three where 0, 1, 2 or 3 eggs hatched.

*(a)*




*(b)*

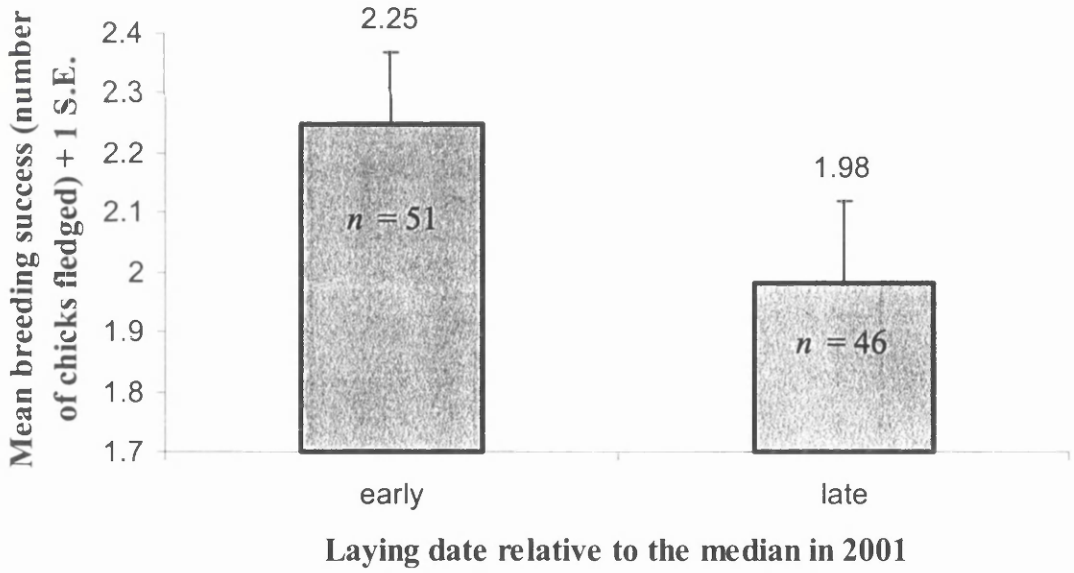



**Figure 4.11** Mean *(a)* fledging success, and *(b)* breeding success from nests at which laying was early (on or before the median laying date of April 29th) or late (after the median laying date) in 2001. Neither of these breeding parameters were significantly different between early- and late-laid clutches.

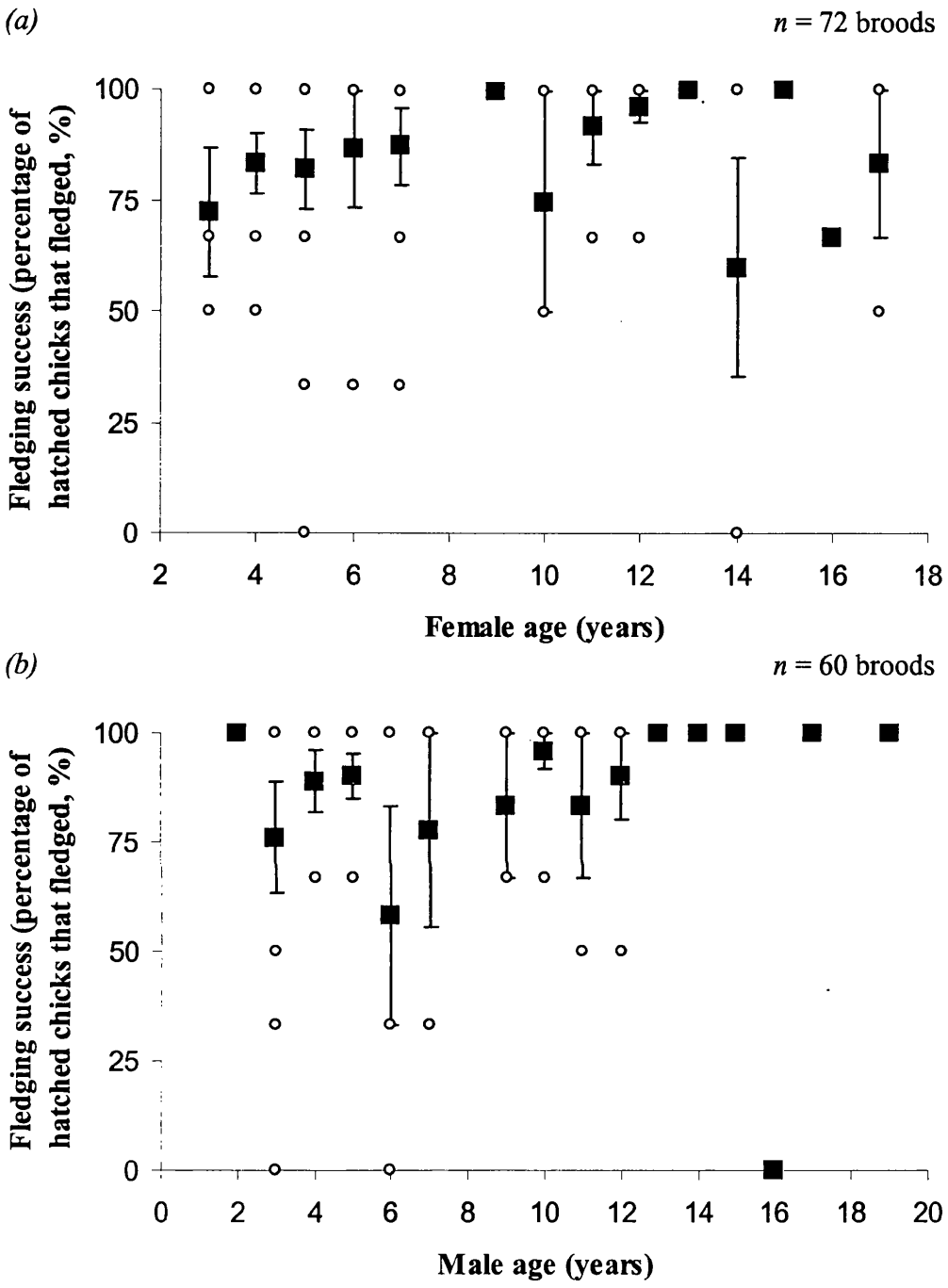



**Figure 4.12** Fledging success in 2001 in relation to the age of the *(a)* female parent, and *(b)* male parent. Open circles show each observation, and the closed squares are the mean fledging success ± 1 S.E. at each age. Sample sizes at each age are shown in table 4.9. Nests at which no eggs hatched are not included in fledging success data. The range of values of fledging success at each nest were: 0% (no chicks fledged), 33% (1/3 fledged), 50% (1/2 fledged), 67% (2/3 fledged) or 100% (all chicks, irrespective of brood size, fledged).

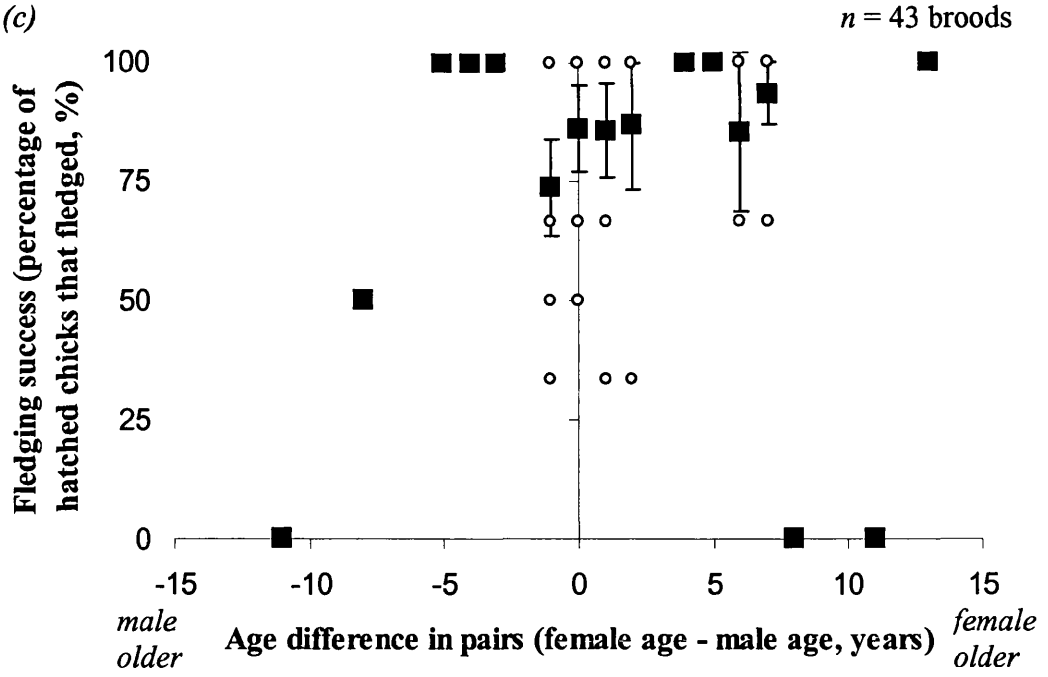



**Figure 4.12** *(c)* Fledging success in relation to the age difference in known age pairs. Open circles show single observations, and the closed squares are the mean fledging success ± 1 S.E. at each age difference (see table 4.9 for sample sizes at each age difference).

**Table 4.9** The sample size at *(a)* each age of female (F) and male (M), and *(b)* each age difference in known age pairs, for fledging success in 2001 as plotted in figure 4.12. In *(b)* only age differences that actually appear in this dataset are shown in the table.

*(a)* Sample size at each age (years)

| Age | 2 yrs | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F | - | 3 | 9 | 13 | 5 | 8 | - | 6 | 2 | 4 | 9 | 3 | 5 | 1 | 1 | 3 | - | - |
| M | 1 | 9 | 6 | 10 | 4 | 3 | - | 2 | 8 | 3 | 5 | 1 | 1 | 2 | 1 | 3 | - | 1 |

*(b)* Sample size (number of pairs) with each age difference between the sexes (female age- male age, years)

| gap | -11 yrs | -8 | -5 | -4 | -3 | -1 | 0 | +1 | +2 | +4 | +5 | +6 | +7 | +8 | +11 | +13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *n* | 1 | 1 | 1 | 1 | 2 | 7 | 6 | 7 | 5 | 1 | 1 | 2 | 5 | 1 | 1 | 1 |

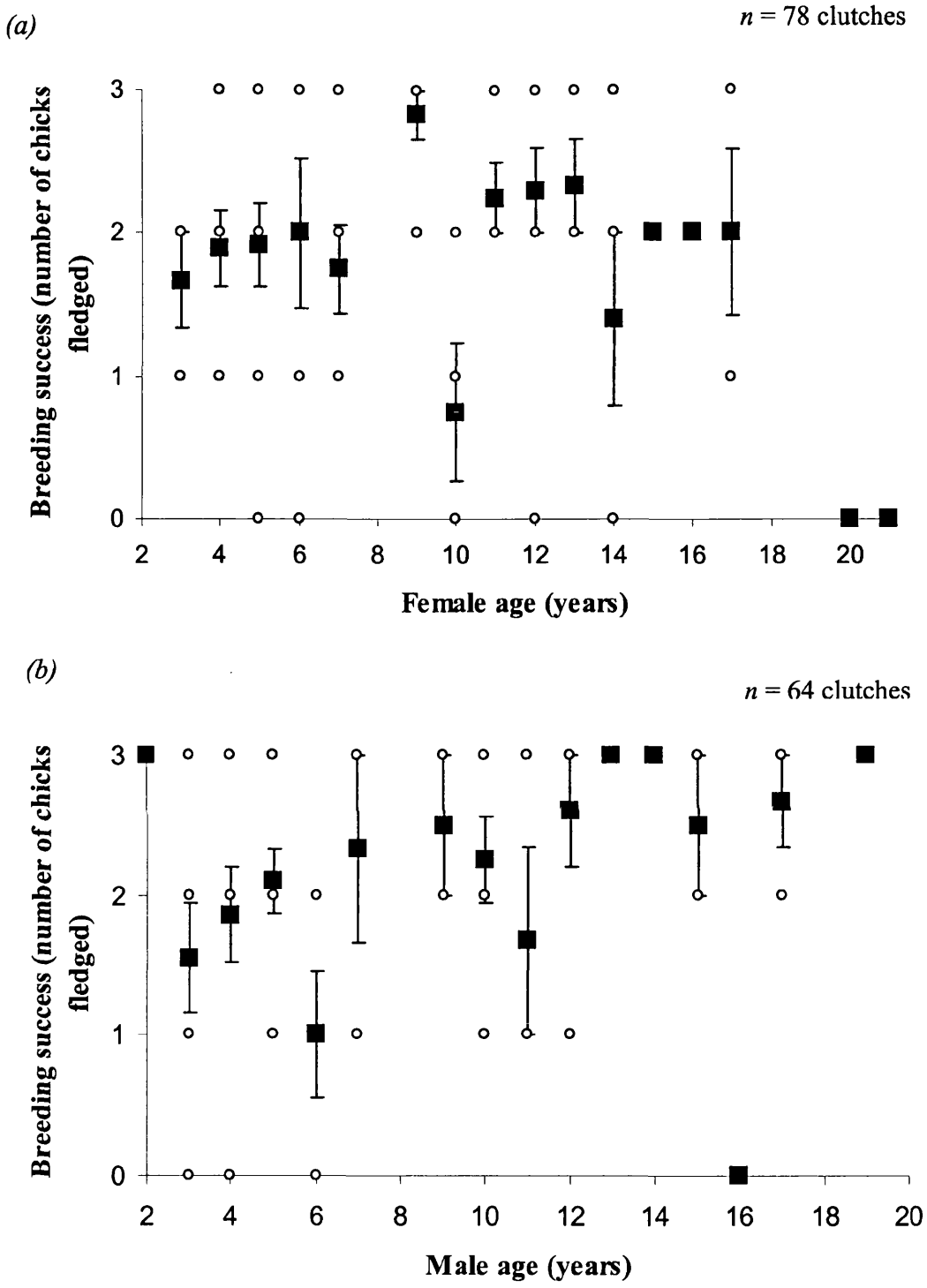



**Figure 4.13** Breeding success in relation to *(a)* the age of the female parent, and *(b)* the age of the male parent. This data includes cases where no eggs hatched. Open circles show single observations, and the closed squares are the mean number of chicks fledged ± 1 S.E. at each age. Sample sizes at each age are the same as for hatching success (table 4.8).

*(c)*

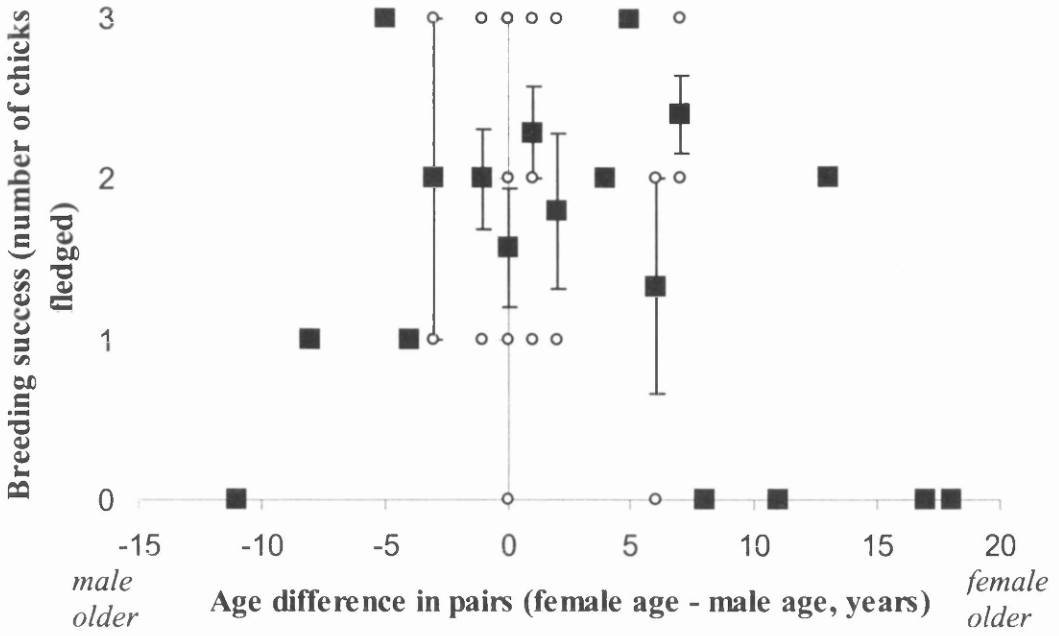



**Figure 4.13** *(c)* Breeding success in relation to the difference in age between pair members in 2001. Open circles show single observations, and the closed squares give the mean number of chicks fledged ± 1 S.E. at each age difference (see table 4.8 for sample sizes of each age difference).

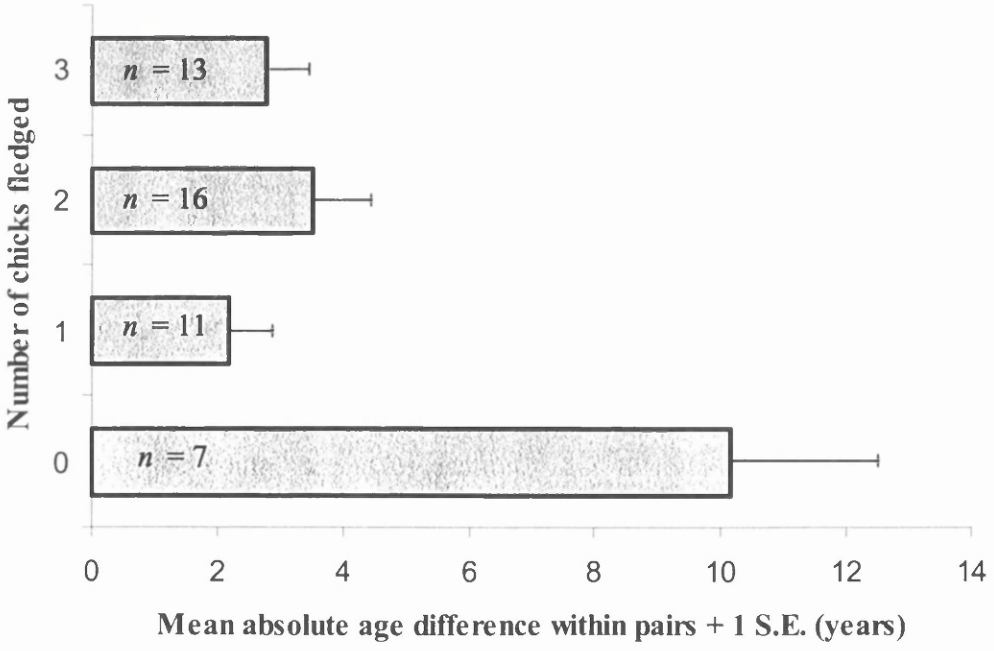
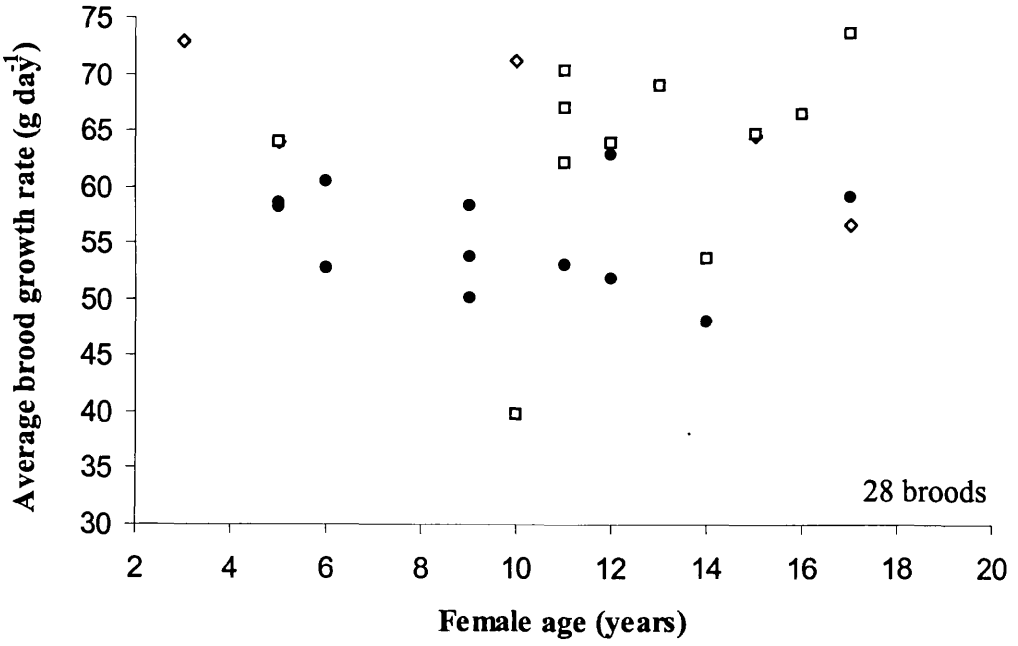



**Figure 4.14** The absolute difference in age between pair members was significantly greater in pairs that did not fledge any chicks.

*(a)*




*(b)*


**Figure 4.15** The average linear growth rate in broods, in relation to the age of the *(a)* female, or *(b)* male parent.

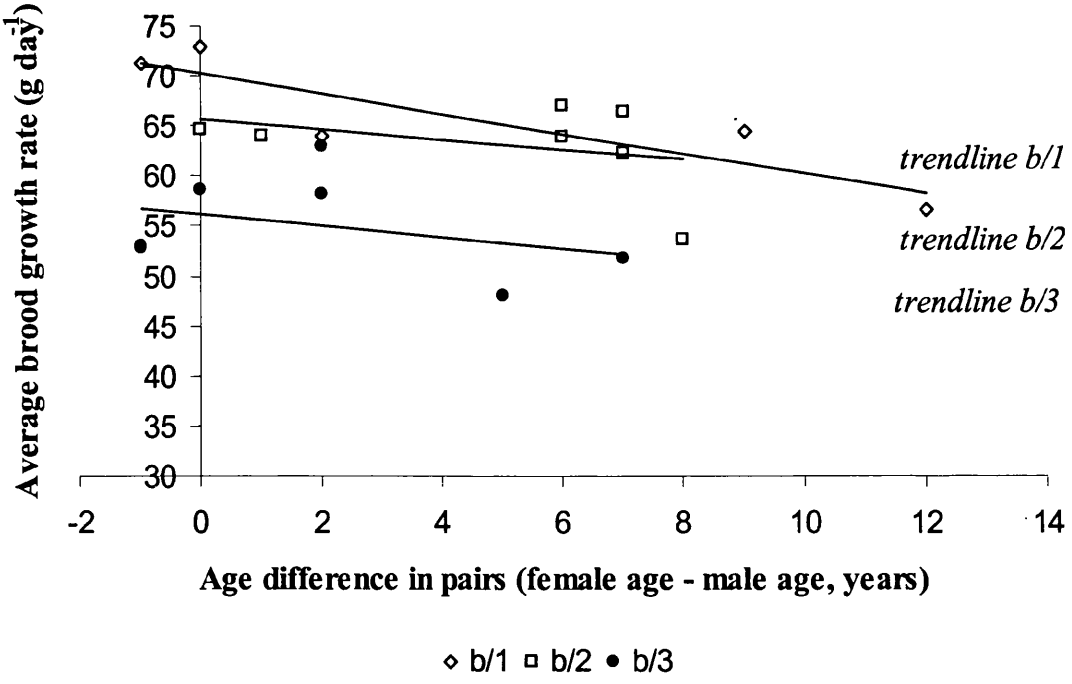



**Figure 4.16** The difference in age of the parents significantly affected the average growth rate of their brood. Thus, for a given brood size, broods raised by pairs in which the female was substantially older than the male had on average lower growth than broods raised by pairs in which the male and female were of similar age. The sample sizes were 5 broods of one, 7 broods of two and 7 broods of three.

| Effect | *F* | *p* |
|---|---|---|
| Laying date[a] | $F_{1,14.5} = 1.62$ | 0.22 |
| **Female age[b]** | **$F_{1,18.3} = 7.82$** | **0.01** |
| **male age[b]** | **$F_{1,18.1} = 2.26$** | **0.15** |
| **hatching order[b]** | **$F_{2,21} = 4.80$** | **0.02** |
| **brood size[b]** | **$F_{2,20.5} = 16.12$** | **< 0.0001** |
| **chick sex[b]** | **$F_{1,25.7} = 18.76$** | **0.0002** |
| **male age × hatching order[b]** | **$F_{2,21.2} = 4.32$** | **0.03** |

[a] Statistics immediately prior to removal of this term.

[b] Statistics for term in the final model.

**Table 4.10** Results of a mixed model analysis of chick growth in 2001 treating brood size, hatching order, and chick sex as fixed factors, female age, male age and laying date as covariates, with brood identity as a random factor (brood identity in the final model: $z = 1.44$, $p = 0.08$). The final model is highlighted in bold.

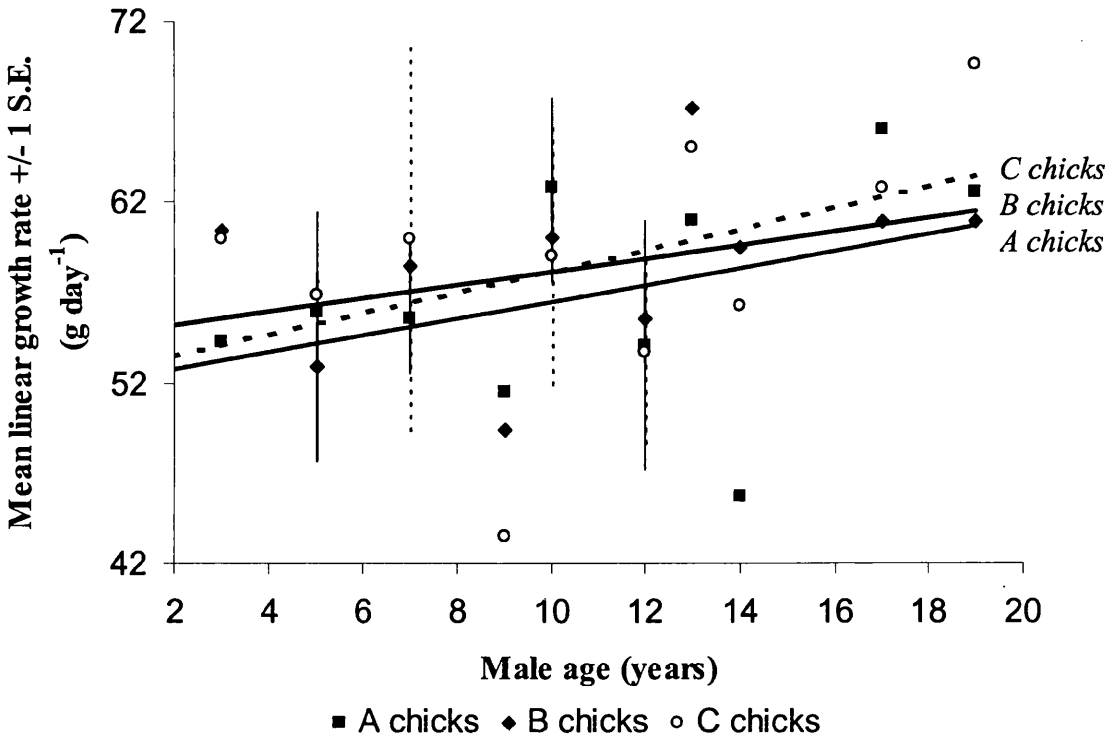
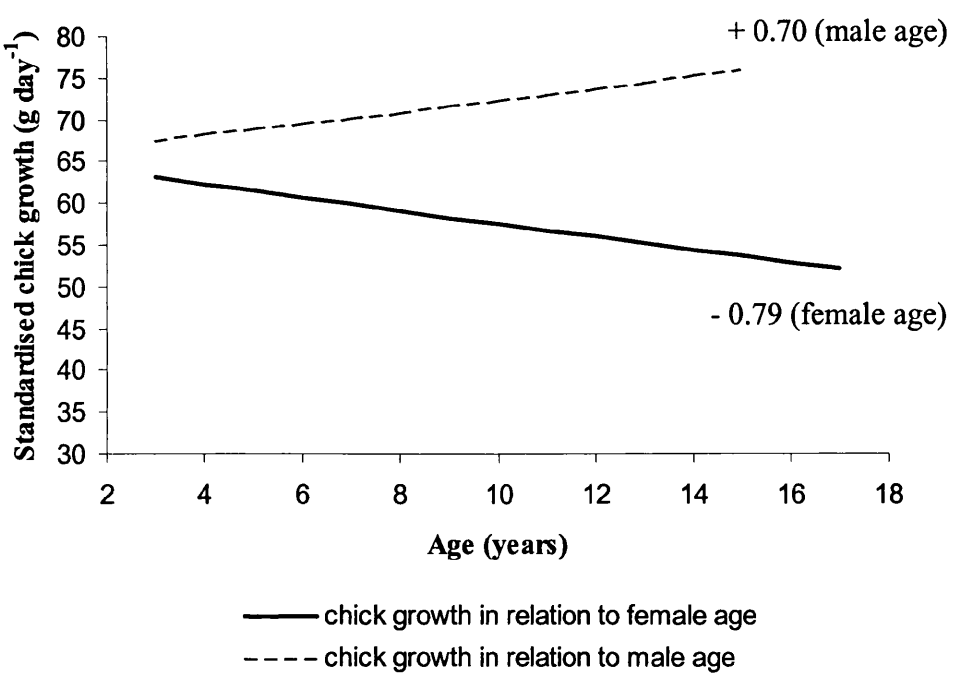



**Figure 4.17** The linear growth rates of A, B and C chicks in broods of three were affected to different extents by male age, although for all three chicks growth was faster when the male parent was old. A trendline has been fitted for the mean growth of each chick in the hatching sequence in relation to paternal age (sample size 15 chicks each A, B and C).

(a)




(b)

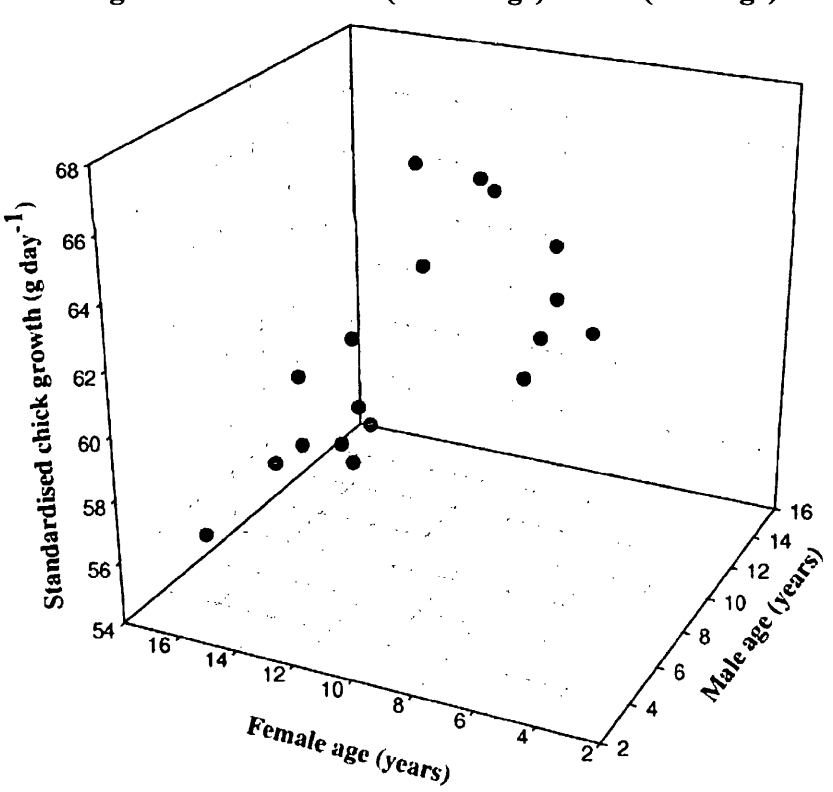



**Figure 4.18** The effect of female and male age (for the age range present in the dataset) on chick growth rate, using the slope estimates from a REML mixed model analysis of chick growth in relation to parental age, incorporating brood identity, brood size, hatching order and chick sex as random effects. *(a)* 2D representation of the slope in relation to the age of each parent *(b)* 3D representation for the observed age combinations in pairs (19 known age pairs).

| Dependent variable | Effect | $F$ | $p$ |
|---|---|---|---|
| Chick condition at 15 days of age | brood size[a] | $F_{2,10.6} = 0.16$ | 0.854 |
| | female age$^{2a}$ | $F_{1,27} = 0.01$ | 0.907 |
| | female age × male age[a] | $F_{1,28} = 1.17$ | 0.290 |
| | male age$^{2a}$ | $F_{1,14.4} = 1.95$ | 0.183 |
| | laying date[a] | $F_{1,17.9} = 1.72$ | 0.206 |
| | female age[a] | $F_{1,20.8} = 3.21$ | 0.088 |
| | **male age[b]** | $\mathbf{F_{1,19.8} = 1.28}$ | **0.272** |
| | **chick sex[b]** | $\mathbf{F_{1,31} = 6.30}$ | **0.018** |
| | **hatching order[b]** | $\mathbf{F_{2,23.2} = 6.70}$ | **0.005** |
| | **male age × hatching order[b]** | $\mathbf{F_{2,22.6} = 4.25}$ | **0.027** |
| Chick condition at 24 days of age | male age$^{2a}$ | $F_{1,6.32} = 0.03$ | 0.876 |
| | female age × male age[a] | $F_{1,9.18} = 0.22$ | 0.652 |
| | laying date[a] | $F_{1,12.8} = 0.03$ | 0.857 |
| | female age$^{2a}$ | $F_{1,15.1} = 3.10$ | 0.098 |
| | female age[a] | $F_{1,14.4} = 0.08$ | 0.777 |
| | **male age[b]** | $\mathbf{F_{1,16.1} = 1.43}$ | **0.249** |
| | **brood size[b]** | $\mathbf{F_{2,19} = 5.59}$ | **0.012** |
| | **chick sex[b]** | $\mathbf{F_{1,25.1} = 7.43}$ | **0.012** |
| | **hatching order[b]** | $\mathbf{F_{2,17.4} = 7.24}$ | **0.005** |
| | **male age × hatching order[b]** | $\mathbf{F_{2,17.6} = 4.19}$ | **0.033** |
| Change in chick condition | female age$^{2a}$ | $F_{1,11} = 0.05$ | 0.832 |
| | male age$^{2a}$ | $F_{1,19} = 3.14$ | 0.093 |
| | female age × male age[a] | $F_{1,21} = 1.46$ | 0.240 |
| | laying date[a] | $F_{1,14.9} = 1.54$ | 0.234 |
| | brood size[a] | $F_{2,24.5} = 2.10$ | 0.144 |
| | female age[a] | $F_{1,19.8} = 1.49$ | 0.237 |
| | chick sex[a] | $F_{1,31.3} = 2.17$ | 0.151 |
| | **male age[b]** | $\mathbf{F_{1,19} = 0.33}$ | **0.571** |
| | **hatching order[b]** | $\mathbf{F_{2,25} = 4.92}$ | **0.016** |
| | **male age × hatching order[b]** | $\mathbf{F_{2,24} = 3.93}$ | **0.033** |

[a]Statistics immediately prior to removal of this term.
[b]Statistics for term in the final model.

**Table 4.11** Results of a REML mixed model analysis of chick condition at 15 days of age, 24 days of age and the change in condition over this period, incorporating the random factor brood identity (see methods and results for further details of analysis). Non-significant terms are shown in the order in which they were removed, and the final model in each case is highlighted in bold. All other two-way interactions were also tested, but none were significant ($p > 0.05$).

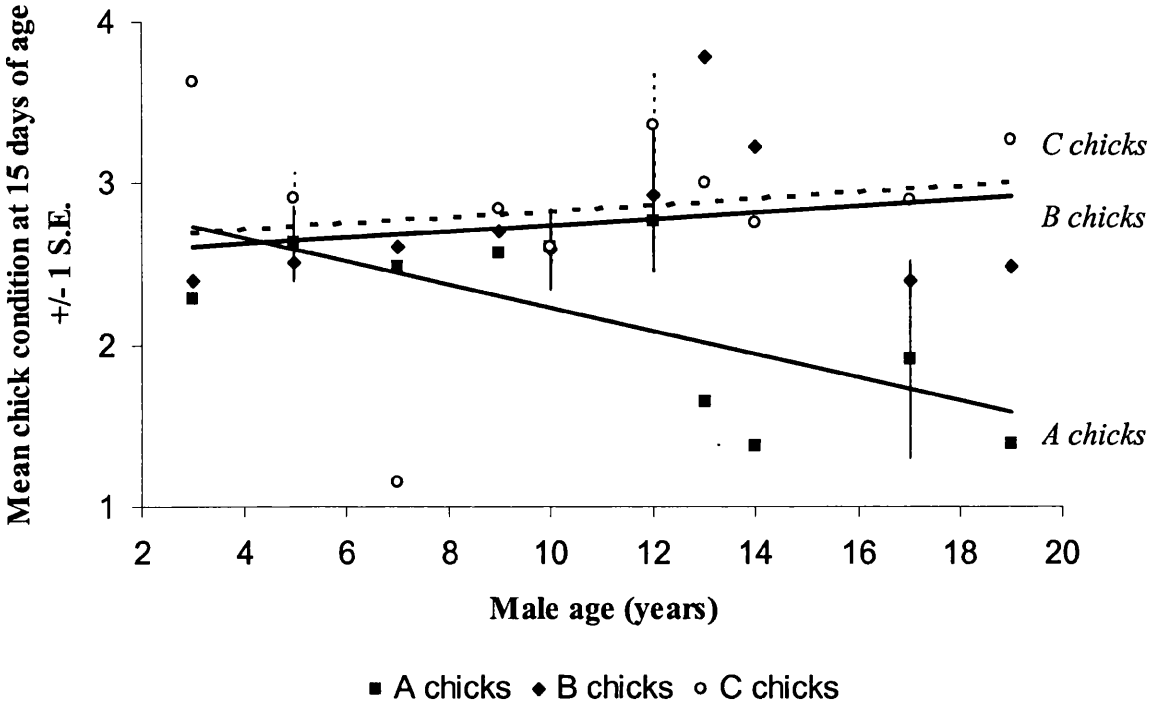



**Figure 4.19** The influence of male age on chick condition at 15 days age varied with hatching order. Trendlines for the mean body condition of A, B and C chicks in broods of three are shown in relation to paternal age (17 chicks each A, B and C).

| Female age (years) | Sample size | | Total |
|---|---|---|---|
| | Known age | Minimum age only known | |
| 3 | 1 | | 1 |
| 4 | 7 | | 7 |
| 5 | 11 | | 11 |
| 6 | 11 | | 11 |
| 7 | 8 | | 8 |
| 8 | 3 | | 3 |
| 9 | 4 | | 4 |
| 10 | 1 | | 1 |
| 11 | 4 | | 4 |
| 12 | 3 | 1 | 4 |
| 13 | 8 | 4 | 12 |
| 14 | 3 | 1 | 4 |
| 15 | 1 | | 1 |
| 17 | 1 | | 1 |
| 18 | 1 | 1 | 2 |
| Total | 67 | 7 | 74 |

**Table 4.12** Sample sizes at each female age for which brood sex ratio was recorded in 2001 or 2002. This dataset contains only broods of three in which all the chicks (and all eggs laid) were sexed. Each female appears only once. There were no 16-year-olds in this dataset, so that age is not included in the table.

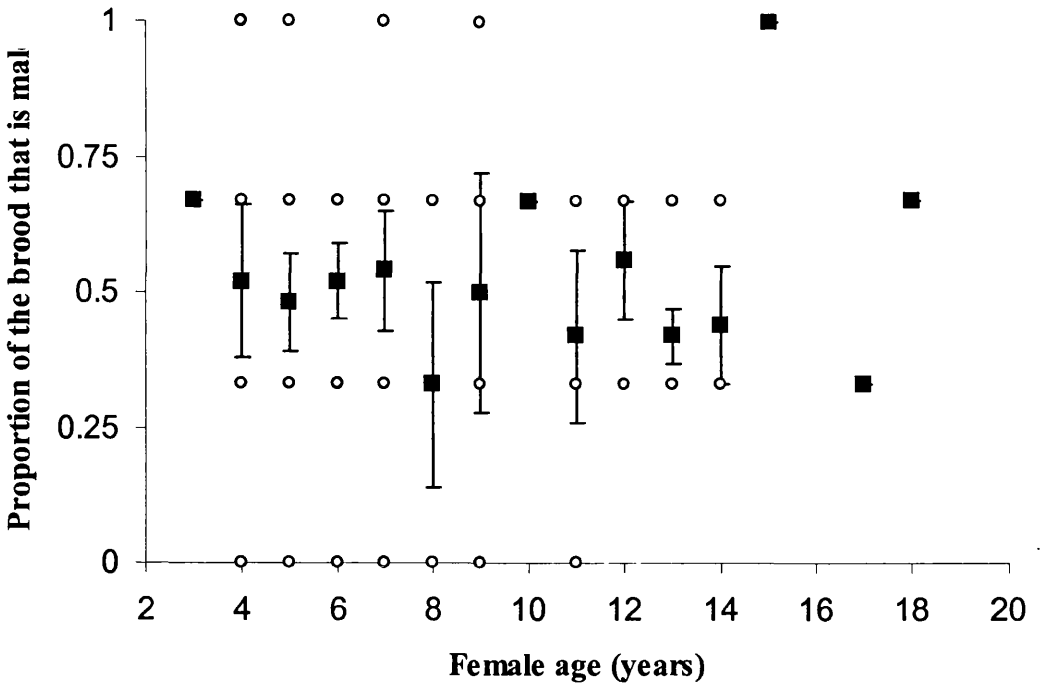



**Figure 4.20** The proportion of broods of three made up of male chicks, in relation to maternal age. The open circles show each brood and the squares the mean proportion ± 1 S.E. at each female age (sample sizes shown in table 4.12).


**Figure 4.21** The mean proportion of broods of three made up of male chicks did not differ between middle-aged (5-9 year old) and old (13+ year old) females.

## Do the physical attributes of adult shags change with age?


### ABSTRACT

In mammals, a suite of senescence-related changes occur in old individuals. These include sometimes obvious changes to external features, such as the grey hair and wrinkles commonly recognised as a sign of ageing in humans. Alterations also occur internally, for instance to the immune system. In adult birds, the outward signs of ageing are generally lacking, but the extent of more subtle senescent change has rarely been investigated. Therefore, the physical traits body size, mass and condition were measured in breeding adult shags of known age, as well as blood parameters and cell-mediated immune response. Despite good sized samples of old birds, there was no relationship between these variables and age, with the exception of a decline in number and increase in size of erythrocytes among the oldest birds. Inter-relationships among these physical attributes were also explored, and they were tested in relation to the reproductive performance of females, to assess their impact on fitness, and whether they are condition-dependent. Implications of the absence of clear senescence-related change in adult shags is discussed, with consideration given to potential sources of bias.

## INTRODUCTION

Senescence can be defined as age-related physiological changes that adversely affect an organism's fitness and reproduction, and increase its chance of mortality (Finch 1990, Rose 1991). The signs of ageing, at least in mammals, are distinct changes in physical appearance and physiological measures. For example, in humans stature and weight decrease from age 40 or 60 years respectively (Arking, 1998). There are also noticeable changes to the skin, associated with changes in collagen structure (Kligman, Grove and Balin, 1985), declining synthesis of bone and loss of bone mineral content, and age-related atrophy of the muscle fibres (Arking, 1998). Telomere loss in some cell types may limit proliferative potential, and lead to an accumulation of senescent cells that could contribute to age-related declines in tissue function (Campisi 1996, chapter 6). Senescence-related changes such as these have however rarely been studied in birds, despite lower survival of old individuals (e.g. lower survival of shags aged over 13 years, Harris *et al.* 1994*b*). This is illustrated by the fact that, while there are a large number of techniques available for determining age in mammals based on physical measures such as tooth wear, there is no reliable means of determining age in adult birds (although counting bone layers has been suggested as a potential method to age dead birds of some species, Klomp and Furness 1992). For this reason, long-term ringing programmes are still relied upon to provide known age study populations of birds.

A number of theories have been proposed to explain the processes of ageing on either a proximate, mechanistic (e.g. molecular changes, mutation accumulation, review Comfort 1979) or ultimate, evolutionary level (Kirkwood and Austad 2000, see chapter 1). Of the former type are "wear and tear" theories (Weismann, 1891), which suggest that ageing results from a gradual wearing down of the somatic tissues as a result of stochastic or systemic changes over time and with use. This idea is contradicted by the fact that organisms raised in protected environments still age, but is consistent with recent evidence showing trade-offs between longevity and costly activities such as reproduction (e.g. *Drosophila*: Fowler and Partridge 1989, humans: Westendorp and Kirkwood 1998, Doblhammer and Oeppen 2003, red-billed choughs, *Pyrrhocorax pyrrhocorax*: Reid *et al.* 2003). There is growing support for oxidative stress as an important mechanism of "wear and tear" (review Finkel and Holbrook, 2000). In evolutionary terms, ageing processes can be explained by the

declining force of selection with age, due to extrinsic mortality reducing the proportion of the population at risk (Charlesworth, 1980). In addition, antagonistic pleiotropy can act: genes that have a beneficial effect early in life, and so on fitness, may persist even if they are disadvantageous for the individual in old age (Williams, 1957). There will be a balance struck between benefits early in life and the costs in old age of such genes. A third, though non-exclusive, evolutionary hypothesis for ageing is the "disposable soma" theory, which concentrates on how resources are allocated between somatic maintenance and reproduction (Kirkwood, 1977). On the basis of these theories, the lack of obvious ageing effects in adult birds, in contrast to mammals, is extremely interesting especially given the unusual longevity of birds for their body size (Lindstedt and Calder 1976, Holmes and Austad 1995*a*, *b*, Holmes, Flückiger and Austad 2001). However, it may be that ageing processes simply have not yet been sufficiently investigated in class *Aves*, or that the relevant measures have not been taken to identify ageing-related deterioration. Evidence of senescence in survival and reproduction has been accumulating in wild animal populations, including birds, since this became the focus of an increasing number of studies.

In the light of these considerations, a number of physical measures were taken from breeding adult shags. Limitations to when adult shags can be easily caught, without risk to their breeding success such as through egg breakage, meant that the sample of adults measured was restricted to only individuals that bred, and were rearing chicks of medium to large size. Unfortunately, this may have biased the sample towards birds in good condition. It must also be kept in mind that, as well as changes within individuals with age, phenotypic distributions may differ among age groups of the population. For instance, this could arise for physical traits that affect survival, and may be relevant to some measurements made in this study, like immune response. In the following section, I set out the predictions, with respect to age and particularly for old birds, for each measure that was taken.

## *What are the predictions for age-related change in physical attributes?*

### *1. Body size*

Body size in birds generally is fixed before first reproduction (O'Connor, 1984), so in breeding adults it is not expected to show age-related change (see chapter 3

introduction). Although changes in size are documented in old humans as a consequence of skeletal or muscular changes, these are perhaps less likely to be observed in wild animals, where individuals are not protected against mortality risk. A more likely scenario is that body size might be an indicator of individual quality, or may be related to varying costs of living or reproducing, such that there is a correlation with survival in the wild. Hence, the phenotypic distribution of body size could differ among age groups. Blanckenhorn (2000) reviewed the potential costs of large size, which include the greater food requirements of large individuals, which can increase mortality under resource limitation. Costs of juvenile growth, and particularly of fast growth (review Metcalfe and Monaghan, 2003), may be additional costs to attaining a large adult body size.

Body size may covary with other selected traits, leading to an uneven distribution with age. For instance, in moorhens where females preferentially mate with males that have large fat reserves (Petrie, 1983), fat males also tend to be small. This may be due to energetic constraints on birds of large body size. Social status, dominance and behaviour may also relate to body size in some species. This may explain the larger body size and differing morphology of older American coots (*Fulica americana*, Alisauskas 1987), which show aggressive territorial behaviour. If different reproductive strategies are adopted by individuals of different size, for instance if age at the first breeding or the level of investment differs, then subsequent lifespan could also be affected, given a trade-off between reproduction and longevity. Natural selection for body size has been documented in Darwin's finches *Geospizinae* (Boag and Grant 1981, Price and Grant, 1984). In this case, the cause of the directional selection was also identified, with environmental conditions affecting the size of seeds available as food, and consequently selection on the bill and body size of finches. This example illustrates how environmental variation among years can influence the phenotypes occurring in different cohorts of a population. Environmental conditions could also differ among individuals, possibly in relation to parental quality. In barnacle geese (*Branta leucopsis*), environmental conditions in the first weeks of life affect not only the growth rate but also the final body size in adulthood (Larsson and Forslund, 1991). In this species, there are differences among cohorts in relation to annual variation in environmental conditions, differences among birds that grew up on different sites, and among individuals born at different

times in the season. Annual differences are however most likely to show up as age-related variation at the population level, while individual variation will be expressed across all age classes.

### 2. Mass and condition

Unlike body size, mass represents the levels of stored reserves in birds and can vary throughout adult life, on a number of temporal scales, including changes during the breeding cycle or among years. As structural body size contributes to mass, measures of body condition are frequently used in which size differences have been taken into account. Lower survival is often associated with lower body condition, and senescent changes may be involved in this relationship. Thus, declining body mass or condition might be expected in old birds (see chapter 3 introduction). The points introduced in the previous section on body size, concerning covariation of traits and differences among cohorts of the population related to changing environmental conditions over time, may also apply to mass or condition.

### 3. Blood parameters

Blood parameters provide an indirect indication of condition, as the system responsible for the mobilisation and transport of nutrients. In some cases blood composition correlates strongly with health and condition (e.g. Gavett and Wakeley, 1986). Both the cellular and plasma portions of the blood can be measured (review Brown, 1996), but in this investigation I concentrate on red blood cells. Haematocrit, the percentage of the total blood volume occupied by erythrocytes, represents the oxygen carrying capacity of the blood and has traditionally been used to assess health. Anaemias, which may be the result of low blood cell production during nutritional stress, destruction of red blood cells by haemolytic disease, blood parasitism, dehydration, toxins, or blood loss, are reflected in low haematocrit values. Thus, diseased animals or those in poor condition are known to have reduced haematocrits, although there is some controversy concerning the ability of this measure to discriminate among clinically normal birds (Dawson and Bortolotti, 1997). An alternative blood measure is mean corpuscular volume (MCV), which is a measure of the average size of red blood cells in the sample. This has been suggested as a better condition index than haematocrit because it involves direct

counts of the number of red blood cells, and so takes into account cell density. A relationship between MCV and hatching date in male great skuas (*Catharacta skua*), and with the number of fledglings produced in both sexes, suggested this measure may be a reliable condition index (Bearhop *et al.* 1999).

In humans, red cell parameters, including red blood cell count, haematocrit and MCV, tend to decline in old age (Inelmen *et al.* 1994, Suwannuruks *et al.* 1997), a pattern confirmed by the longitudinal study of individuals aged 60 years or more (Ohhara *et al.* 1994). The erythrocytes of 70-90 year olds are larger and less dense than in 20-40 year olds (Danon, Bologna and Gavendo, 1992). These studies have also highlighted that different outcomes may be obtained from longitudinal versus cross-sectional investigation of blood parameters; in one case although MCV declined in individuals with age, cross-sectional comparisons revealed the opposite trend. This suggests a selective process favouring individuals with high values of MCV may operate, as well as ageing-related declines occurring on an individual level. A reduced ability to recover from haematological stress has also been documented in elderly humans (Globerson 1999, 2001).

In relation to old birds, the predictions for changes in blood parameters are not entirely clear. As has been discussed elsewhere (chapters 1, 3, 4), there are conflicting hypotheses ("constraint" vs. "restraint") concerning the ability of old individuals to reproduce, and the levels of reproductive effort expected towards the end of life. If old shags are constrained, this may well be reflected in lower blood values. On the other hand, greater effort by old individuals, even if this is not evident in reproductive output, may correspond to high values for these blood parameters because the birds are working harder. In addition, there are many variables that can potentially confound blood measures, such as physiological changes due to handling stress at sampling. The importance of these variables is considered further in the discussion.

### *4. Immunity*

The immune system fulfils a crucial self-maintenance function, guarding against infection by pathogens and parasites. There has been recent interest in immunocompetence in birds as a potential fitness-related trait that may trade-off with other aspects of the life-history, such as reproductive effort (Folstad and Karter 1992,

Norris and Evans 2000). The incidence and level of parasitic infection is often higher among reproducing individuals, and with increased reproductive investment (Festa-Bianchet 1989, Norris, Anwar and Read 1994, Richner, Christe and Oppliger 1995, Sheldon and Verhulst 1996). It has been shown experimentally in zebra finches (*Taeniopygia guttata*) that reduced immunocompetence relates to the increased activity levels that accompany reproduction (Deerenberg *et al.* 1997). Given that mounting an immune response is costly or competes for finite resources (reviews Råberg *et al.* 1998, Norris and Evans 2000), ageing-related deterioration might be expected to impact on this attribute.

Ageing-related changes in immune response are reflected in humans as a high incidence of severe infections, a poor protective effect of vaccination, and increasing autoimmune activity in the elderly (Grubeck-Loebenstein and Wick, 2002). Autopsy data has shown that the majority of deaths in the over-80s result from infections (Horiuchi and Wilmoth, 1997). Among the alterations to the immune system that occur with human ageing (review Malaguarnera *et al.* 2001) are a reduction in the proliferative capacity of T-lymphocytes, and in the response of B-lymphocytes to antigens. Some immune changes are likely to be secondary to other age-associated modifications (review Effros, 2001), such as changes in the levels of sex hormones. In old birds senescence might be expected to constrain the mounting or extent of an immune response. However, there are very few studies of immunocompetence in wild birds with respect to senescence. Apanius and Nisbet (2003) measured serum immunoglobulin G (IgG) levels in old common terns (*Sterna hirundo*), but found no relationship between this measure of immunity and age. Their finding is in contrast to the pattern in mammals, which typically show elevated IgG levels at old age as a consequence of B-cell defects and declining regulation by T-cell mediated cytokines (Grubeck-Loebenstein and Wick, 2002). In contrast, an age-related deterioration in humoral immune function was seen among breeding females in a wild population of collared flycatchers *Ficedula albicollis* (Cichon, Sendecka and Gustafsson 2003). Lozano and Lank (2003) also reported immunosenescence in both sexes of the ruff (*Philomachus pugnax*), with lower cell-mediated immunity in older birds.

Increased longevity of mice artificially selected for high antibody responses (Salazar *et al.* 1995) highlights the importance of the immune system for survival. It also points out that old members of a population may have survived so long because

they possess particularly healthy or efficient immune systems. Thus, selection based on immunocompetence would predict a greater response of old birds to an immune challenge, in comparison to younger individuals in the population, the opposite prediction to that based on senescence-related declines.

In ecological studies of birds, two main techniques have been used to measure immunocompetence (reviewed in Norris and Evans, 2000). These are monitoring techniques in which immunity-associated cells or proteins are assayed to provide a measure of an individual's current health and immune status at sampling, and challenge techniques in which an individual's response to a novel immune challenge is tested. Challenge techniques routinely used in birds are tests of the humoral immune system (e.g. antibody production in response to sheep red blood cells), or cell-mediated immunity (e.g. mitogenic response to phytohaemagluttinin (PHA)). These are both tests of acquired immunity. In the present study, cell-mediated immune response to PHA was measured in adult shags of different age, in order to test the hypothesis that old breeding birds show declining immune responsiveness.

Developed as a protocol in poultry science (Goto *et al.* 1978), the PHA test has now been applied in numerous ecological studies (e.g. Hõrak *et al.* 1999, Johnsen *et al.* 2000, Tella *et al.* 2000, 2001). It provides a measure of the proliferative response potential of circulating T lymphocytes, and is usually applied at the wing or foot web, depending on the species. The test involves challenging one web with the immunostimulant PHA and injecting the other web with a control substance that does not elicit an immune response. Smits, Bortolotti and Tella (1999) provide a recent overview of this technique. They have also suggested modifying the method to eliminate the "control" injection (but see Siva-Jothy and Ryder 2001, Smits, Bortolotti and Tella 2001).

### *Inter-relationships among physical traits, and with reproductive performance*

Physical or physiological traits may be correlated with one another, particularly if these traits reflect inherent differences in the quality of individuals or their current condition. On that assumption, an old individual displaying a low value for one aspect of physical health would also show low values of the other variables measured. However, this is a simplistic view, as there may be antagonistic relationships between some physical traits. This possibility has been discussed

recently in relation to the different components of the immune system (Norris and Evans, 2000). Resource limitation could also lead to trade-offs between factors such as immunocompetence and growth, possibly affecting final body size (but see Hõrak *et al.* 2000). Current condition may be a consequence of previous investment in reproduction. Thus, potential inter-relationships among the physical attributes of breeding adults, and with reproductive performance, are interesting and are also considered here.

# METHODS

## Collection of data in the field, and analysis in relation to age

### 1. Size and mass

Towards the end of the season in 2001, 2002 and 2003, when all birds were on medium to large chicks, adults were caught at the nest. Birds were weighed using a 2.5 kg (to the nearest 20g) or 5kg (to the nearest 25g) Pesola spring balance. Head and bill length was measured to the nearest millimetre using a 22cm measure constructed for this purpose, wing length to the nearest mm with a standard wing rule, and tarsus length with callipers (to 0.1 mm).

The product of the three linear size measures ($\times 10^{-6}$) was used as an overall score of structural body size. The product of the linear measurements correlates with their PCA score in both sexes (males: $r = 0.66$, $n = 37$, $p < 0.001$, females: $r = 0.99$, $n = 82$, $p < 0.001$).

For 33 individuals that were measured in two years and three individuals measured in all three years, cases were removed at random so that each individual appeared only once in the data before cross-sectional comparisons were made. The number of different individuals measured was 119, of which 106 were of exact known age (33 males, 73 females). The sample size differs between the sexes because females were preferentially caught in 2002, as a means of maximising the sample of that sex. The age range of known age birds was 5 to 19 years for males and 2 to 20 years for females. General linear models (GLM) were used to test for differences in size or mass among birds of different age (covariate), with the factors sex (fixed) and year (random) included.

Changes in mass within individuals were tested when measures had been taken in more than one year. As there were only three birds measured in three years, two years' measurements were selected to maximise the age difference for those cases, and they were then analysed with the individuals that had been measured twice, using paired $t$ tests. The total number of individuals in this longitudinal comparison was 36 (14 males, 22 females). However, as some individuals were measured in 2001 and 2002 ($n = 5$), some in 2002 and 2003 ($n = 11$) and some in 2001 and 2003 ($n = 20$), these three categories were analysed separately to allow for differences in

environmental conditions among years, and with respect to the length of time between measures. For each category, the change in mass within individuals was plotted in relation to their age when first measured, in order to see whether the pattern of mass change varied with age.

### 2. Blood parameters

Blood parameters tested for their relationship to age were a mean count of red blood cells (mRBC, cells per $mm^3 \times 10^{-6}$), mean haematocrit (%) and mean corpuscular volume (MCV, $\mu m^3$). All three measures were made from a single blood sample per individual, taken at the same point as size and mass measurements. Blood was taken (under Home Office licence) from the tarsus using 23G needles and drawn into EDTA-coated syringes to prevent clotting. Between 0.5ml and 1ml of blood was generally taken from adult shags. Blood parameters were measured in the laboratory, within three hours of collection of each blood sample. To measure haematocrit, blood was drawn into two fine capillaries, which were then sealed at one end. Placing the sealed end at the outer rim of a rotor, the capillaries were spun in a centrifuge (10-RPM for 8 minutes), so separating blood within the capillary into a clear plasma portion and a red cell portion. Callipers were used to measure the length of the capillary containing the red blood cells, and the total length of plasma plus cells. Haematocrit is the percentage of the total filled capillary length made up by the red blood cell portion. The mean haematocrit of two capillaries per sample was used. MCV is the mean size of red blood cells in the sample, calculated (after Campbell 1988, Bearhop *et al.* 1999) by: MCV ($\mu m^3$) = [haematocrit (%)/ mean erythrocyte count ($10^6/\mu l$)] $\times$ 10. Four erythrocyte counts were made for each sample, to give the mean for this calculation. Erythrocyte counts involved diluting the blood 1 in 200 with Natt and Herrick's staining solution (Campbell, 1988) in a diluting pipette. The pipette was shaken to mix and approximately a quarter of the volume then discarded. An ordinary Neubauer counting chamber was filled from the mixture remaining in the pipette, and the red blood cells viewed and counted under $\times$10 magnification (further details in Baker *et al.* 1966).

As the peak of the MCV distribution was skewed to the left, this variable was base-10 log transformed. Mean haematocrit was arcsine transformed before analysis. MCV correlates with mean haematocrit ($r$ = 0.33, $n$ = 99, $p$ = 0.001), and more

strongly with the red blood cell count ($r$ = -0.85, $n$ = 99, $p < 0.001$). However, there is no significant correlation between the red blood cell count and mean haematocrit ($r = 0.18$, $n = 99$, $p = 0.081$).

These measures were collected in both 2001 and 2002. The total number of different individuals measured was 100 (22 males, 78 females), although in one case there was insufficient blood to carry out all the measurements, so only haematocrit was recorded. A total of six individuals were measured in both years. Cross-sectional comparisons of blood parameters were made after randomly selecting one case per individual from those with measures taken in both years. In that data, the sample of known age individuals was 90 (20 males, 70 females), and the overall age range was 5 to 20 years. Paired $t$ tests were used to compare measurements of the same individuals in each year, and the change in blood measures within birds were plotted in relation to their age when first measured.

### 3. Cell-mediated immune response

In 2001 and 2002, inflammatory response to the antigen phytohaemagglutinin (PHA) was measured in the foot web of adults 24 ± 2 hours after injection, with respect to a negative control, injection of phosphate-buffered saline solution (PBS). PBS does not stimulate an immune response. PHA scores calculated using PBS injection as a negative control were compared to scores when PBS measurements were not taken into account (after Smits, Bortolotti and Tella, 1999).

The precise procedure was as follows. After mass and size measurements, and a blood sample, had been taken from an adult shag, the thickness of the innermost foot web on both feet was measured to the nearest 0.05mm using a spessimeter (K50 feeler gauge with C type feelers, Coventry Gauges Ltd, Poole, U.K.). Measurements were taken three times on each web and the mean of these values used. The measurement site on both webs was marked with a permanent marker. After swabbing the area with alcohol, 0.05ml of PBS was injected slowly into one foot web at the centre of the marked area using a sterile microfine needle (29G). In the same way, 0.05ml of a 5mg $ml^{-1}$ solution of PHA (Sigma Aldrich Co. Ltd., Dorset, U.K.) in PBS was injected into the foot web of the other foot (method and PHA concentration after Saino *et al.* 1997, Hõrak *et al.* 2000, procedure under Home Office licence). This dose of PHA (250µg) and injection volume (50µl) is in line

with applications of this test in other species (e.g. Smits, Bortolotti and Tella, 1999), in relation to the size of shags and the thickness of their foot web. Following injection, the bird was released. Upon recapture after 24 (±2) hours, the thicknesses of both foot webs were measured again. I took all measurements of foot web thickness. The increase in thickness of the PHA injected web measures cell-mediated inflammatory response.

The main challenge in applying the PHA test is taking accurate measurements of web thickness, and this is thought to be the most error-prone part of the test (Smits, Bortolotti and Tella, 1999). Thus, to assess measurement reliability, the repeatability of the three thickness measures taken on each PHA injected web, post-injection, was calculated (after Becker 1984, Lessells and Boag 1987). The changes in thickness of the foot webs injected with PBS were also examined, to confirm that there was no response to the PBS solution in shags.

A total of 63 different birds were tested, with two individuals measured in both years. Of these birds, 55 were of exact known age (10 male, 45 female). PHA response among birds of different age was tested in a GLM with sex and year (age as a covariate, sex a fixed factor, and year a random factor).

### *Investigating the relationships between these physical traits*

In addition to testing whether size, mass, blood measures or cell-mediated immunity change with age among adult shags, I investigated whether these physical traits are related to one another. In GLM analyses, each blood parameter was tested for effects of sex and either structural size, mass, or mass divided by size, plus interactions. Correlations were used to test for relationships between PHA response and size, mass and blood measures.

### *Are the physical characteristics of adults related to their reproductive performance?*

Laying date, the number of hatchlings, and the number of fledglings produced by females were compared in relation to female size, mass, blood parameters and cell-mediated immunity. Correlation tests were used to compare laying date to characteristics of the female parent, while differences in the physical attributes of females that hatched or fledged different numbers of chicks were assessed by

oneway ANOVAs. Only 2001 data was used in these analyses. 2002 data were excluded because a clutch exchange experiment carried out that year may have biased the sample of females (females laying clutches of three were selected and matched for laying date), and meant that females did not raise their own offspring. Laying dates and reproductive success were not monitored in 2003. The total number of females measured in 2001, for which reproductive performance was also recorded, was 25. The physical attributes of females in relation to clutch size and volume is examined in chapter 3. All means are shown ± 1 S.E.

# RESULTS

## Physical characteristics in relation to age

Figure 5.1 shows each of the physical traits that were measured in adults, plotted in relation to age for each sex.

### 1. Structural size

Variation in structural size among birds was small (mean C.V. in the three linear size measures, treating the sexes separately, ranged from 1.6 to 2.7 %). There was no effect of age on body size in a GLM including sex and year (age: $F_{1,101} = 0.96$, $p = 0.33$, sex: $F_{1,102} = 104.12$, $p < 0.001$, year: $F_{2,102} = 4.15$, $p = 0.018$, all interactions $p > 0.05$). Adult males are larger than females in all three measures of size (head and bill length: $t_{117} = 4.09$, $p < 0.001$, wing length: $t_{117} = 8.47$, $p < 0.001$, tarsus length: $t_{117} = 7.90$, $p < 0.001$). Differences among years, though significant, were not great (figure 5.2).

### 2. Mass

There was greater variation among individuals in mass than in structural size (mean C.V. mass for all three years in males = 5.5%, in females = 6.0%,). However, age did not explain a significant portion of this variation, when tested with sex and year (age: $F_{1,101} = 0.19$, $p = 0.67$, year: $F_{2,102} = 0.81$, $p = 0.45$, sex : $F_{1,104} = 122.9$, $p < 0.001$, all interactions $p > 0.05$). Age was not related to body condition (mass divided by body size), when year was taken into account (age: $F_{1,102} = 0.15$, $p = 0.70$, sex : $F_{1,101} = 0.03$, $p = 0.86$, year: $F_{2,103} = 5.41$, $p = 0.006$, all interactions $p > 0.05$). Neither the logistic nor quadratic relationship between body condition and age were significant (regressions, logistic: $F_{1,104} = 1.32$, $p = 0.25$ quadratic: $F_{2,103} = 0.65$, $p = 0.52$). The mass of individual birds did not change significantly between 2002 and 2003 (paired $t$ tests: $t_{10} = 0.65$, $p = 0.53$), or between 2001 and 2003 ($t_{19} = 0.14$, $p = 0.89$). In contrast, for birds measured in both 2001 and 2002, mass increased significantly between years (2001 1632 ± 36 g, 2002 1685 ± 36 g, $t_4 = 3.34$, $p = 0.029$). Figure 5.3 shows the change in mass within individuals for each of these pairs of years, plotted in relation to their age when first weighed.

### 3. Blood parameters

When age was not taken into account, there was no significant difference in any of the blood parameters between the sexes (mRBC: males 1.66 ± 0.06 × $10^{-6}$ cells per $mm^3$, females 1.55 ± 0.04 × $10^{-6}$ cells per $mm^3$ , $t_{97}$ = 1.33, $p$ = 0.19, mean haematocrit: males 46 ± 0.006 %, females 44 ± 0.004 %, $t_{98}$ = 1.63, $p$ = 0.11, MCV: males 282 ± 1 $\mu m^3$, females 288 ± 1 $\mu m^3$ , $t_{97}$ = 0.42, $p$ = 0.67), or between years (mRBC: 2001 1.57 ± 0.06 × $10^{-6}$ cells per $mm^3$ , 2002 1.58 ± 0.04 × $10^{-6}$ cells per $mm^3$ , $t_{97}$ = 0.03, $p$ = 0.97, mean haematocrit: 2001 43 ± 0.010 %, 2002 45 ± 0.004 %, $t_{98}$ = 1.88, $p$ = 0.063, MCV: 2001 282 ± 1 $\mu m^3$, 2002 295 ± 1 $\mu m^3$, $t_{97}$ = 0.65, $p$ = 0.52). In order to control for age, and test for interactions, these factors were also included with the covariate age in a GLM on each blood parameter. In most cases, age was not significant ($p$ > 0.05). However, there was a significant interaction between age and sex in MCV (age: $F_{1,85}$ = 2.23, $p$ = 0.14, sex: $F_{1,85}$ = 5.78, $p$ = 0.018, age × sex: $F_{1,85}$ = 5.38, $p$ = 0.023). Testing males and females separately for MCV, this interaction arises because there is a significant positive relationship between MCV and age in males ($F_{1,18}$ = 6.04, $p$ = 0.024), but no relationship in females ($F_{1,67}$ = 0.88, $p$ = 0.35). In the GLM for mean haematocrit, sex and year were both significant (sex: $F_{1,87}$ = 7.91, $p$ = 0.006, year: $F_{1,87}$ = 9.69, $p$ = 0.003), although they explained less than 10% of the variation (*adj.* $R^2$ = 0.093). Males had higher haematocrit values than females (46% compared to 44%), and mean haematocrit was also higher in 2002 (46%) than 2001 (44%). In plots of the blood parameters against age, only mRBC showed any indication of decline in the oldest individuals (figure 5.1*d*). Although the quadratic regression was not significant ($F_{2,86}$ = 1.86, $p$ = 0.16), there was a significant negative correlation between mRBC and age in birds aged 15 years or older ($r$ = -0.51, $n$ = 20, $p$ = 0.021, figure 5.4). This decline was of approximately 37 × $10^{-6}$ cells per $mm^3$ between the ages of 15 and 20 years. In a longitudinal analysis of the six individuals measured in both 2001 and 2002, there was no significant difference in their blood parameters between years (paired $t$ tests, mRBC: $t_5$ = 1.69, $p$ = 0.15, mean haematocrit: $t_5$ = 0.030, $p$ = 0.98, MCV : $t_5$ = 1.41, $p$ = 0.22) and no pattern of change in relation to age (figure 5.5)

### 4. Cell-mediated immune response

PHA scores calculated by subtracting the change in foot web thickness of the PBS-injected foot from the thickness change induced by PHA were highly correlated with scores that did not take PBS measurements into account ($r = 0.86$, $n = 63$, $p < 0.001$). Moreover, the coefficient of variation was similar for both methods of calculating PHA score (*C.V.* using PBS change as a negative control = 26%, without using PBS measurements = 25%). Thus, in the following analyses PHA scores calculated by subtracting the PBS foot web thickness change have been used. By both methods of calculation, the coefficient of variation was fairly high, indicating that individuals differ in their level of response.

Overall, average inflammation due to PHA was 1.00 ± 0.03 mm. The mean change in thickness of the control, PBS-injected, web was negligible (0.02 ± 0.02 mm, maximum change recorded of 0.45 mm). The repeatability of thickness measures of each PHA-injected web, taken post-injection, was high ($r$ (95% *C.I.*) = 0.87 (0.81, 0.92), $F_{62,126} = 21.46$, $p < 0.001$), indicating low measurement error (8-19%). PHA scores did not correlate with the time between antigen injection and post-injection measurement of the foot web thickness in either sex (males: $r = 0.17$, $n = 11$, $p = 0.63$, females: $r = -0.006$, $n = 52$, $p = 0.97$).

There was no overall difference between the sexes (males: 0.87 ± 0.06 mm, females: 1.03 ± 0.04 mm, $t_{61} = 1.91$, $p = 0.061$), or between years (2001: 0.93 ± 0.06 mm, 2002: 1.04 ± 0.04 mm, $t_{61} = 1.52$, $p = 0.13$) in the amount of inflammation of the foot web caused by PHA injection. This was also the case when age was controlled for in a GLM of sex, year and age ($p > 0.05$). There was no relationship between age and PHA response ($F_{1,53} = 1.46$, $p = 0.23$). Too few birds (2) were measured in both years to test for differences in PHA response between years or in relation to exposure to the antigen. Both the birds that were measured twice were old (over 16 years in 2001) females. One of these females showed very similar responses to the antigen in both years (0.88 mm inflammation in 2001, 0.87 mm inflammation in 2002), while the second bird showed a greater response in 2002 (0.77 mm inflammation in 2001, 1.10 mm inflammation in 2002).

## Inter-relationships of physical traits

### 1. Are blood parameters related to body size or mass?

There was no significant effect of structural size, mass or body condition on any of the three blood measures, when tested in a GLM with sex. Sex, and the interaction terms for each size or mass measure with sex were also not significant (in all cases $p > 0.05$).

### 2. Is PHA response related to body size or mass?

The amount of PHA-induced inflammation did not correlate with structural body size ($r = 0.07$, $n = 63$, $p = 0.58$), with mass ($r = 0.07$, $n = 63$, $p = 0.60$), or with body condition ($r = 0.17$, $n = 63$, $p = 0.18$) in adult birds. There was also no indication of a logistic or quadratic relationship between these variables (for both types of regression $p > 0.05$).

### 3. Is PHA response related to blood parameters?

The PHA score was not related to mean red blood cell count ($r = 0.03$, $n = 62$, $p = 0.83$), mean haematocrit ($r = 0.14$, $n = 62$, $p = 0.28$) or MCV ($r = 0.04$, $n = 62$, $p = 0.75$).

## Do physical characteristics of adults relate to their reproductive performance?

### 1. Laying date

Laying date did not correlate with female body size ($r = 0.27$, $n = 21$, $p = 0.24$), mass ($r = 0.39$, $n = 22$, $p = 0.07$), or body condition ($r = 0.12$, $n = 22$, $p = 0.61$) in 2001. As previous analyses have shown a relationship between laying date and age, female mass was also tested for an interaction with age in a GLM. Neither age, mass nor the interaction term were significant in 2001 ($n = 21$, $p > 0.05$). None of the three blood parameters measured in females correlated with laying date (laying date and mRBC: $r = 0.38$, $n = 20$, $p = 0.10$, laying date and mean haematocrit: $r = 0.16$, $n = 21$, $p = 0.49$, laying date and MCV: $r = 0.34$, $n = 20$, $p = 0.14$). There was also no correlation between laying date and response to PHA in females ($r = 0.12$, $n = 9$, $p = 0.77$).

### 2. Number of chicks produced

There was no significant difference in any of the physical attributes among females that produced 1, 2 or 3 chicks at hatching or 0, 1, 2 or 3 chicks at fledging (in all cases $p > 0.05$). There was also no relationship between these female characteristics and a measure of rearing ability, the number of chicks fledged/ number of chicks at hatching, after cases where no chicks hatched were excluded ($p > 0.05$ for all adult attributes).

## DISCUSSION

There is no clear relationship between age in adult shags and their body size, mass, body condition, haematocrit, or cell-mediated immune response. A positive correlation between mean corpuscular volume and age was observed in males, but not in females. Differences between the sexes in blood parameters have been previously reported in humans (e.g. Ohhara *et al.* 1994), but the precise reasons for these remain unclear. In both sexes, erythrocyte counts declined with age among birds aged 15 years or older. This is approximately the age at which survival falls significantly in these birds (at 13 years of age, Harris *et al.* 1994*b*). This finding also parallels the declining red blood cell counts of old humans (see introduction). There were no significant relationships among these potential condition-related traits, and neither did they relate to the date on which females laid their eggs, or the number of chicks produced at hatching or fledging. This calls into question their validity as measures of individual state. These results are discussed in relation to the relevance of these measures in breeding adult shags, particularly with regard to senescence. Possible confounding variables and sources of bias that may mask the detection of age-related changes are also identified.

There was very little variation in body size among adult shags, once the difference between the sexes in size (males being approximately 14% larger) was taken into account. This may partly be due to the biased sample that was measured: all shags that were caught had returned to the colony, had bred, and had raised chicks to medium or large size that year. Thus, this sample comprised individuals at the high end of the quality and condition spectrum, which may reduce the chances of detecting variation in condition-related traits. In addition, the productivity of shags on the Isle of May in the years 2001, 2002 and 2003 was high (Wilson, Wanless and Harris 2002, Wilson *et al.* 2002, 2003), suggesting environmental conditions in those years were favourable. This could also hinder the detection of age-specific costs (e.g. Tinbergen, van Balen and van Eck, 1985). The size of adults did not vary with age. Although a significant difference in body size among years was found, this difference was extremely small and is likely to reflect differences in measurement error among years.

Breeding adults showed greater variation in mass than in structural size, but again this variation was not explained by age. There was some indication that body

condition differed among years, which might relate to differences in food supply, either over-winter or at the breeding colony. While individuals showed a significant difference in their mass between 2001 and 2002, all increased in mass between these years, and the magnitude of the mass change did not relate to their age.

There was no overall difference between the sexes in their blood parameters, and no overall differences among years, when age was not taken into account. However, a GLM including age, sex and year revealed a significant interaction between sex and age in mean corpuscular volume, with only males showing a positive correlation of this measure to age. It is somewhat unclear what the best interpretation of this result is, as there are uncertainties concerning what constitutes optimal blood cell size, and the temporal scale to which blood values relate. In addition, as these results come from comparisons among individuals of different age (cross-sectional analysis), they may reflect population level processes of differential mortality, rather than alterations with age in individuals.

Higher values of MCV equate to larger red blood cells on average, which can result from regenerative anaemias or previously depressed erythropoiesis. Bird species with higher metabolic rates tend to have smaller erythrocytes (Hartman and Lessler, 1963), though whether this pattern also occurs intra-specifically with regard to metabolic efficiency is unknown. Erythrocyte size could be an indicator of proximate condition or inherent differences among individuals (Bearhop *et al.* 1999). Variation in MCV might relate to the time in the season at which individuals breed. However, as younger shags tend to breed later than older, more experienced birds, and there are advantages to breeding early (Coulson, Potts and Horobin 1969, Potts, Coulson and Deans 1980, Aebischer 1993, Harris *et al.* 1994*b*, Aebischer, Potts and Coulson 1995, Daunt *et al.* 1999), the prediction based on laying date in this case (a negative correlation) is in the opposite direction to the pattern observed. In addition, no relationship between the laying dates of females and their blood parameters were seen. Another interpretation is that a decline in red blood cell production occurs with age, towards fewer, larger cells, as in humans. This is supported by the negative relationship between erythrocyte count and age in shags of 15 years or more, although this does not explain the difference between the sexes in MCV with age. Sex differences in blood parameters could relate to differences in other aspects of physiology, including metabolism in relation to size (male shags are the larger sex)

or hormonal differences. The change in red blood cell count was not detected in a longitudinal comparison, but this was only across one year. It is likely that age-related changes to blood values in individuals occur gradually, over at least several years. There was no difference in haematocrit with age in either sex, so the proportion of the blood containing red cells does not differ. Differences in haematocrit among years and between the sexes explained only a small amount of the variation in that measure. As there is scant information on avian haematological function, whether the age-related changes that were observed are adaptive, or represent constraints imposed on the system, remains an open question.

Adult shags varied in their level of cell-mediated immune response to PHA, but this variation was not due to age, sex or year differences. This finding is unlikely to be a type two error resulting from measurement inaccuracy, as the repeatability of web thickness measures was high. There was no correlation between PHA score and the time between injection of the antigen and post-injection measurement, suggesting the period for re-capture of test individuals (± 2 hours around the 24-hour mark) was appropriate. The PBS control did not cause any reaction in shags, as changes in the thickness of control webs were negligible. PHA scores calculated by subtracting the PBS thickness change were strongly correlated to the PHA score when the PBS control was disregarded. Thus, for this species I would agree with Smits, Bortolotti and Tella (1999) that the PBS control can be eliminated from the protocol. However, this should only be done if the researcher is satisfied that the injection procedure is sterile and proficiently carried out, so that no additional swelling results from the procedure *per se*. Coefficients of variation of the PHA scores were very similar between the two methods of calculation, suggesting that eliminating the PBS control would not improve that aspect. Therefore, the main advantage of modifying the protocol in this case would be the reduction in handling time of the birds. Changes in cell-mediated immunity in individual shags as they grew older were not systematically measured because of the adaptive nature of the acquired immune response. Rather, PHA was used because it is a novel mitogen, to which these birds have not previously been exposed.

Variation in the immune response to PHA among adult shags remains unexplained. However, there are a number of possible confounding variables that may have hampered identification of sex- or age-related differences among birds.

Some of these also relate to variation in the other traits that were measured. Firstly, although all adults were measured when rearing medium to large chicks, which occurred in June or July each year, reproductive investment, effort and work levels will have differed among individuals. This, and fine-scale differences in the stage of the breeding cycle, could be reflected in hormonal differences among birds, which are known to have immune-modulating effects. A variety of other stressors may also vary from one individual to the next, including food supply, parasites, and diseases past and present. Variation in the stress response at handling could also affect PHA test results, given the immunosuppressive nature of corticosterone (Smits, Bortolotti and Tella, 2001). A study on the PHA immune response in captive zebra finches found that activity preceding this test somehow triggered physiological events that suppressed the reaction (Ewenson, Zann and Flannery, 2003). Only one component of immunity was measured in the present study (other aspects being innate and humoral immunity). As there could be a trade-off between different immune system components, Norris & Evans (2000) argued that more than one should be tested in ecological studies, although this is difficult to achieve in practice. Other components of immunocompetence might show senescence-related change. On the evidence so far, changes in cell-mediated immunity either are too subtle to be detected by the PHA test, do not occur in old shags, or occur only very late in life. Alternatively, those birds that show immunosenescence may not appear at the breeding colony, or may not breed successfully.

No inter-relationships among the traits measured in adults were seen, and in addition these measures in females were not related to their reproductive performance (including clutch volume, chapter 3). Thus, there is no current evidence that these measures actually relate to quality or condition in the European shag, though they are condition-dependent in other species (e.g. Weimerskirch 1992, Wiebe and Martin 1998, Tveraa *et al.* 1998, but see Dawson and Bortolotti 1997). Perhaps high levels of variation among individuals in each measure disguised these relationships. To date, the only attribute of breeding adult shags that has been linked to their reproductive performance and hence current condition, other than age, is crest size (Daunt *et al.* 2003). In a year of overall poor breeding performance, birds that did not subsequently produce a clutch had significantly smaller crests during courtship than those that did. There was also a significant relationship between

ornament size and laying date, with early breeders having larger crests. However, crest size did not relate to age in either sex, supporting the idea that although condition varies among individual shags, it does not appear to decline with age.

On one hand, given the differences in survival (Harris *et al.* 1994*b*) and reproductive performance, such as egg size, occurring with age in the shag, the lack of change in physical or physiological parameters in old age is surprising. On the other hand, this finding ties in with the long-standing (but increasingly challenged) belief that wild animals do not age (Nice 1943, Lack 1954, Williams 1992), and the lack of conspicuous signs of ageing in birds. Diseases of ageing, such as atherosclerosis and diabetes, and reproductive senescence are both documented in captive birds (Holmes and Austad, 1995*a*, *b*), which indicates that the same processes of ageing can occur in birds as in mammals. However, there may be differences between captive and wild birds. Firstly, activity levels and diet will differ in captivity from that in the wild, which may contribute to the incidence of ageing-related disease. Secondly, captive animals can be observed right up to the point of death. As already mentioned, the oldest wild birds, in which senescent changes might be evident, may not be present at the breeding colony or may not establish a breeding site, thus making their detection less likely and biasing samples towards younger and healthier individuals. Lastly, the birds that have traditionally been kept in captivity are different species than those commonly studied in the wild. Notably, captive birds are often more short-lived species, such as chicken or quail (an exception to this is parrots, which are both long-lived and frequently kept in captivity). Senescence theory predicts that high levels of extrinsic mortality, such as predation, lead to the evolution of life histories with high fecundity, short lifespans and comparatively fast rates of ageing (Medawar 1952, Williams 1957, Edney and Gill 1967, Charlesworth 1980, Partridge and Barton 1993). This applies to most species of birds kept in captivity, and contrasts to the life histories of long-lived wild species, which have evolved under different circumstances, and in which reproduction is slower and senescence may be delayed. This theoretical pattern has also recently been observed in practice in two natural populations of guppies *Poecilia reticulata* (Bryant and Reznick, 2004).

On a proximate level, the greater longevity of birds as a class, for their size and metabolic rate in comparison to mammals, has been linked to lower levels of free

radical production, particularly at the mitochondria, and higher levels of circulating anti-oxidants (reviews Perez-Campo *et al.* 1998, Holmes, Flückiger and Austad 2001, Barja 2002). Reactive oxygen species have been suggested to play a causative role in ageing since Harman (1956, 1972) proposed the free radical theory of ageing. There may also be variation among bird species in their levels of oxidative stress, repair, or protective mechanisms, linked to their respective maximum lifespans. Thus, the lack of signs of "wear and tear" in old individuals of long-lived birds, including the shag, may reflect special protective mechanisms at the molecular level. In the following chapter, this possibility is investigated by measuring telomere length change, a process influenced by oxidative stress, in known age shags.

## FIGURES

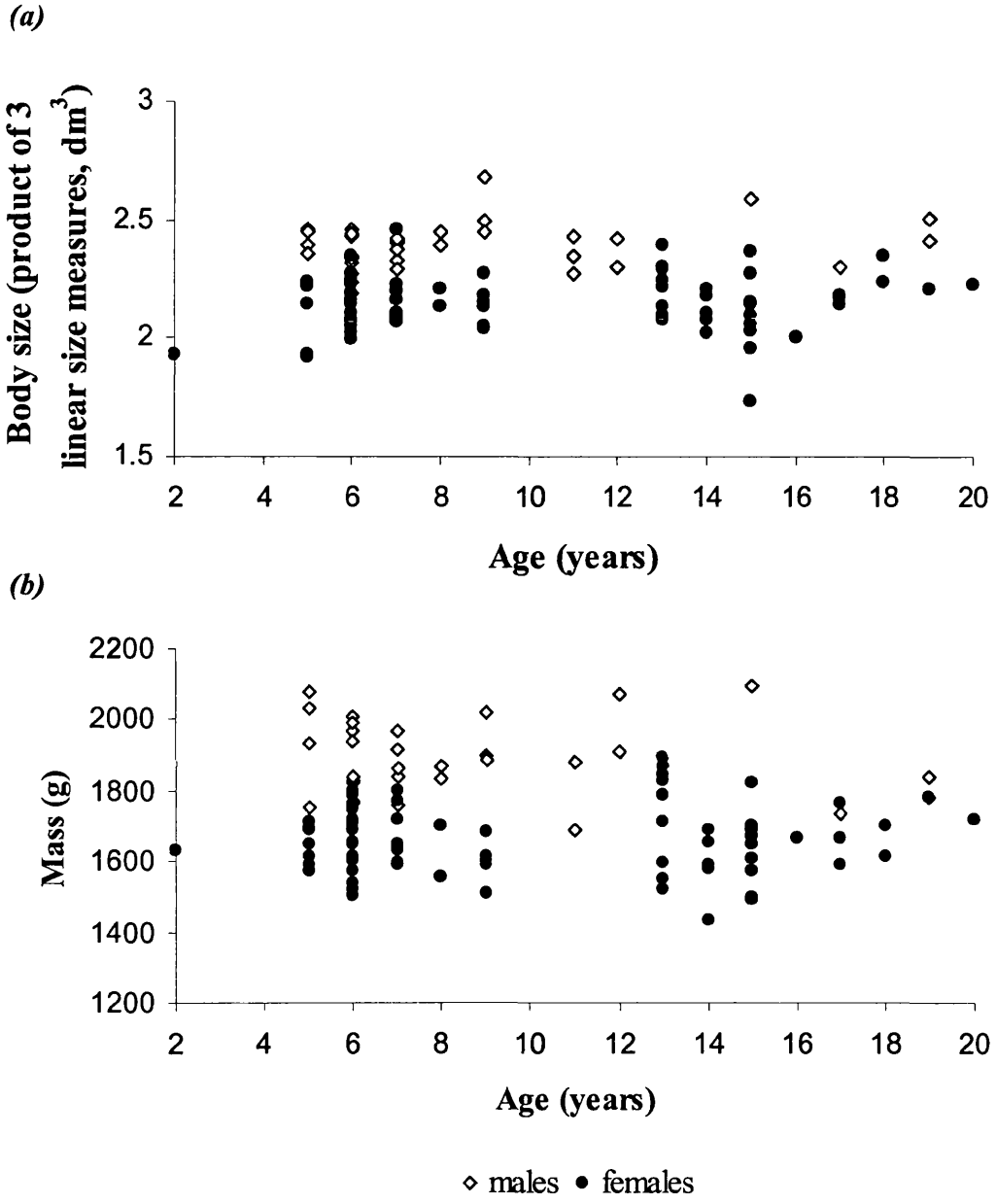
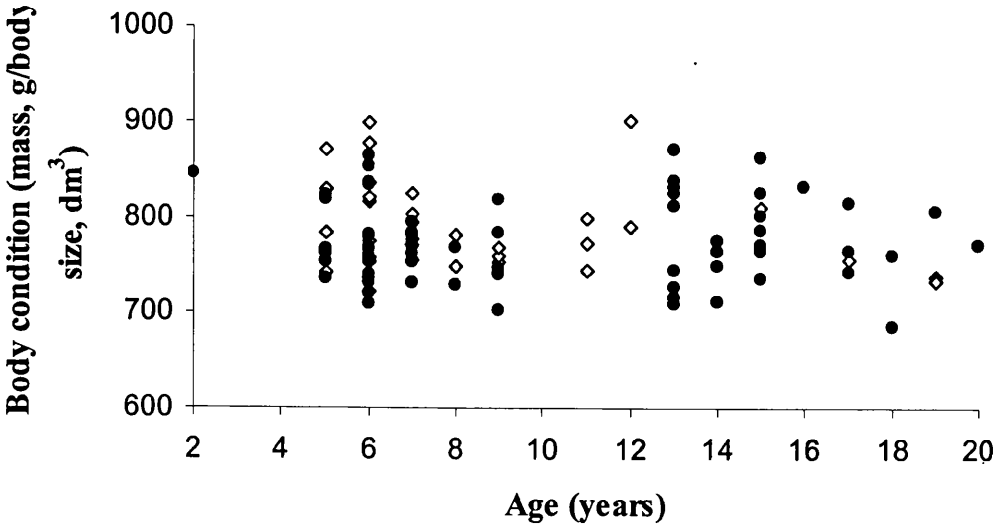
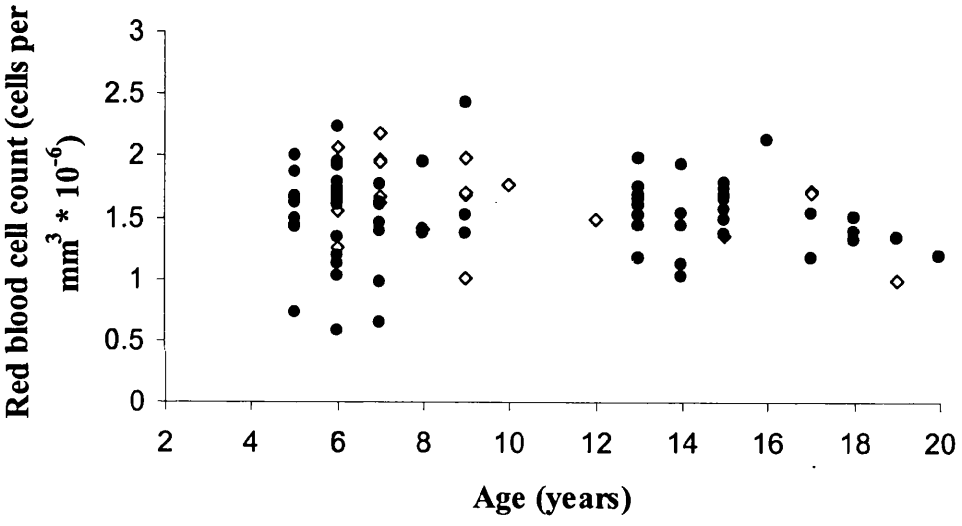



**Figure 5.1** *(a)* Body size and *(b)* mass of adults (cross-sectional data) plotted in relation to age and sex. This figure continues on the following pages, to show body condition, blood parameters and PHA response with respect to age. Sample sizes are shown after the final graph in this figure.

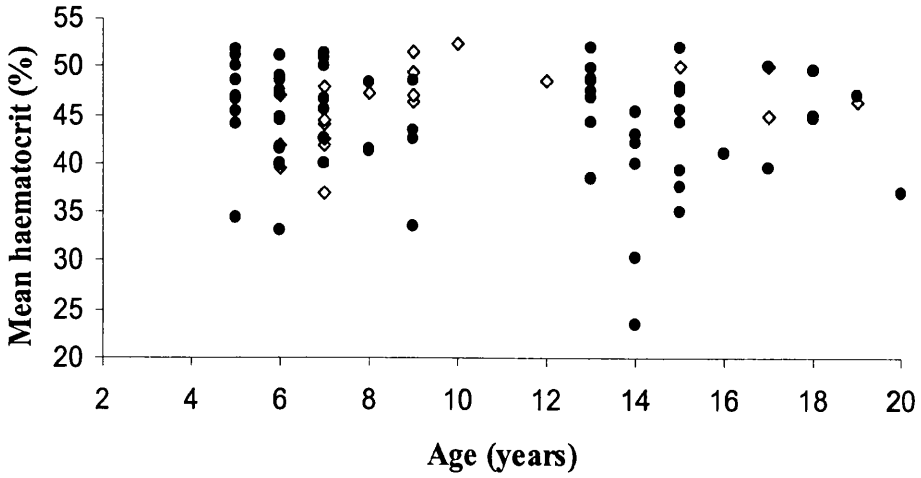
*(c)*




*(d)*



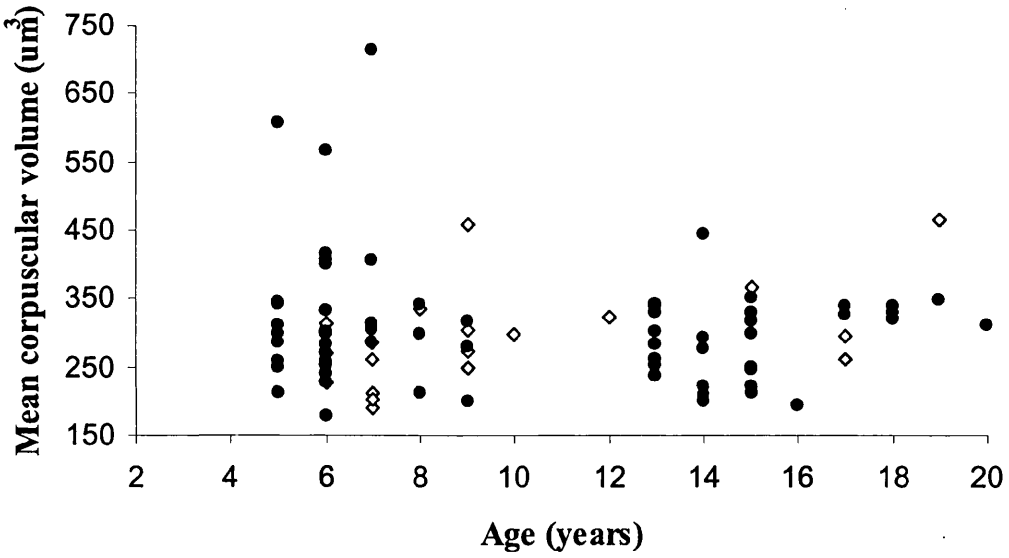

*(e)*



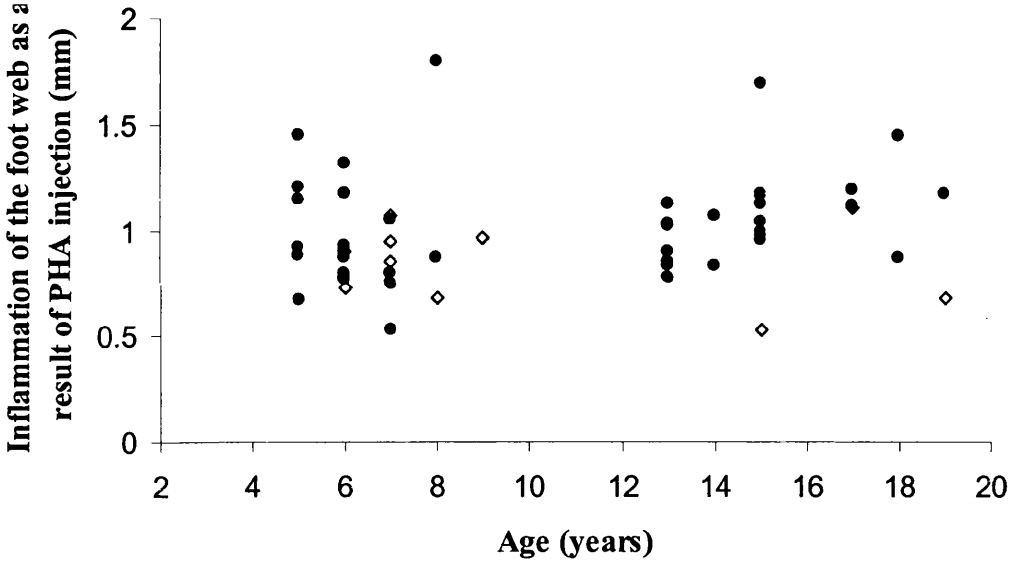

◇ males • females

**Figure 5.1** *(c)* body condition, *(d)* mean red blood cell count, and *(e)* mean haematocrit in relation to the age and sex of adults (cross-sectional data).

*(f)*




*(g)*




◇ males • females

**Figure 5.1** *(f)* mean corpuscular volume, and *(g)* cell-mediated immune response in relation to age for males and females. Cross-sectional data, in which each individual appears only once, is shown.

**Sample size for each graph:**

| sex | *n* | | | |
|---|---|---|---|---|
| | Body size, mass and condition | mRBC, MCV | haematocrit | PHA score |
| males | 33 | 20 | 20 | 10 |
| females | 73 | 69 | 70 | 45 |

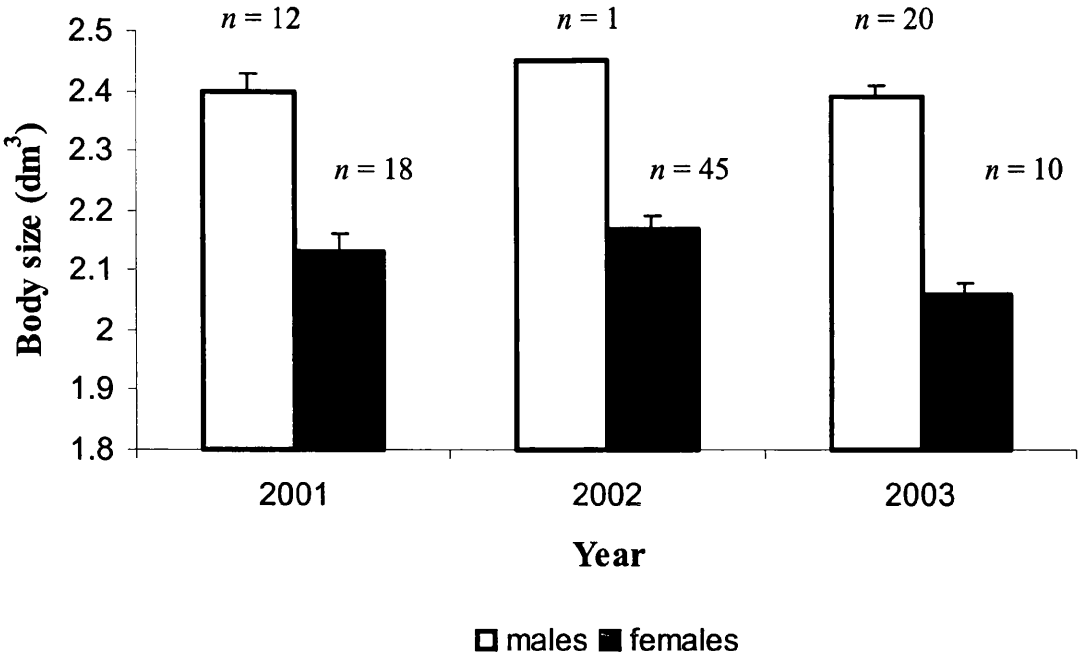



**Figure 5.2** Differences in adult body size among years and between the sexes, in the cross-sectional data. Males are significantly larger than females. Differences among years are very small.

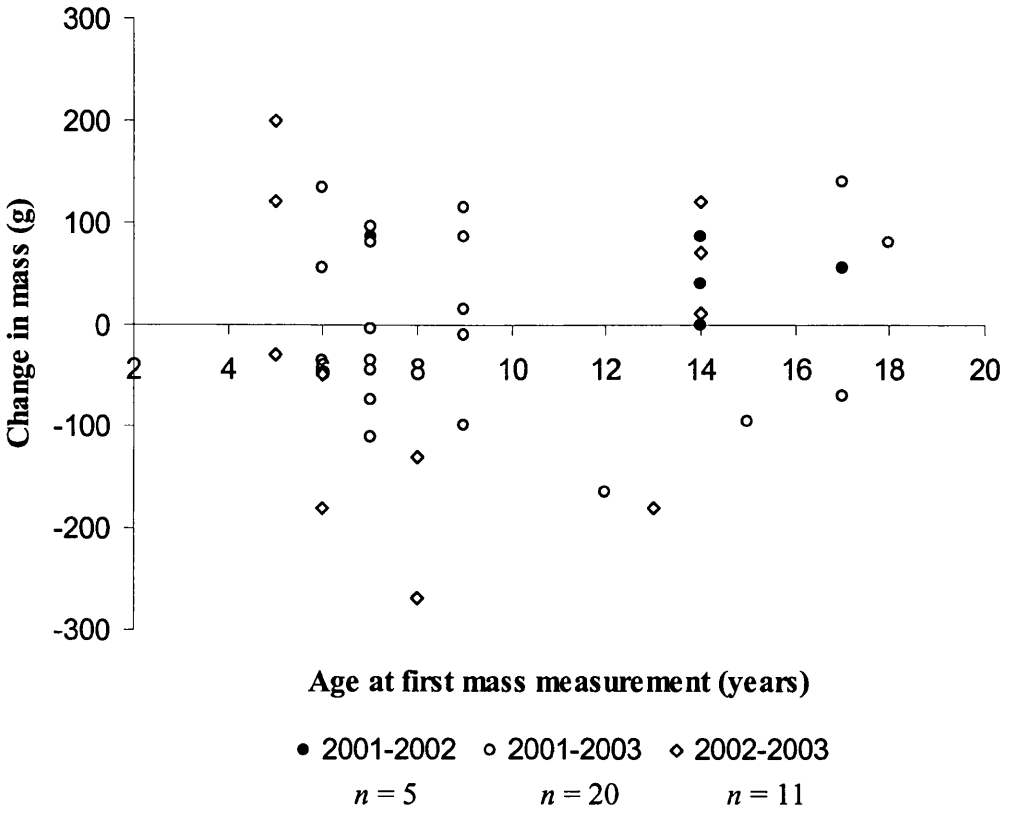



**Figure 5.3** The change in mass within adults measured twice, either in 2001 and 2002, 2001 and 2003 or 2002 and 2003. Only for those measured in 2001 and 2002 was the difference in mass between years significant. However, between 2001 and 2002 all birds sampled showed an increase in mass, and there was no overall pattern of mass change with respect to age.

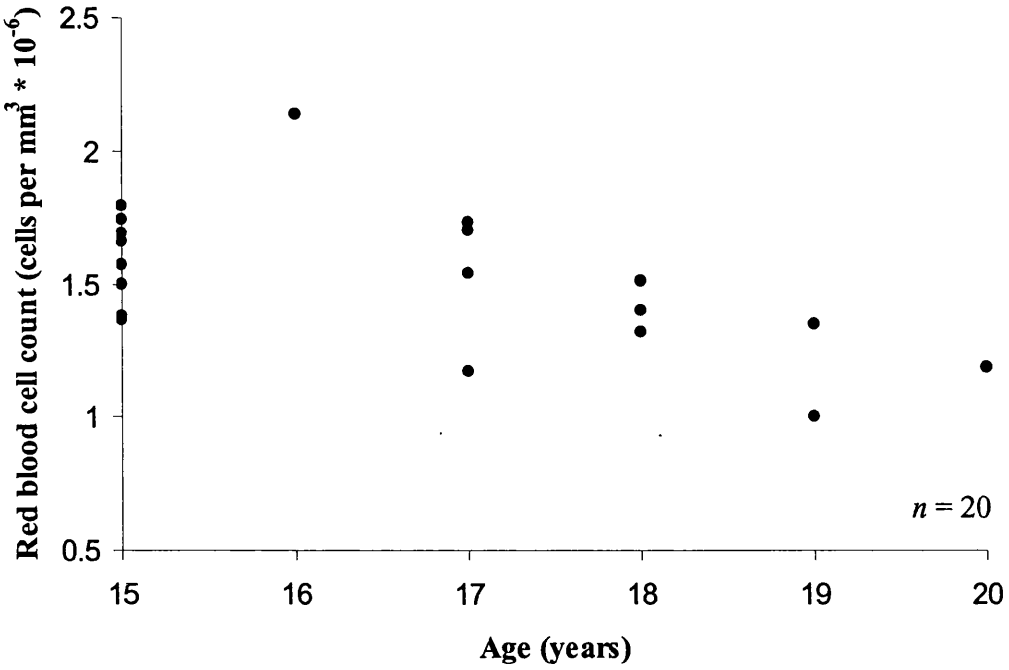



**Figure 5.4** There was a significant negative correlation between red blood cell count and age in birds aged 15 years or older.

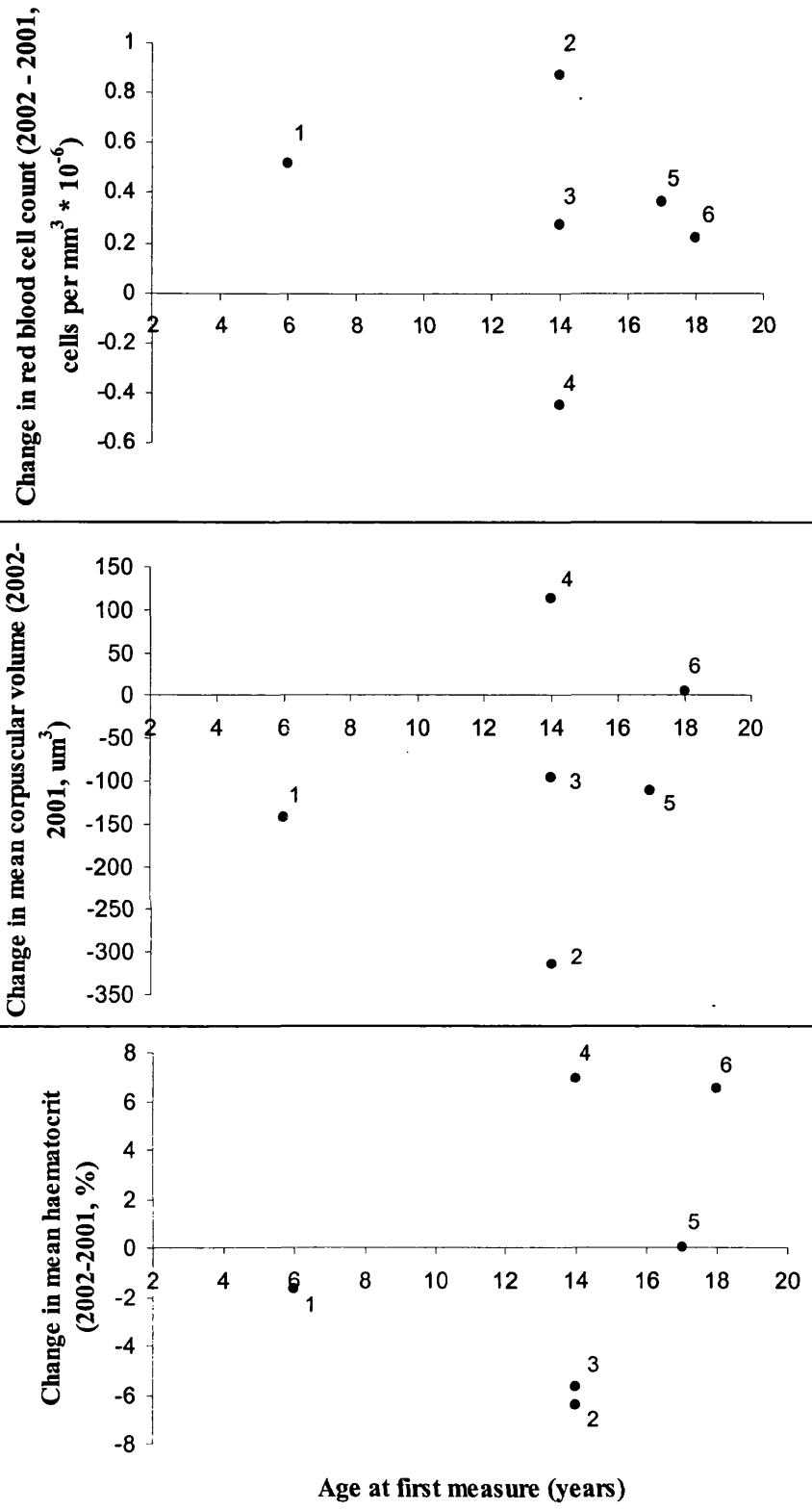



**Figure 5.5** The change in each blood parameter within individuals of different age measured in 2001 and 2002. The individual plotted at 18 years of age was ringed as an adult, and thus 18 years is the minimum age for that bird. All other birds shown are of exact known age. The number attached to each data point identifies the same individual in each graph.

# Telomeres in blood cells of the European shag shorten most in early life: comparisons among and within birds of known age, and in relation to environmental effects

## ABSTRACT

Telomere length in somatic cells has been shown to shorten with age in a variety of mammals, and recent studies in birds have revealed similar declines for several species. Here telomere length in the blood cells of a long-lived seabird, the European shag, is assessed both by comparing mean length among individuals of different age and serial blood samples taken between 2 and 6 years apart for the same individual. This is the first study to take a direct longitudinal approach to age-related telomere attrition in birds. It is demonstrated that telomere length declines between nestlings and breeding adults. However, within adults telomere length was not related to age. This mirrors findings for human nucleated blood cells, where the rate of telomere sequence loss is greatest for young children. The amount of telomere attrition in juveniles could provide a link between lifespan and early conditions, such as nutrition or growth rate. Nest effects on telomere length, which may act via the shared genetic or environmental background of siblings, are examined. As many environmental conditions will also be common to chicks hatching in the same year, in addition the effects of birth cohort on telomere length are considered. Potential environmental effects acting on telomere length at the individual level are investigated by longitudinal comparison. It is demonstrated that both the date on which eggs are laid and chick mass in relation to skeletal size can influence subsequent telomere shortening in European shags.

## INTRODUCTION

Telomeres are repetitive DNA sequences that lie at the ends of linear chromosomes and protect coding DNA from attrition during cell replication. The sequence of six base pairs that make up each telomeric repeat, $(TTAGGG)_n$, is highly conserved among vertebrates (Meyne, Ratliff and Moyzis, 1989), but the number of repeats varies among species. Telomeres prevent recognition of the chromosome termini as broken ends, as well as serving a role in nuclear architecture (reviewed in McEachern, Krauskopf and Blackburn, 2000). The shortening of telomeres at each cell division, in the absence of the enzyme telomerase, has been proposed to act as a 'mitotic clock' mechanism that triggers cell senescence at a critical length (Hayflick and Moorhead 1961, Olovnikov 1973, Levy *et al.* 1992, Vaziri *et al.* 1994, Allsopp and Harley 1995). (Telomerase, present in germline and cancer tissues but downregulated in most somatic cells, is a ribonucleoprotein complex that catalyses addition of telomeric repeats to chromosome ends (reviewed in Morin 1996, Urquidi, Tarin and Goodison 2000).) The rate of telomere shortening, at least in cultured human cells, depends on the length of the single-stranded 3' telomeric overhang (Huffman *et al.* 2000). Initiation of cellular replicative senescence by shortened telomeres appears to relate to loss of their protective shield of telomeric-binding proteins, at least in human fibroblasts (Karlseder, Smogorzewska and de Lange, 2002). Whether 3' overhangs and telomere binding proteins are present and act in a similar manner in avian cells has not yet been investigated.

Telomeres in proliferative tissues are thus expected to shorten as an individual gets older, and this has been observed for human cells *in vivo* and with culture *in vitro* (Harley, Futcher and Greider 1990, Hastie *et al.* 1990, Lindsey *et al.* 1991). It has also been found for some tissues of other mammalian species, for example the mouse *Mus spretus* (Coviello-McLaughlin and Prowse, 1997), and donkey *Equus asinus* (Argyle *et al.* 2003). (Recent results from birds are discussed below.) Via cellular senescence, telomere shortening has been causally linked to tissue and organismal ageing (Campisi 1996, Rudolph *et al.* 1999, Kirkwood 2002, Bird, Ostler and Faragher 2003). Silencing effects on genes located close to telomeric repeats in yeast (Gottschling *et al.* 1990, Renauld *et al.* 1993, Kyrion *et al.* 1993) and in humans (Baur *et al.* 2001), and the dependence of these effects on telomere length, suggest gene expression could also be modified by telomere shortening throughout

the replicative lifespan. In this way, telomere length can have phenotypic effects before the point of cellular senescence. Changes caused to gene expression rather than telomere loss *per se* are believed to have the most profound effects *in vivo* (Campisi 1998, Oshima *et al.* 1995).

While most of the research on telomere dynamics with age has been carried out on human tissues, there has been a recent increase in studies on telomere length in birds. This has been initiated for 2 main reasons. Firstly, to investigate the use of telomere length as a marker of chronological age in birds, as an alternative to the long-term ringing of individuals. Fortunately avian red blood cells are nucleated, which means sufficient amounts of DNA for telomere assays can be obtained by non-destructive sampling. Secondly, there has been interest in comparing the telomeres of birds to those in humans and other animals, given the unusual longevity of class *Aves* and of several particularly long-lived species within the class (Lindstedt and Calder 1976, Finch 1990, Holmes and Austad 1995*a*, *b*).

Lejnine, Makarov and Langmore (1995) confirmed that, at least in the chicken *Gallus domesticus*, telomeres consist of repeats of the highly conserved sequence TTAGGG, and on some chromosomes telomeres are of similar length to those in humans, at 3-10 kilobases. Venkatesan and Price (1998) identified telomere restriction fragments (TRFs) of 8-20 kb in chicken red blood cells and demonstrated an overall decline in telomere length with increased population doubling of chicken embryonic fibroblast cultures. Delany, Krupkin and Miller (2000) and Taylor and Delany (2000) examined intra-individual differences in telomere length in chickens and found that the mean TRF length in embryonic tissues was always greater than that in adults. In these studies the average telomere length of somatic tissue was compared to that of germline tissue within individuals, rather than using a direct longitudinal comparison of telomere length from the same tissue at different ages. Interestingly, the authors noted substantial variation between individuals of the same age for telomere lengths in the same tissue, and that this heterogeneity between individuals complicated inter-age comparisons. Similar heterogeneity in telomere length between same-aged individuals was reported in the mouse (Prowse and Greider 1995, Coviello-McLaughlin & Prowse, 1997) and between human foetuses (Youngren *et al.* 1998, Okuda *et al.* 2002) and adults (Slagboom, Droog and Boomsma, 1994).

Using DNA extracted from the blood of different aged zebra finches *Taeniopygia guttata*, and so a cross-sectional approach, Haussmann and Vleck (2002) found a significant decrease in telomere length with age for a sample of 27 birds, with no difference between the sexes. They reported an approximate reduction of 516 base pairs per year in zebra finch erythrocytes and suggested that telomere length thus could provide a means of estimating age in these birds. Age explained 54% of the variation in telomere length. However, no longitudinal assessment was published with these results so it remains to be seen whether changes in telomere length with age within individuals are consistent with the overall cross-sectional pattern, or whether telomere dynamics vary among individuals. There was overlap in the telomere lengths of the 3 age-groups these authors assigned, and comparisons between these groups by Tukey's difference test found that only the oldest age group ('old' birds > 18 months old) differed from the 2 younger classes ('juveniles' < 4 months old, 'young' birds 11-15 months old). In other words, the juvenile and young zebra finches did not differ in their mean telomere lengths. As zebra finches can breed at 2-3 months of age (Zann, 1996) the youngest age group may have contained both reproductively mature and immature individuals. No information is given in the paper regarding the breeding status of the sampled individuals. Nothing is known about the constancy of the rate of telomeric loss over time in birds, that is whether repeats are lost at the same rate throughout life.

Telomere length in 4 more avian species has now been studied. In addition to the zebra finch, the telomeres of tree swallows (*Tachycineta bicolor*), Adélie penguins (*Pygoscelis adeliae*), common terns (*Sterna hirundo*) and Leach's storm petrels (*Oceanodroma leucorhoa*) have been measured (Haussmann *et al.* 2003, Vleck, Haussmann and Vleck 2003, Haussmann, Vleck and Nisbet 2003). Cross-sectional comparisons were again used for these 4 species and in all but the Leach's storm petrel telomeres decreased in length with advancing age at sampling (Haussmann *et al.* 2003). In the petrel, which the authors report to be the most long-lived of these species, the oldest individuals displayed longer telomeres than the youngest. Haussmann *et al.* discuss these results in terms of different rates of telomeric attrition occurring in species of differing lifespans, suggesting that the extreme longevity of Leach's storm petrels may in part be due to their 'escape' from the normal telomere shortening processes. Telomerase activity is a potential

mechanism by which telomere length could be maintained, and very recent work has found relatively high telomerase activity in the bone marrow of this species, as well as in common terns which are also long-lived. Moreover, this telomerase activity did not decrease with age (Haussmann *et al.* 2004). Within-individual comparisons were not made in the telomere length or telomerase study, so it is also possible that the differences observed among age groups for the petrel are due to selective mortality. There is a case for selective mortality operating on the basis of telomere length given the link between short telomeres and replicative senescence. There is also growing evidence in humans that the susceptibility to age-related diseases is greater for individuals with shorter telomeres (e.g. Samani *et al.* 2001, von Zglinicki *et al.* 2000, Jeanclos *et al.* 2000). Among over-60 year olds, those with shorter blood telomeres have poorer survival, in part due to their higher mortality from heart disease and infectious disease (Cawthon *et al.* 2003).

There is a need for longitudinal studies of telomere length to address within-individual changes with age, to overcome the problems of large inter-individual telomere length variation at birth, and of selective mortality. Arking (1998) recognised that while ageing processes can be inferred from population data, it is necessary to ultimately refer to their expression in individuals. He described the main drawbacks of cross-sectional analyses. Firstly, they assume changes in the average value from one group to the next accurately reflect changes in single individuals over time. However, the phenotypic composition of age classes may differ. Secondly, environmental changes are confounded with the effects of age. Furthermore, differences in individual rates of change cannot be examined by cross-sectional comparison, making it difficult to identify factors influencing the rate or direction of that change.

There have been some longitudinal studies of telomere length with age in animals other than birds. A longitudinal study of telomere length in feline blood cells compared serial samples taken over the course of a year for newborn kittens, 2 year old and 10 year old domestic cats (Brummendorf *et al.* 2002). The rate of telomeric shortening was greatest in the kittens. Ideally, serial samples should be collected over a greater time course so that the rate can actually be compared within a single animal as it ages. However, time constraints often limit longitudinal studies. Zeichner *et al.* (1999) did inspect telomere length changes over a wider age range

within individuals, assessing repeat blood samples taken from 9 human infants over the first 3 years of life and 2 adults followed for 8 and 10 years. Ethical considerations have previously limited such studies in humans, but in this case samples were all obtained initially for clinical reasons. The rate of telomere shortening between 1 and 36 months of age was on average 270 base pairs per year, compared to 49 base pairs per year in the 2 adults, who were measured between the ages of 28 to 36 years and 30 to 40 years respectively. The only other longitudinal comparisons of telomere length published to date relate to cell replication in various disease states, or the consequences of treatment, in most cases for human immunodeficiency virus (HIV) in human patients, chimpanzees or monkeys (Feng *et al.* 1998, Feng *et al.* 1999, Shibata *et al.* 1999, Wolthers *et al.* 1999, Adelfalk *et al.* 2001, Natarajan *et al.* 2002, Franco *et al.* 2003).

Rapid telomere attrition in early life, compared to that in adults, is also observed in cross-sections of the human population. It has been described by Frenck, Blackburn and Shannon (1998), Rufer *et al.* (1999) and by Friedrich *et al.* (2001), all working on blood cell types. Frenck, Blackburn and Shannon describe the greatest rate of attrition occurring before the age of 4 years, followed by a plateau in young adulthood and then gradual shortening later in life. They noted that the longest telomeres were found in children under 18 months of age, and this result was confirmed by Rufer *et al.* who observed very rapid loss in the first year of life and subsequent shortening at a 30-fold lower rate. Friedrich *et al.* investigated changes even earlier, in gestation, by comparing the telomere lengths of preterm and fullterm neonates. They showed that a rapid and significant decline in telomere length occurs between 27 and 32 weeks gestation, even exceeding the rate in young children. The extremely fast decline in blood cell telomere length early in life is consistent with the extensive somatic growth and maturation that occurs during that period, but there is disagreement on whether different rates of loss also reflect differential regulation of telomere length. Frenck, Blackburn and Shannon point to changes in telomere length regulation by both negative and positive factors through life, such that telomeric sequence is not lost at the same rate with each cell division. In contrast, Rufer *et al.* believe that constant loss at each division, together with known ontogeny-related differences in primitive hematopoietic cells, can explain the data.

Arising from the large variation in telomere length between individuals is the question of to what extent this is genetically determined. The level of heritability of telomere length in birds is unknown, and Haussmann and Vleck (2002) did not report the relatedness of the donor finches in their study. However, research in humans suggests telomere length in blood is largely determined by genetic influences (Slagboom, Droog and Boomsma 1994, Jeanclos *et al.* 2000), although it is not clear whether this relates to differences between individuals in germline telomere length (see Allsopp *et al.* 1992), turnover rates of cells or the amount of telomeric sequence lost per cell division. Genetic control has also been suggested to explain strain-specific differences in telomere length in mice (Kipling and Cooke 1990, Zhu *et al.* 1998, Manning *et al.* 2002) and to play a role in yeast telomere length and stability (Carson and Hartwell 1985, Lundblad and Szostak 1989). However, longevity is influenced by differences in environment and lifestyle, and is not solely under genetic control (e.g. Marmot *et al.* 1975, Marmot and Smith 1989). It may be that some of these non-genetic differences in life expectancy also act on telomere dynamics.

It has been demonstrated that mild oxidative stress can accelerate telomere shortening *in vitro*, probably via accumulation of single-strand breaks in the telomeres (von Zglinicki *et al.* 1995, Petersen, Saretzki and von Zglinicki 1998, von Zglinicki, Pilger and Sitte 2000). Telomeres are predisposed as targets for oxidative damage due to their multiple GGG-triplets (Retèl *et al.* 1993, Saito *et al.* 1995, Hall, Holmlin and Barton 1996). An inverse correlation exists between anti-oxidant capacity and the rate of telomere shortening for human fibroblast strains (Lorenz *et al.* 2001, Saretzki and von Zglinicki 2002). In addition, anti-oxidative agents are able to extend replicative life in culture by slowing the rate of telomere attrition (Furumoto *et al.* 1998, Serra *et al.* 2000, Serra *et al.* 2003, Kashino *et al.* 2003). A correlation between oxidative stress and telomere shortening in people with inherited respiratory chain disorders (Oexle and Zwirner, 1997) suggests this relationship also holds *in vivo*. For these reasons, Saretzki and von Zglinicki (2002) describe telomere length as a cumulative indicator of the oxidative damage a tissue has experienced through life, and of the ability of that individual to cope with oxidative stress. So, telomere shortening may partly reflect past oxidative history. Relating this to the findings of variation in the rate of telomere attrition with age, it can be

postulated that periods of high oxidative stress may be responsible for phases of rapid telomeric loss, for instance the rapid loss during childhood. Differences among individuals in the rates of telomere loss may relate to differences in the level of nutrition or growth rate, which may partly determine oxidative damage for instance via effects on levels of diet-derived anti-oxidants (Beckman and Ames 1998, Blount *et al.* 2003). Faster growth is linked to higher levels of oxidative stress (Merry 1995, Rollo 2002). This idea was outlined by Jennings, Ozanne and Hales in a recent review (2000).

As the phase of life with most rapid loss of telomere repeats appears to be childhood or gestation, it may be that the action of environmental factors at that time is particularly crucial for later life and longevity. Environmental effects acting early in life can have delayed downstream effects (Lindström 1999, Metcalfe and Monaghan 2001, Lummaa and Clutton-Brock 2002, Metcalfe and Monaghan 2003). For example, lifespan in rats has been linked to levels of maternal nutrition, such that females undernourished during gestation produce offspring with lower life expectancy (Desai and Hales, 1997). There is also a relationship between death from coronary heart disease and catch-up growth occurring before the age of 7 years in men (Eriksson *et al.* 1999). Telomeres may play a role in this process, and in rats growth retardation in foetal life followed by postnatal catch-up growth has been shown to be associated with a shorter life span and shorter kidney telomeres (Jennings *et al.* 1999). Aviv, Levy and Mangel (2003) constructed a model predicting the probability of surviving disease-free to age 65 years based upon telomere dynamics and the growth rate between birth and 11 years of age. In that model, more rapid growth in childhood was related to a lower probability of remaining disease-free in later life.

In the present study, blood cell telomere length in the European shag *Phalacrocorax aristotelis*, a long-lived seabird for which telomere length has not previously been measured, is investigated. This species was studied because there was access to a breeding population that has been the subject of long-term monitoring and ringing, and thus in which a large proportion of the birds are of known age. Both cross-sectional analysis of telomere length in relation to age and sex, and a longitudinal comparison of telomere length within individual birds sampled as chicks and again as adults, was carried out. Telomere lengths among

siblings, between parents and offspring, and the effects on telomere length of year of hatching and environmental conditions in the hatching year were examined.

# METHODS

## 1. Blood sampling and DNA extraction

Blood was collected from adult and nestling shags on the Isle of May, Scotland (56°11'N, 02°33'W) under UK Home Office licence, by superficial venipuncture of the tarsus during the breeding season in 1997, 1998, 1999, 2001, 2002 and 2003. A total of 81 shag chicks and 71 adults were sampled, ranging in age from 1 day to 20 years old. 16 chicks, all from different broods and different nest sites even across years, were measured again as adults after 2, 5 or 6 years had passed. Chick whole blood samples were stored in 90% ethanol at -20°C until DNA could be extracted. Adult samples were spun to separate plasma and cells. The blood cells were subsequently stored in liquid nitrogen until they could be transferred to a –80°C freezer, where they remained until DNA extraction. For both nestling and adult shags DNA from whole blood was used in the telomere length assay, and therefore our measures of telomere length refer to all nucleated blood cells present. In birds, red blood cells are nucleated and make up the largest portion of the blood.

DNA was isolated from all blood samples by the same procedure. First, blood was digested with proteinase K (10-15 µl blood with 300 µl SET, 30 µl 10% SDS and 12.5-15 µl proteinase K overnight at 55°C with agitation). DNA was then extracted using a standard phenol/chloroform procedure for isolating large DNA molecules, and precipitated using ethanol and sodium acetate (modified from Sambrook, Fritsch and Maniatis, 1989). DNA yields and integrity were verified by gel electrophoresis. The concentration of DNA per sample was quantified by spectrophotometry.

## 2. Production of the TRF image

Telomere restriction fragment (TRF) length was measured by southern blot hybridisation following the protocol outlined in the TeloTAGGG Telomere Length Assay Kit (Roche Diagnostics Ltd., East Sussex, UK), and mainly using the supplied reagents. TRF length measurement is the traditional method for estimating telomere length. The fragments produced comprise the terminal telomeric repeats plus a subtelomeric portion. TRF lengths measured by this traditional approach have been

shown to correlate with those determined by other methods including quantitative fluorescence *in situ* hybridisation (Q-FISH) (Lansdorp *et al.* 1996, Hultdin *et al.* 1998) and quantitative polymerase chain reaction (PCR) (Cawthon, 2002). For simplicity, TRF length is referred to simply as telomere length in the results and discussion.

The procedure used was as follows. First, 2μg of DNA per sample was digested for 16 hours at 37°C with the enzymes *Rsa*I and *Hinf*I (10U/μl, Invitrogen Ltd., Paisley, UK); 0.6μl of each enzyme was added per sample, such that the final concentration was 6U enzyme mix per μg of DNA. Fragments from digestion were run on a 0.8% agarose gel at 120V for 4 hours, with a digoxigenin (DIG)-labelled molecular weight marker (23.1-0.12 kb, Roche Diagnostics Ltd., East Sussex, UK) loaded in the first and last lanes of the gel. A second gel loaded with a small fraction of each sample and a standard 1 kb ladder (Invitrogen Ltd., Paisley, UK) was examined by ethidium bromide staining and photographed to check for complete digestion of DNA. Following that check, the main gel was denatured to separate the DNA into single strands (following TeloTAGGG Telomere Length Assay Instruction manual, Roche). The fragments were transferred to a Hybond N+ nylon membrane (Amersham Pharmacia Biotech. Ltd., Bucks, UK) by overnight southern blot transfer in 20 × SSC at room temperature. DNA was fixed to the membrane by UV cross-linking (optimal cross-link, Spectrolinker™ XL-1000, Spectronics Corporation). Hybridisation to a DIG-labelled probe (5' $(TTAGGG)_4$ 3') was carried out at 42°C in a hybridisation oven (Hybaid maxi) for 16 hours. Later, hybridisation time was reduced to 3 hours. The membrane was washed in 2 × SSC, 0.01% SDS at room temperature and then in 0.2 × SSC, 0.1% SDS at 50°C. It was incubated for 30 minutes at room temperature with a DIG-specific antibody, which was supplied covalently coupled to alkaline phosphatase (anti-DIG-AP, DIG Luminescent Detection Kit, Roche Diagnostics Ltd., East Sussex, UK). Following that, a chemiluminescent substrate CSPD (DIG Luminescent Detection Kit, Roche Diagnostics Ltd., East Sussex, UK), which is metabolised by alkaline phosphatase, was added to the membrane thus allowing visualisation of the TRF smears. Membranes were exposed to autoradiography film to form a permanent image of the telomere fragments in each lane.

In a small number of cases, the TRF smear extended well above the uppermost band of the molecular weight marker, and above the probable limit of size resolution for the gel (see Sambrook, Fritsch and Maniatis, 1989). Therefore, those samples were re-run on a 1% agarose gel in pulse-field for 22 hours at 6V/cm (40-90s switch time, CHEF-DR II, Bio-Rad Laboratories Ltd., Hertfordshire, UK). A 48.5 - 8.3 kb marker (CHEF DNA standards, Bio-Rad Laboratories Ltd., Hertfordshire, UK) was loaded on the pulse-field gels, in addition to a 23.1-0.125 kb standard (λDNA/Hind III fragments, Invitrogen Ltd., Paisley, UK). However, this procedure was only necessary for 5 samples out of the total 170 tested, suggesting that constant field electrophoresis with a 23.1-0.12 kb standard was generally appropriate for measuring shag telomere restriction fragments.

### 3. *Measurement of TRF length from autoradiography film*

After scanning each image, the intensity of the TRF smears at different molecular sizes was measured using TotalLab software (TotalLab from Phoretix, version 1.10, ©1996- 2000 Nonlinear Dynamics Ltd., Newcastle-upon-Tyne, UK). The mean TRF length per lane was calculated using the formula: $\text{meanTRF}L = \sum (OD_l) / \sum (OD_l L_l)$, where $OD_l$ and $L_l$ are the signal intensity and the TRF length respectively, at position 1 on the image. The background signal was subtracted from the signal intensity measurements for each lane before calculation. Median TRF length was calculated for a sub-sample of 26 lanes, drawn from 2 separate gels. However, as no difference was apparent between mean and median values (paired-samples t test, $t(25) = -1.4$, $p > 0.05$), mean TRF length was used in all subsequent analyses. Peak TRF length, which corresponds to the size of TRF occurring most often in the sample or the modal length, and TRF range, the range of lengths exhibited, were also measured for every lane. An average of the peak TRF length for the 2 lanes run per sample was calculated, and the modal values reported in the results refer to this average value. Mean TRF length averaged across 2 lanes per sample is referred to simply as telomere length in the results and discussion. The percentage signal intensity within 5 size regions defined by the molecular weight standard was also recorded for each lane, to illustrate how the distribution of telomere lengths differs between samples (after Jennings *et al.* 1999, Cherif *et al.* 2003). Peak, range and distribution measures are alternatives to mean TRF length, and may be particularly

revealing if it is the shortest telomeres rather than the average length which is important for loss of function and triggering cell cycle arrest, as has been suggested (Hemann *et al.* 2001).

For each blood sample, duplicate digests were run on the same gel and the average of these 2 mean TRF lengths used as the estimate of telomere length. The repeatability of mean TRF length, which is the proportion of the variation found between different blood samples compared to that between duplicates of the same sample (calculated as recommended by Lessells and Boag, 1987), was high ($F_{146,147}$ = 87.44, $p < 0.001$, $r$ (95% CI) = 0.98 (0.97, 0.98), confidence interval calculated as Becker, 1984). In order to assess between-gel differences, a sub-set of blood samples were run on 2 separate gels ($n$ = 18). Repeatability when duplicates were run on separate gels was lower ($F_{17, 18}$ = 4.27, $p$ = 0.002, $r$ (95%CI) = 0.62 (0.24, 0.84)).

The analysis of the scanned images was carried out blind with respect to donor age and sex. An independent second observer also analysed each image. Mean TRF lengths calculated for a lane by different observers were highly correlated (Pearson correlation coefficients $r$ = 0.97, $n$ = 165, $p < 0.001$). The average of 2 lanes measured by one observer was used in analyses.

### *4. Comparing the telomere lengths of related individuals*

Twelve pairs of apparent siblings (i.e. from the same brood) occurred among the nestling shags for which telomere length was measured. However, the difference in telomere length between siblings did not differ from that between randomly assigned non-sibling pairs ($t_{22}$ = 1.72, $p > 0.05$). The values of individuals, rather than brood averages, have been used in analyses. An effect of nest on mean TRF length in the sibling data was also tested for, and the repeatability of mean TRF length in nests calculated (after Lessells and Boag, 1987 and Becker, 1984). Of the adult shags for which it was possible to check the presence of siblings (86%), none had a sibling present in the data. This was not known for the remaining 14%, as they were either ringed as adults ($n$ = 10) or brood size was not recorded at ringing ($n$ = 1). No siblings were included in the longitudinal data. For a subset of the data, the telomere length of offspring was compared to that of their parents.

### 5. Measurement of environmental conditions

In order to examine potential effects of conditions in the natal year on telomere length, environmental condition measures in each of the hatching years included in the telomere data were compiled. This data forms part of the long-term monitoring of shag breeding productivity on the Isle of May, recently as part of the Joint Nature Conservation Committee's (JNCC) Seabird Monitoring Programme (e.g. Wilson, Wanless and Harris, 2002). As part of this monitoring, breeding attempts by shags are followed from nest building up to chick fledging for 16 designated sites around the island. Mean plot success is then calculated as the number of chicks fledged per incubated nest, averaged across these sites. Each year there is also a concerted effort to ring a large proportion of the new shag chicks. From the ringing records the total number of chicks ringed, average brood size at ringing, and median ringing date were obtained. Breeding success declines as median ringing date advances (Harris *et al.* unpublished data). The first factor from a principal components analysis (PCA) on the variables mean plot success, total number of young ringed and average brood size at ringing explained approximately 84% of the variation in these measures, and was available for 18 separate cohorts. This factor describes differences in breeding success and cohort size. Median ringing date was also included with these variables to provide another factor (75% variation explained, available for 17 cohorts), which also incorporated differences in the timing of breeding. These 2 principal component factors were used as indicators of the environmental conditions present in each hatching year. Whether mean telomere length was related to the factor score for the year in which an individual hatched was investigated.

## RESULTS

For simplicity, in the following section and discussion the term telomere length has been used. This refers to the mean TRF length averaged across 2 replicate digests of the same blood sample.

### *Blood cell telomere length in the shag*

Telomere length in shags ranged from 6.1 to 14.1 kilobases. The overall average telomere length was 9.6 +/- 0.13 kb (mean +/- s.e.m., $n$ = 169). The smallest detectable fragment size was 1.0 kb and the largest over 23.0 kb. Figure 6.1 provides an example of a typical autoradiograph, and table 6.1 gives the corresponding values of mean TRF length, peak TRF length and TRF range measured for each lane.

### *Telomere lengths of related individuals*

There was no significant effect of nest on telomere length in the sibling data ($F_{11,12}$ = 1.55, $p > 0.05$), even after sex was included in the analysis (sex $F_{1,11} = 1.52$, $p > 0.05$, nest $F_{11,11} = 1.58$, $p > 0.05$). The repeatability of telomere length within a brood was 0.214 (95% C.I. –0.365, 0.682). Therefore, the mean telomere lengths of chicks from the same nest are not strongly associated. However, comparison of telomere length between parents and their offspring (for a small proportion of our sample, identifying in the dataset parents of 2001- and 2002-sampled chicks) showed a significant positive correlation, regardless of at what age parents were sampled ($r_s$ = 0.57, $n$ = 18, $p$ = 0.01, see figure 6.2). A mixture of male and female parents' telomere lengths were present in this analysis (in 11/18 cases the male parent's, 7/18 cases the female parent's telomere length was compared to that of their offspring). As some of the chicks included in this parent-offspring comparison were from the same nest, the analysis was also repeated using only 1 chick from each nest, which reduced the sample size by 4. There was still a significant positive correlation between parent and offspring telomere length ($r_s = 0.57$, $n = 14$, $p = 0.03$).

### *Cross-sectional analysis of telomere length with regard to age*

In the cross-sectional data, telomere length appears to decline with age (reduced major axis regression: slope = - 0.30 ± 0.02 (s.d.) kb $yr^{-1}$, intercept = 10.96 ± 0.27

(s.d.) kb, $R$ = -0.59, $p < 0.001$, see figure 6.3*a*). However, when chicks were removed from the dataset and only birds aged between 2 and 20 years assessed, there was no significant effect of age on telomere length ($R$ = -0.08, $p > 0.05$). On average, nestling shags had telomeres of 10.7 ± 0.1 kb (mean ± 1 s.e.), which was significantly greater than that of adults at 8.4 ± 0.2 kb (see figure 6.3*b*). There was no effect of sex on telomere length ($F_{1,148}$ = 0.003, $p$ = 0.96), and the interaction between sex and the factor 'chick or adult' was also not significant ($F_{1,147}$ = 0.65, $p$ = 0.42). The modal TRF length was 12.2 kb in chicks and 9.2 kb in adult shags (means with 95% C.I.: chicks 12.2 (11.5, 12.9), adults 9.2 (8.7, 9.7), mean difference with 95% C.I. = 3.0 (2.2, 3.8)). So the most frequently occurring fragment, as well as the mean fragment length, was shorter in adults than chicks. A distribution plot of signal intensity for 5 fragment size regions in chicks, compared to that for adults (figure 6.3*c*), shows that adult blood cells have a lower proportion of telomeres of sizes 23.1 - 9.4 kb and a higher proportion of shorter fragments 6.6 - 4.4 kb long.

### *The effects of birth cohort and environmental conditions*

The potential impact of environmental conditions during early development on subsequent phenotypic and life history traits was described in the introduction to this chapter. As individuals born in the same year will to some extent have a common environmental history, there may be an effect of cohort or environmental conditions in the natal year on telomere length. Cohort effects may also obscure any relationship between telomere length and age. In this section these issues are addressed by considering three main questions: ***(a)*** whether the difference in telomere length between chicks and adults can be partly explained by the year of hatching, ***(b)*** whether there is an effect of age on telomere length in adults when the random effect 'year of hatching' is controlled for, and ***(c)*** if any relationship exists between telomere length and environmental conditions in the natal year.

***(a)*** In the data set including both chicks and adults, year of hatching had a significant effect on telomere length ($F_{19,131}$ = 5.12, $p < 0.001$). However, as a highly significant correlation exists between year of hatching and age ($r_s$ = -0.93, $n$ = 144, $p < 0.001$), this may simply reflect the contrast between chicks and adults due to age. Year of hatching is not exactly equivalent to age because birds that hatched in the same year were sampled in different years. For example, if two birds hatched

in 1996 one might be sampled in 1999 at the age of 3 years while the other was sampled in 2002 at 6 years of age (see table 6.2). The greater the number of different years in which sampling occurs, the weaker the correlation between hatching year and age will become, but it appears that sampling would have to be spread over a great many years in order to separate these two variables.

To address this problem, cases were categorised as chick or adult and those categories used as the fixed factor in a General Linear Model (GLM, in SPSS) to test for effects on telomere length with year hatched as a random factor. Neither the interaction term ($F_{2,128} = 0.72$, $p = 0.49$) nor the main effect 'year hatched' ($F_{19,130} = 0.35$, $p = 0.99$) were significant. The category 'chick or adult' remained significant ($F_{1,130} = 13.22$, $p < 0.001$).

While both PCA factors for environmental conditions in the year of hatching were significantly correlated with telomere length across the complete age range of birds tested (factor for mean plot success, number of young ringed and brood size $r_s = 0.38$, $n = 134$, $p < 0.001$, factor also including median ringing date $r_s = 0.43$, $n = 134$, $p < 0.001$), this correlation did not hold either within the adults alone ($r_s = -0.08$, $n = 53$, $p = 0.58$, $r_s = -0.12$, $n = 53$, $p = 0.40$, respectively) or within the chicks (for both factors $r_s = 0.19$, $n = 81$, $p = 0.09$). The highest positive factor scores occurred in 2001, and then 2002, which may explain this result. Approximately two thirds of the chicks sampled hatched in 1 of these 2 highly productive years, but only 1 adult in the dataset (of a total of 53 adults for which factor scores could be calculated) hatched then. When either PCA factor was included as a covariate with the fixed factor 'chick or adult', they had no significant effect on telomere length (factor incorporating mean plot success, number of young ringed and brood size $F_{1,131} = 3.37$, $p = 0.07$, factor including median ringing date $F_{1,131} = 2.62$, $p = 0.11$). The fixed factor 'chick or adult' remained significant. Similarly, both age and the PCA factors are significant in a multiple regression with telomere length in the full dataset including chicks and adults (age $p < 0.001$, both factors $p < 0.01$), but when either chicks or adults are analysed separately no significant effect is found (in both cases $p > 0.05$).

*(b)* Removing chicks from the dataset, it was tested whether an effect of age on telomere length in adult birds occurred when the random effect 'hatching year' was taken into account. In this case the effects of the covariate 'age at sampling' and the

random factor 'year of hatching' were tested. Neither were found to have a significant effect on telomere length (age $F_{1,44} = 0.03, p = 0.87$, year hatched $F_{17,44} = 0.20, p > 0.9$).

*(c)* Overall, variance in mean telomere length did not differ between birth cohorts (Levene's test $F_{18, 125} = 1.00, p = 0.46$). Telomere lengths for different years of hatching were compared within ages. Within the group of birds sampled as chicks there were 4 cohorts present (hatched in 1997, 1998, 2001 and 2002). There was no effect of year of hatching on telomere length within this group ($F_{3, 77} = 1.57, p > 0.05$). Within 6 year olds, which was the adult age at which the largest number of different hatching years were found and for which 3 cohorts were present in the dataset (1995, 1996, 1997), there was also no effect of hatching year on telomere length ($F_{2,13} = 0.25, p > 0.05$). The PCA factors describing environmental conditions did not correlate with telomere length ($r_s = 0.28$, $n = 16$, $p = 0.30$) within this age group. Looking solely within the adults, multiple regressions of mean TRF length on the variables age and either of the 2 PCA factors confirmed these results. Neither age nor natal environmental descriptors had a significant effect on telomere length in adult life (in all cases $p > 0.05$). Figure 6.4 shows the factor 1 scores, and the values of mean plot success, number of young ringed, brood size at ringing and median ringing date which were included in these principal components analyses for the hatching years of the chicks and the 6 year old birds.

### *Longitudinal analysis of telomere length*

Telomere length declined within individuals as they grew older (paired t test: $t_{15} = 6.8, p < 0.001$). For 16 birds sampled as nestlings and again as adults, with an intervening period of 2, 5 or 6 years, telomere length was reduced from an average of 10.2 ± 0.3 kb to 8.3 ± 0.3 kb (figure 6.5*a*). A similar reduction was seen in the modal length within individuals ($t_{15} = 2.61, p = 0.02$, mean difference with 95% C.I. of the difference 3.0 (0.5, 5.5)). Separate plots for these birds showing TRF signal intensity at 5 size regions as chicks and as adults indicated a decrease in the proportion of fragments of size 23.1 - 9.4 kb and an increase in 6.6 - 4.4 kb fragments with age (see figure 6.5*c* for a selection of the plots).

In all but 1 case, telomere length declined. The smallest decline was of 130 base pairs and the largest 3770 base pairs, while rates of attrition ranged between 22 and

1157 base pairs per year (table 6.3). The difference in telomere length for the individual that showed an increase was 290 base pairs. However, it should be borne in mind that changes in telomere length below 1 kb fall within the bounds of measurement error, and hence for those cases the possibility that no change occurred cannot be discounted. If only individuals where the absolute difference between telomere length as a chick and as an adult was greater than 1 kb are considered, 12 individuals remain. These 12 birds all experienced telomere shortening of between 1.4 and 3.8 kb. So, while the general pattern was clearly for telomere length to decline between nestling and adult life (mean rate of loss for all 16 cases = -392 bp $yr^{-1}$), there was variation in telomere length as a chick (C.V. = 13%), the amount of telomere length change (C.V. = 59%), and the rate of change (C.V.= 75%, see figure 6.5*b*).

The rate of telomere length change between chick and adult life correlated with the age at which telomeres were measured as an adult ($r_s$ = -0.57, $n$ = 16, $p$ = 0.02). The rate of change was lower for birds sampled for the second time at 6 years of age ($n$ = 11, mean -281 bp $yr^{-1}$) than for birds sampled at 5 years ($n$ = 4, mean –504 bp $yr^{-1}$), although this difference was not significant ($t_{13}$ = -1.87, $p$ = 0.08). The rate of telomere length change for the single individual sampled for the second time at 2 years of age was -1157 bp $yr^{-1}$. This correlation between the rate of telomere change and the number of years between sampling suggests the rate of telomere loss is not constant over time. If there were a pattern of constant telomere attrition across the first 6 years of life, then the rate of change would be expected to be the same irrespective of how many years of ageing were monitored. This interpretation fits with the cross-sectional results which showed no detectable change in telomere length with age among adults. Therefore, in the following analyses the absolute difference in telomere length rather than the rate of telomere change was used, as this allows for variation in the amount of change occurring at different ages.

Variables that might explain differences among individuals in telomere length change were examined. No effect of sex on the magnitude of the change in telomere length was found ($F_{1,14}$ = 0.32, $p$ = 0.58). There was also no correlation between the amount of change in telomere length and growth rate during the linear phase of growth, between 8 and 30 days of age (Pearson correlation coefficient = -0.30, $p$ = 0.30). As the chicks in the longitudinal dataset were blood sampled at between 23

and 36 days of age, the linear growth rate corresponds to their growth at or just preceding sampling. Growth rate also did not correlate with telomere length as a chick (Pearson correlation coefficient = 0.29, $p$ = 0.32) or as an adult (Pearson correlation coefficient = 0.04, $p$ = 0.89). Similarly, no link between the peak mass of chicks, calculated from logistic growth curves, and the change in telomere length was seen (Pearson correlation coefficient = -0.38, $p$ = 0.20). There was no effect of hatching order ($F_{2,11}$ = 1.00, $p$ = 0.40), brood size at hatching ($F_{3,12}$ = 1.03, $p$ = 0.42), or brood size at fledging ($F_{2,13}$ = 1.18, $p$ = 0.34) on telomere length change, and none of these factors influenced telomere length as a chick (in all cases p > 0.05).

Both chick telomere length (Pearson correlation coefficient = -0.58, $p$ = 0.02) and mass controlled for skeletal size (mass/wing at 30 days of age, Pearson correlation coefficient = -0.62, $p$ = 0.01) correlated with the change in telomere length between chick and adult life. When these two variables were included in a multiple regression with the laying date of the eggs from which these individuals hatched, significant independent effects of all three on telomere length change later in life were found (laying date $F_{1,11}$ = 5.21, $p$ = 0.04, chick telomere length $F_{1,11}$ = 5.29, $p$ = 0.04, mass/wing length $F_{1,11}$ = 8.76, $p$ = 0.01). These three variables together explained 57% of the variance in telomere change. The interaction terms were not significant ($p$ > 0.05). Those shags with long telomeres as a chick, and chicks that were heavier in relation to their skeletal size, showed more telomere loss later in life (figures 6.5*d* and 6.5*e* respectively). Birds that hatched from late laid eggs also showed greater declines in telomere length than individuals that hatched earlier (figure 6.5*f*). The bird from the earliest egg in our data, laid March 21$^{st}$, exhibited total telomere loss of 130 base pairs over 6 years. In contrast, the individual from the latest egg, laid May 28$^{th}$, showed total telomeric attrition of 2210 base pairs over 2 years. Standardising laying dates by subtracting from each the median laying date for the respective year increased the significance of all three variables, as well as the proportion of the variance explained (telomere length as a chick $F_{1,11}$ = 7.36, $p$ = 0.020, body mass corrected for size $F_{1,11}$ = 12.31, $p$ = 0.005, date on which eggs were laid standardised for the median laying date in each year $F_{1,11}$ = 6.54, $p$ = 0.027, *adj.* $R^2$ = 61%, see figure 6.6). Neither laying date nor mass controlled for size were related to telomere length as a chick ($p$ > 0.05).

## DISCUSSION

The main findings of this study are that the telomeres of shag blood cells do not decline in length with age among breeding adults, but a significant difference in telomere length is observed between chicks and adults. This difference was confirmed in a longitudinal dataset, which also suggested there is variation among individuals in the rate of telomere change. A number of potential explanatory variables for differences in the amount of telomere length change were investigated, and it was found that telomere length as a chick, mass in relation to skeletal size, and the date on which individuals were laid as eggs all have independent effects. The repeatability of telomere length within broods was low, although there was a positive correlation between the telomere length of chicks and that of their parents. No difference was found between the sexes in telomere length or the rate of telomere length change. No effect on telomere length of natal year or measures of environmental conditions in the natal year was seen.

### *Telomere length in shag blood cells, in comparison to other animals*

Telomere length in shags ranged from 6.1 to 14.1 kb. This falls within the spectrum of telomere lengths recorded for other birds (see figure 6.7). The only bird species investigated so far for which there is no overlap with the shag in telomere length is Leach's storm petrel, with longer telomeres (Haussmann *et al.* 2003). Avian telomeres are similar in size to those of several mammals, including donkeys (7.4-20.5 kb, Argyle *et al.* 2003), dogs (9.7-23 kb, Nasir *et al.* 2001, McKevitt *et al.* 2002) and humans (4-15 kb, Allshire *et al.* 1988, Allshire, Dempster and Hastie 1989, Harley, Futcher and Greider 1990, Hastie *et al.* 1990). The exception among mammals is rodents which display substantially longer telomeres, and among which there is also greater overall length variation. For example, the rat (*Rattus rattus* and Fischer 344) has telomeres 15-100 kb long (Golubovskaya *et al.* 1999), while some strains of the mouse *Mus musculus* have telomeres up to 150 kb (Kipling and Cooke 1990, Starling *et al.* 1990, Prowse and Greider 1995, but see Hemann and Greider 2000). With regard to species lifespan it is not absolute telomere length, but the rate of change with age that appears to be important and this relationship is further discussed later.

It could be argued that telomere loss in blood cells may not be very important for ageing or senescence due to the short-lived and non-proliferative nature of these cells in the circulation. In birds, red blood cells are in circulation for approximately 28-35 days (chicken), which represents a turnover rate around double that of humans (at 50-60 days). Avian bursal lymphocytes are even more short-lived at only 15 days. However, blood cell telomere length is believed to reflect the length of telomeres in the hematopoietic stem cells (Rufer *et al.* 1999, Brummendorf *et al.* 2002). Candidate human stem cells purified from adult bone marrow have been found to have shorter telomeres than cells from foetal liver or umbilical cord blood, and loss of telomeric DNA was observed on proliferation of blood precursor cell cultures (Vaziri *et al.* 1994, Engelhardt *et al.* 1997). In addition, accelerated telomere shortening compared to healthy individuals has been observed for diseases in which increased stem cell turnover occurs, such as chronic myelogenous leukaemia (Brummendorf *et al.* 2000) and aplastic anaemia (Brummendorf *et al.* 2001), as well as in recipients of bone marrow transplants (Notaro *et al.* 1997, Mathioudakis *et al.* 2000, Rufer *et al.* 2001). This suggests that hematopoietic stem cells are limited in their ability to divide and that this decreases with age. The high turnover rate of blood cells means that telomeres might be expected to shorten more rapidly in hematopoietic stem cells than in other tissues. In humans, low levels of telomerase activity have been identified in blood precursors (Hiyama *et al.* 1995, Broccoli, Young and de Lange 1995, Yui, Chiu and Lansdorp 1998) but this activity is not sufficient to prevent telomere shortening (Vaziri *et al.* 1994, Engelhardt *et al.* 1997). Blood has the obvious advantages that it is easy to collect, sampling is non-destructive, and that it is often collected for other reasons.

### *The heritability of telomere length in the shag*

The repeatability of telomere length within broods was low, and therefore the telomere lengths of siblings are not particularly alike. Repeatability also sets an upper limit for the heritability of a trait (Falconer, 1960). This finding suggests that heritable genetic influences on telomere length and its regulation are not great in the shag, but also that environmental factors shared by nestlings did not contribute greatly to their telomere lengths. This may be due to differences among chicks in the same brood, for instance in their growth rate, although it seems unlikely that these

differences would be greater than those among broods. This result was also surprising given previous work showing a large genetic component contributing to telomere length in humans and mice. This was shown by twin studies in humans (Slagboom, Droog and Boomsma 1994, Rufer *et al.* 1999, Jeanclos *et al.* 2000), by the finding of characteristic telomere lengths for specific chromosome arms in different tissues of the same person (Martens *et al.* 1998), and by strain-specific telomere length variation in mice (Kipling and Cooke, 1990). However, the sample size of related individuals tested was small.

An alternative way of looking at this was to compare the telomere length or rates of telomere loss between parents and their offspring. When this was done for a small portion of our sample, a significant positive correlation between the telomere length of chicks and that of their parents was found. So this method, in contrast to the sibling comparison, suggests that telomere length is at least in part genetically determined. Clearly the sample sizes available here for assessing the extent of the heritability of telomere length in the shag are insufficient. Recent research in humans on the inheritance of telomere length suggests it may be X-linked (Nawrot *et al.* 2004); parent-offspring comparisons in the shag therefore may be improved by separating them into father-son, father-daughter, mother-son and mother-daughter contrasts. One problem is the lack of accurate pedigree information in the shag. As 18% extra-pair paternity has previously been found in this species on the Isle of May (Graves *et al.* 1992), it may not be adequate to assume adults occupying a particular nest site are the biological parents of the chicks at that site, especially in assigning paternity. Further work would be required to ascertain the level of genetic determination of telomere length or shortening rate in this bird.

### *No sex difference in telomere length or shortening rate in the shag*

There was no difference between the sexes in telomere length, or in their rates of intra-individual telomere length change. In most tests of sex-specific survival in adult shags no difference has been found between the sexes (Potts, Coulson and Deans 1980, Harris *et al.* 1994*b*, but see Potts 1969). Given that one sex is not more long-lived than the other, a sex difference in telomere length or its regulation is not expected. On the other hand, as the shag is a sexually dimorphic species in which adult males are on average about 20% heavier than females (Snow, 1960), it is

possible that differences in growth between the sexes could affect telomere dynamics. No sex difference in telomere length has yet been reported for any bird, although tests of this have only been published for the zebra finch (Haussmann and Vleck, 2002).

Females have been shown to have longer telomeres than males in humans (Jeanclos *et al.* 2000, Benetos *et al.* 2001), although not all studies have found any gender difference (e.g. Vaziri *et al.* 1993). The difference is not present at birth in humans (Okuda *et al.* 2002), suggesting that it arises from different rates of telomeric attrition later in life. Okuda *et al.* propose that the more gradual telomere loss in females may be due to effects of oestrogen, which can both stimulate telomerase (Kyo *et al.* 1999, Misiti *et al.* 2000) and reduce levels of oxidative stress (Tang and Subbiah 1996, Romer *et al.* 1997). Women produce fewer reactive oxygen species than men, and may metabolise them better because of this hormone (Aviv, 2002). Alternatively, the difference may be linked to telomere-regulating alleles carried on the X chromosome, in conjunction with differential X-inactivation in heterozygous women (Nawrot *et al.* 2004). Interestingly, one study on rats found different patterns of telomerase activity with age in males compared to females (Leri *et al.* 2000). While a decrease in telomerase activity with age in ventricular myocytes was observed in male rats, females showed increasing activity in the same tissue type as they grew older. Alternative explanations for the gender difference are that it could relate to differences in growth rate or metabolism between the sexes. The telomeres of males have also been found to be shorter than those of age-matched females in the mouse *M. spretus* (Coviello-McLaughlin and Prowse, 1997) and in the rat (Cherif *et al.* 2003) for a number of different tissues. In the latter of these studies, the sex difference in telomere length was only found after 3 months of age, and was not detected in 21 day old rats or at any age in the brain, in which cell number is fixed at birth. So this study again suggests that it is not the initial telomere length that differs between the sexes but the rate at which telomeric repeats are lost. The less rapid telomeric shortening in females, compared to males, ties in with their longer average lifespans in humans (Gavrilov and Gavrilova 1991, Guralnik, Balfour and Volpato 2000, Franceschi *et al.* 2000) and rats (Hales *et al.* 1996).

It must be borne in mind that longevity is affected by many factors acting throughout life, and I am not suggesting here that telomere length is responsible for the gender gap in longevity observed in humans. Survival rate may or may not relate to differences in biological ageing, depending in part on the levels of extrinsic mortality. The sex difference in humans, while a consistent trend, is not found in all populations (Gavrilov and Gavrilova, 1991) and moreover varies widely among populations (Smith, 1993), suggesting it can be modified by socio-economic factors. In addition, it has been estimated that a large proportion of the greater mortality of human males can be attributed to a higher incidence in that sex of high-risk behaviours (Waldron 1987, or see Arking 1998 for further discussion).

### *Telomere length and age*

There was no relationship between telomere length and age among breeding adult shags. While differences among cohorts, which shared the same natal environmental conditions, might have been obscuring a pattern of telomere loss with age in adults no evidence of this was found. Controlling for the year of hatching did not reveal a relationship between telomere length and age in the adult dataset. The only significant difference in length occurred between the telomeres of chicks (aged 1-39 days) and those of adults (aged 2-20 years) which were shorter. This is in contrast to the consistent age-related declines reported for the zebra finch, tree swallow, Adélie penguin, and common tern (Haussmann *et al.* 2003), where telomere length was found to decrease across the full range of ages studied. However, anomalous results reported for Leach's storm petrel, in which older birds displayed longer telomeres than younger individuals in a cross-sectional analysis (Haussmann *et al.* 2003), may suggest that telomeric attrition with age is not obligate in all bird species.

Positive regulation of telomere length is a possible explanation for the lack of telomere shortening among adults in shags and the increase in telomere length with age in Leach's storm petrel, but requires confirmation by longitudinal study. Preferably it should also be backed up by molecular work looking at the mechanism of such regulation. Telomere length can be maintained by telomerase or in some tissue types by alternative lengthening mechanisms. A recent investigation of telomerase activity in the bone marrow of four bird species (the short-lived zebra finch and tree swallow, and the long-lived common tern and Leach's storm petrel)

found relatively high telomerase activity in the two long-lived species, which did not decrease with age. In contrast, in the short-lived birds there was downregulation of telomerase in adult life (Haussmann *et al.* 2004). This suggests telomerase may be responsible for maintaining telomere length in long-lived birds. The presence of other lengthening processes in avian blood cells has not yet been tested. The telomerase-independent mechanism of telomere maintenance 'Alternative Lengthening of Telomeres' (ALT) has been observed in human cancers, immortalised human cell lines and telomerase-null mouse cell lines (Bryan *et al.* 1995, Bryan and Reddel 1997, Bryan *et al.* 1997, Henson *et al.* 2002), but has not been described for any normal tissues. The exact mechanism by which ALT acts has not been elucidated, but one possibility is non-reciprocal recombination between telomeres, as occurs in several yeast species (Pluta and Zakian 1989, Wang and Zakian 1990, Lundblad and Blackburn 1993, McEachern and Blackburn 1996).

The failure to demonstrate telomere attrition with age in adult shags could reflect problems of using cross-sectional analysis to address this question, or with the method used to measure telomeres. In both cases, possible problems arise from heterogeneity among individuals. In cross-sectional comparisons, unlike longitudinal analyses, there is no inherent control of factors unique to individuals. As this study was conducted in a wild population of birds, past and present conditions to which individuals have been subjected have in no way been controlled and in addition are not fully known. For instance, the incidence of disease within this population is not known. Within-individual variation in telomere length between different chromosomes (Martens *et al.* 1998) and even between the 2 alleles of a single chromosome (Baird *et al.* 2003) may also be contributing to the background noise in our data. The possibility cannot be dismissed that, although cross-sectional analysis of mean TRF length was able to pick up the large difference produced by rapid loss early on, it was not sensitive enough to identify small changes during adult life due to more gradual attrition.

Using the traditional method of estimating telomere length from TRF size, relative mean TRF lengths of individuals can vary by as much as 5% depending on the particular restriction enzymes used (Cawthon, 2002), which suggests the existence of subtelomeric restriction site polymorphisms. This may confound the identification of factors accounting for inter-individual variation in the mean length

of true telomeric repeats. In humans the sub-telomeric region is estimated to be 2.5-4 kb long (Allshire, Dempster and Hastie 1989, Levy *et al.* 1992), and its mean length may vary by up to 2 kb between individuals (Hultdin *et al.* 1998, Cawthon 2002). Techniques for measuring telomeric DNA *per se* have recently been developed to eliminate this problem (Bryant *et al.* 1997, Norwood and Dimitrov 1998, Nakamura *et al.* 1999), for instance by quantitative PCR (e.g. Cawthon 2002, see methods review by Lauzon *et al.* 2000) or single telomere length analysis of individual chromosomes (STELA, Baird *et al.* 2003).

Our results may be explained by changes in the rate of telomere loss through life. As the only difference in telomere length found in shag blood cells was between chicks and adults, it may be that rapid shortening occurs in the chick or juvenile phase followed by no or extremely gradual changes in later life. This is supported by the steep decline in telomere length observed for the single individual sampled for the second time at 2 years of age in the longitudinal dataset, and the correlation between the rate of telomere change and the number of years over which the change is assessed. Rapid telomere attrition early in life has already been reported for humans (Frenck, Blackburn and Shannon 1998, Rufer *et al.* 1999, Zeichner *et al.* 1999, Friedrich *et al.* 2001) and domestic cats (Brummendorf *et al.* 2002), as described in the introduction. This rapid shortening most likely relates to the large amount of somatic growth and development that occurs during childhood. In humans, the proliferation rate of granulocyte-macrophage progenitor cells is significantly higher in neonates compared to adults (Christensen, Harper and Rothstein, 1986). Human hematopoietic stem cell turnover has been estimated to be of approximately 15 population doublings in the first 6 month of life, followed by 5-6 additional cell divisions until adulthood (Rufer *et al.* 1999). A similar pattern of high turnover early in life was estimated for cats, with around 30 population doublings in the first year of life (Brummendorf *et al.* 2002). Rapid telomere loss in childhood could also be caused by differential regulation of telomere length through life or by differences in environmental conditions, such as oxidative stress levels.

The longitudinal results comparing the telomere lengths of individuals at under 1 year of age to that at 2, 5 or 6 years old show the same pattern of telomeric loss between chick and adult phases as seen in the cross-sectional analysis. This is the first study in birds to confirm a cross-sectional trend of telomere loss with age by

longitudinal comparisons within the same tissue. Looking at the longitudinal results in more detail, the most rapid reduction in mean telomere length is seen in the blood cells of the individual sampled for the second time at 2 years of age, that is the individual for which the sampling interval was shortest. Unfortunately, only one 2-year-old was sampled in the longitudinal dataset so it is not possible to discriminate between unusually rapid telomere loss in this particular bird and the possibility that most loss generally occurs before 2 years of age. In addition, if mortality is higher for individuals with very rapid telomere loss, samples of older birds will be biased towards those with less rapid telomeric attrition. Looking at serial samples taken during the chick period would allow further definition of the age at which telomere loss is most rapid in this bird.

### *Explanatory variables for differences in the rate of telomere shortening*

As well as confirming the result from cross-sectional analysis of a decline in telomere length between chicks and adults, longitudinal analysis also exposed differences among individuals in the amount of telomere change. This raised the question of what might be responsible for these differences. Growth rate is one factor likely to influence telomere attrition, via cell division to increase cell number. Nutritional levels might also influence telomere shortening, either via growth rate or by modifying the amount of oxidative damage, for instance if diet-derived anti-oxidants vary. A number of potential explanatory variables for differences in telomere length change within the longitudinal dataset were analysed.

Sex, nestling growth rate during the linear phase at 8-30 days of age, the peak mass of chicks, the order in which they hatched and brood size were all found to be unrelated to the magnitude of telomere loss in this longitudinal sample. This may in part be due to the relatively small sample size assessed. Individuals in the sample were likely to differ in a number of potential explanatory variables, for instance be of different sex, from broods of different size and undergoing different rates of growth. To give an indication of the amount of variation in these variables, growth rates ranged from 49 to 67 grams per day and peak mass from 1392 to 1794 g. There were 9 first-hatched 'A' chicks, 3 second-hatched 'B' chicks and 2 third-hatched 'C' chicks, with hatching order unknown for the 2 remaining individuals. Brood sizes at

fledging included 2 cases with 1 chick only, 9 where 2 chicks fledged and 5 that fledged 3 chicks.

Greater telomere loss in individuals laid as eggs later in the season was intriguing. This relationship could be linked to growth differences. Faster growth means greater rates of cell division, plus levels of oxidative stress are higher when growth is fast (Rollo, 2002). However, in most cases the offspring of birds that breed earliest grow faster (Harris 1980, Birkhead and Nettleship 1982, Gaston *et al.* 1983, Weimerskirch 1990, Cooch *et al.* 1991, Larsson and Forslund 1991, Sedinger and Flint 1991, Kersten and Brenninkmeijer 1995, Lepage, Gauthier and Reed 1998). This also appears to be the case in shags as analysis of a wider dataset ($n$ = 112) from 2001 revealed a significant negative correlation between the linear growth rate of chicks and the date on which they were laid as eggs in that year ($r_s$ = -0.32, $p$ = 0.001). Therefore, greater cell proliferation and hence more speedy telomere loss would be expected to occur in chicks that hatched earlier, not later. However, the relationship between laying date and growth rate is not simple, as birds that lay at different times in the season may also differ in quality. For instance in shags young pairs, and especially first-time breeders, tend to breed later in the season (Potts, Coulson and Deans 1980, Aebischer 1993, Daunt *et al.* 1999). These pairs also lay smaller eggs (Coulson, Potts and Horobin 1969), and have lower overall breeding success than older pairs (Potts, Coulson and Deans 1980, Aebischer 1993, Aebicher, Potts and Coulson 1995, Daunt *et al.* 1999). The apparent effect of laying date on the amount of telomere loss could therefore tie in with differences in parental genotype, which could affect offspring via inheritance or via parental rearing capabilities. It potentially could also relate to differences in diet across the breeding season, or reflect egg differences. Oxidative stress varies with levels of diet-derived anti-oxidants (Beckman and Ames, 1998), and eggs can also differ in their anti-oxidant properties depending on the diet of the laying bird (Speake *et al.* 1999). So, if late-laid eggs give rise to chicks in poor condition, the anti-oxidant defences of these chicks may also be compromised incurring a higher rate of telomere loss via oxidative damage. Interestingly, there is evidence that post-fledging survival to breeding age of early-hatched shags is much higher than that of late-hatched individuals (Harris *et al.* 1994*a*).

Despite the lack of any relationship between linear growth rate or peak mass and telomere change, it was found that the mass of chicks at 30 days of age, when controlled for skeletal size, had a significant effect on subsequent telomere loss. Chicks that were heavy for their size showed greater telomere loss later in life. As the growth rate measure and peak mass did not take into account skeletal size, it may be that the type of growth rather than simply the rate of mass gain is important for telomere dynamics. In addition, because mass controlled for size at 30 days did not affect the telomere length at that age, but only the change in telomere length that was observed later, it appears that this variable is having delayed effects on telomere dynamics.

Telomere length as a chick correlated with longitudinal telomere length change, such that those individuals with greater telomere length at the first measurement were found to have the greatest telomere loss. This may relate to the mechanism of telomere shortening at the molecular level, if more telomeric repeats are lost from longer arrays, either passively or in a regulated fashion. However, work on telomere dynamics in yeast *Saccharomyces cerevisiae* suggests that the degradation rates of telomeres, in the absence of telomerase activity, are constant and independent of length (Marcand, Brevet and Gilson, 1999). Interestingly, telomerase-mediated elongation rates in yeast were shown in that study to be influenced by telomere length, with inhibition of telomerase by the elongating telomere. If telomerase is active in the hematopoietic stem cells or blood cell precursors of the shag, then this mechanism could explain a pattern of greater telomere length reduction in individuals with longer starting lengths. Given that there was no decrease in telomere length with age among adult shags, it may be that telomerase is only active in adults and can maintain telomere length at that time. A possible scenario would be as follows. In chicks with no active telomerase there is rapid shortening, presumably at a constant rate per cell division. After activation or upregulation of telomerase later in life, the level of telomere elongation depends on the length of the telomere (as for yeast). At that stage individuals in which telomere length is greater will show repression of telomerase, less elongation, and so greater telomeric loss overall as a consequence of base-line degradation. In contrast individuals with short telomeres upon activation or upregulation of telomerase would not repress its action and so elongation processes would counteract shortening and the shortening rate

would be less. Of course, this supposition relies completely on results from one other, distantly related, organism, and as such is only a very tentative potential explanation for the observed pattern.

Recent work on telomere shortening in cultured human fibroblasts suggests another possible mechanism at the molecular level for this pattern. It has been shown that overexpression of telomeric repeat binding factor 2 (TRF2), a telomere-associated protein, increases the rate of telomere shortening (Karlseder, Smogorzewska and de Lange, 2002). Baird *et al.* (2003) suggest faster erosion might therefore occur at longer telomeres because they have more TRF2 bound although, at least in telomerase-negative human fibroblasts, they did not observe such a trend. It is not known whether TRF2 or a homologous protein is present in avian cells. It should also be kept in mind that a higher chance of recording a decline occurs when the initial measurement is of large magnitude.

The relationships between the amount of telomere change and starting length, between chick mass controlled for size and subsequent telomeric shortening, and the effect of laying date on telomere dynamics all warrant further investigation. This should be via both longitudinal comparisons within larger samples of individuals, and if possible by experimental manipulation of the variables proposed to influence telomere loss. The latter would be more easily performed in a captive species of bird.

### *No effect of hatching year or natal environmental conditions on telomere length*

Although the effects of laying date and chick mass/ wing length on telomere change in the longitudinal data suggest that telomere length can be influenced by environmental conditions, there was no evidence of environmental effects at the cohort level. It was shown that the difference in telomere length between chicks and adults could not be explained by the different years of hatching occurring in these 2 groups, or the specific environmental conditions found in the year that a bird hatched. The most parsimonious explanation remains that this contrast is due to age. It may be that conditions experienced as a chick shorten telomeres rapidly, but a change in these conditions at or during adulthood means attrition becomes negligible. This explanation fits with what has previously been observed in humans.

Alternatively, telomere length regulatory factors in hematopoietic precursor cells may be altered as these birds age, for instance telomerase activity, as discussed in the previous section.

No evidence that environmental conditions in the year of hatching influence telomere length as a chick or later in life was found. However, it became obvious during examination of this data that in order to test for cohort effects a larger sample size would be preferable. In particular it would be useful to have sampled in a greater number of years (even though sampling for this study did span 6 different years), such that the correlation between age and year of hatching was reduced. There may not have been a large enough contrast between the environmental conditions in each hatching year in our data. In addition, the reproductive strategy of the shag, which is long-lived and will skip breeding in exceptionally poor years, may make cohort effects particularly difficult to detect. From our results, it appears that the relative date on which an individual hatched within the season has a greater impact on their subsequent telomere change than the year in which they were born.

### *Telomere length and lifespan*

No relationship has been found between telomere length and species lifespan (Hemann and Greider 2000, Vleck, Haussmann and Vleck 2003). However, Rohme (1981) showed that the replicative capacity of fibroblasts relates to species longevity for a range of mammals. It has now also been demonstrated that the telomere rate of change (TROC) correlates with species maximum lifespans in both birds and mammals (Haussmann *et al.* 2003). This relationship was revealed using rates of telomere loss calculated from regressions of telomere length on age in cross-sectional comparisons. Our data suggest that the rate of telomere shortening in shags may not be constant throughout life, and consequently that TROC will depend on the particular age distribution being assessed. The large heterogeneity among individuals in telomere change observed in the longitudinal shag data also brings into question how meaningful a measure average TROC is. However, out of interest the average reduction in telomere length between shag chicks and adults was compared to the reduction reported for other avian species with regard to maximum lifespan.

European shags can live for at least 30 years (longevity record of 30 years, 6 months, Clark *et al.* 2002). In 2003 a 23 year old individual was seen on the Isle of May (personal observation), so these birds do approach the species longevity record at that site. The regression equation given in Haussmann *et al.* predicts, based on a maximum longevity of 30 years, that telomere length in shags should show a reduction of only 22 base pairs per year. This is more than 10-fold less than the rate of shortening I recorded (slope for the overall regression of telomere length on age = -300 bp $yr^{-1}$, average rate of telomere length change from longitudinal comparison = -392 bp $yr^{-1}$). Based on our shortening rates and Haussmann *et al.*'s regression equation, the maximum lifespan of shags would be predicted as between 11 and 16 years (see figure 6.8). Cross-sectional analysis of telomere length with age in the wandering albatross (*Diomedea exulans*) gave an average rate of telomere change of –160 bp $yr^{-1}$ (Hall *et al.* 2004). This also differs widely from what Haussmann *et al.*'s regression equation predicts for this bird. Even using a conservative estimate of maximum lifespan for wandering albatrosses of 50 years (Croxall and Prince 1990, Jouventin *et al.* 1999), the prediction is for their telomeres to lengthen by 368 bp $yr^{-1}$, which would result in extremely long telomeres in the oldest albatrosses. Lengthening of telomeres in the albatross also contradicts an observed pattern of loss between chicks and adults. As illustrated in figure 6.8, results from the shag and wandering albatross do not support the hypothesis that long-lived birds have escaped telomeric constraints on cellular replicative lifespan, instead suggesting a levelling-off of the relationship between TROC and maximum lifespan as TROC approaches zero.

In Haussmann *et al.*'s study reported maximum lifespans in the wild were used, which are based on sightings of banded birds, as is the estimate of maximum lifespan in shags. Gavrilov and Gavrilova in their 1991 book discuss the drawbacks of using these measures of the oldest living organism as 'species-specific lifespan'. Among the potential problems is the fact that the magnitude of the greatest lifespan grows with an increase in the number of observations, and so if there are differences in the size of the banded population for different species maximum lifespan estimates may not be comparable. Additionally, the age of the oldest living organism is not an invariant measure for lifespan in a particular species, for instance in humans it has been shown to differ over time for a population of a single country as socio-

economic and living conditions change. A further problem with the analysis is that phylogeny was not controlled.

### *Applications of telomere length investigation in birds*

Investigating variance in telomere length and telomere rates of change among species and among individuals within a species, as a means of exploring factors responsible for biological ageing, is perhaps the most interesting application of telomere studies in birds. The recent finding of differences in bone marrow telomerase activity between short- and long-lived bird species (Haussmann *et al.* 2004) suggests maximum lifespan may be linked to the regulation of this enzyme through life and the consequent rate of telomere shortening. If this is the case, the question is raised of how these long-lived birds avoid the increased risk of cancer associated with the presence of telomerase (Harley *et al.* 1994). This aspect of telomere study in birds is of greater interest, and likely to offer more insight at this time, than the use of telomere length as a marker of chronological age.

Estimating chronological age from telomere length suffers from a number of problems. Firstly, age cannot be estimated very accurately using this method. For instance, in the common tern Haussmann, Vleck and Nisbet (2003) reported a standard error of estimate of 4.7 years from the regression predicting age from telomere length, and were only able to class birds into 3 broad age categories with 80% reliability. In addition, the studies published so far that attempt to measure age by telomere length have all based their estimates on cross-sectional comparisons, and have not yet illustrated that telomeres shorten in the same manner within individuals as they age for those species. The scale of the variability in telomere length change with age among individuals is unknown. So little is currently known about the factors affecting telomere length in birds that to use this measure as equivalent to chronological age, when age can be determined accurately by ringing, seems risky. Although age determination by ringing requires long-term and intensive ringing effort, individuals are often ringed for other purposes anyway, for instance for individual recognition in behaviour or migration studies. As well as these drawbacks telomere length measurement, at least using southern blot hybridisation, is not a particularly quick and easy method to perform. The protocol is in fact quite time-consuming when large numbers of samples are to be measured, hence recent

advances in methodology such as Q- and flow- FISH. Fairly high quality genomic DNA samples are needed, and the method also often requires modifications before reliable results can be produced, for instance to deal with interstitial telomeric sequence as has been the case in zebra finches (Venkatesan and Price 1998, Whitaker 2002 unpublished PhD thesis).

## FIGURES

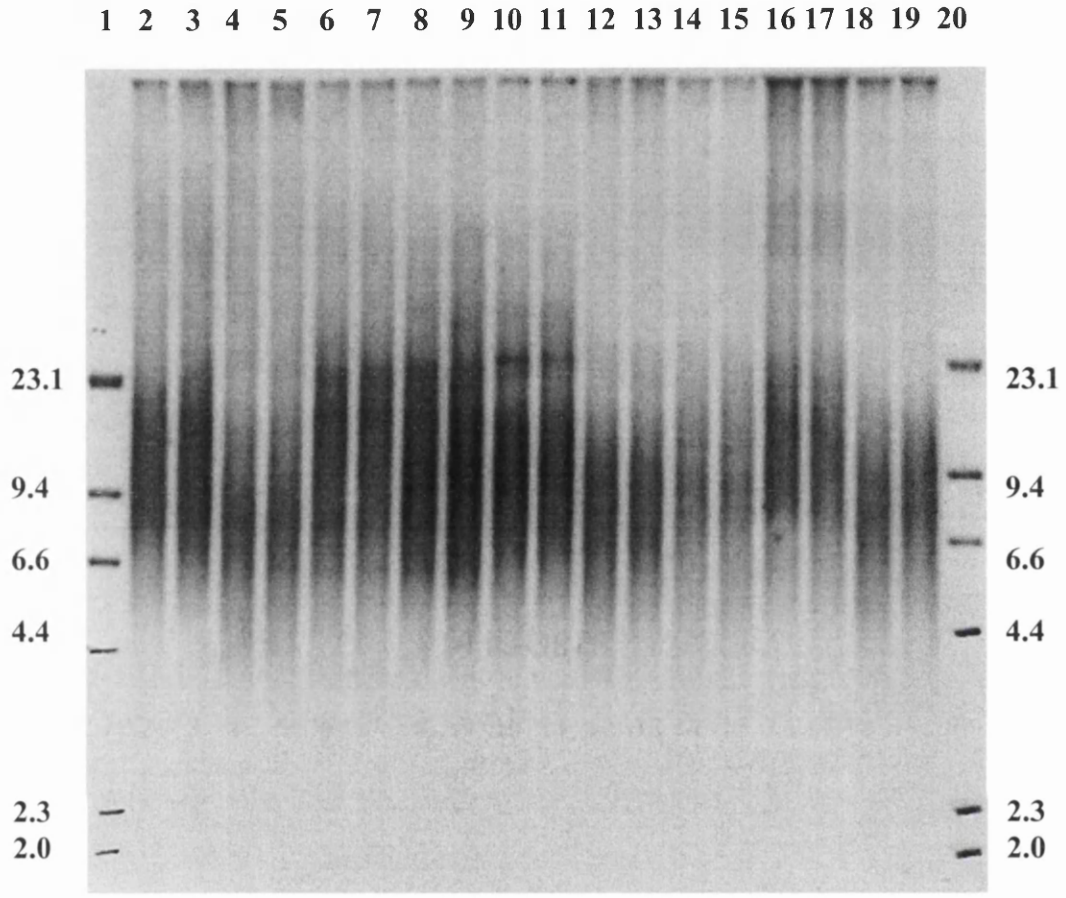



**Figure 6.1** A typical autoradiograph image of telomere restriction fragments from shag blood cells. The size distribution of TRFs appears as a smear in each lane. Molecular weight markers (values in kilobases) are in *lanes 1 and 20.* Duplicate samples appear in adjacent lanes: *lanes 2 and 3* male 7 year old, *4 and 5* female 7 year old, *6 and 7* female 14 year old, *8 and 9* male 6 year old, *10 and 11* male of 14 years minimum, *12 and 13* male 7 year old, *14 and 15* female 14 year old, *16 and 17* female 9 year old, *18 and 19* male 9 year old. Refer to table 6.1 for the corresponding values of mean, peak and range TRF lengths.

| Sample | Lane | Age (years) | Sex | Mean TRF length (kb) | Peak TRF length (kb) | Range of TRF lengths (kb) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Smallest fragment | Largest fragment [b] |
| 1 | 2 | 7 | Male | 10.3 | 8.8 | 3.8 | >23 |
| | 3 | | | 10.2 | 8.9 | 4.0 | >23 |
| 2 | 4 | 7 | Female | 7.9 | 8.3 | 3.0 | 22.6 |
| | 5 | | | 8.0 | 8.1 | 3.4 | 22.2 |
| 3 | 6 | 14 | Female | 10.6 | 16.8 | 3.7 | >23 |
| | 7 | | | 10.7 | 16.8 | 3.6 | >23 |
| 4 | 8 | 6 | Male | 9.6 | 10.5 | 3.7 | >23 |
| | 9 | | | 9.4 | 10.0 | 3.5 | >23 |
| 5 | 10 | 14[a] | Male | 10.0 | 10.4 | 4.2 | >23 |
| | 11 | | | 10.3 | 10.6 | 3.9 | >23 |
| 6 | 12 | 7 | Male | 7.8 | 9.0 | 3.8 | >23 |
| | 13 | | | 8.4 | 9.4 | 4.0 | >23 |
| 7 | 14 | 14 | Female | 8.7 | 9.2 | 3.7 | >23 |
| | 15 | | | 8.6 | 9.4 | 3.9 | >23 |
| 8 | 16 | 9 | Female | 11.1 | 10.4 | 4.9 | >23 |
| | 17 | | | 10.4 | 10.3 | 4.3 | >23 |
| 9 | 18 | 9 | Male | 7.5 | 8.4 | 3.7 | 19.4 |
| | 19 | | | 7.8 | 7.8 | 3.9 | 19.8 |

[a] for this bird only the minimum age is known, because it was ringed as an adult.

[b] it is not possible to accurately estimate fragment size above the uppermost limit of the molecular weight standard. Therefore all telomere fragments of greater size are denoted > 23 kb.

**Table 6.1** Mean, peak and range TRF lengths for lanes 2-19 in Figure 6.1.

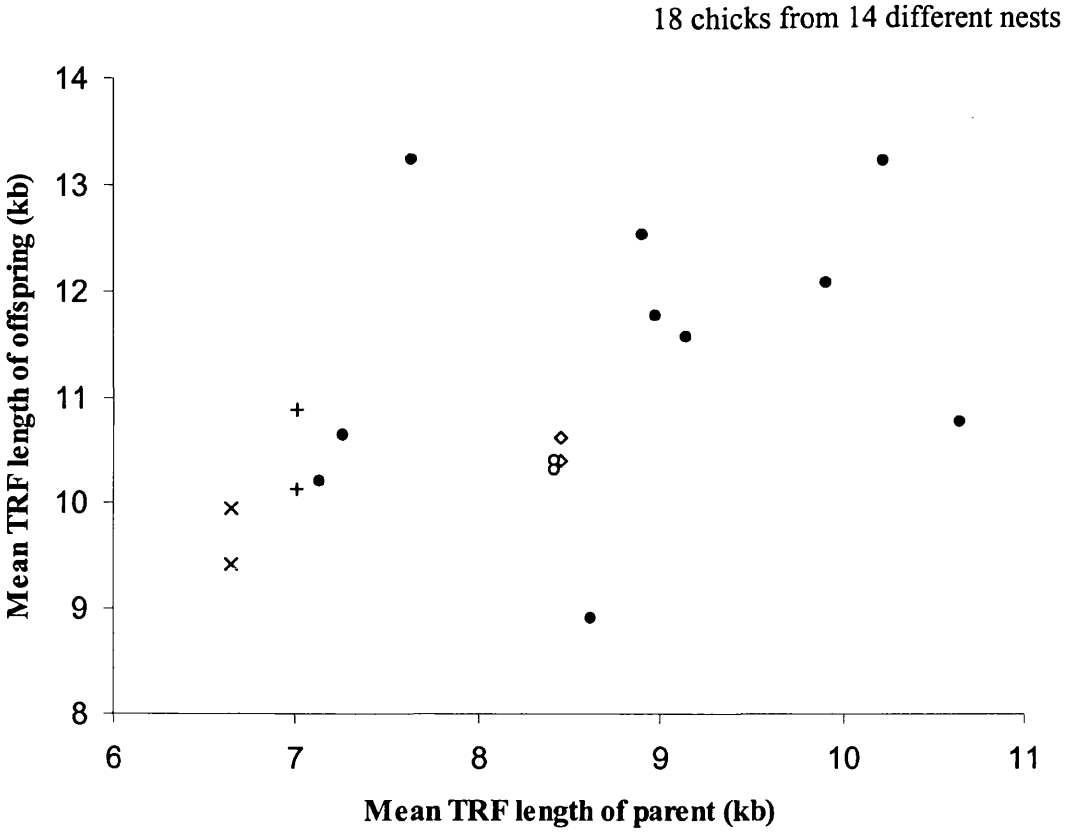
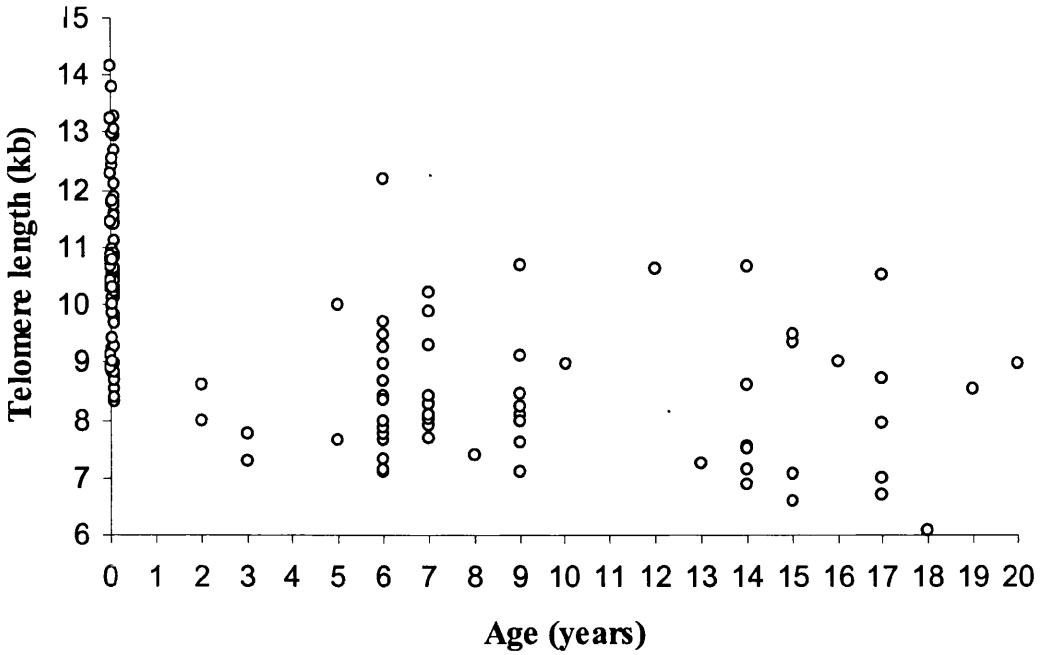
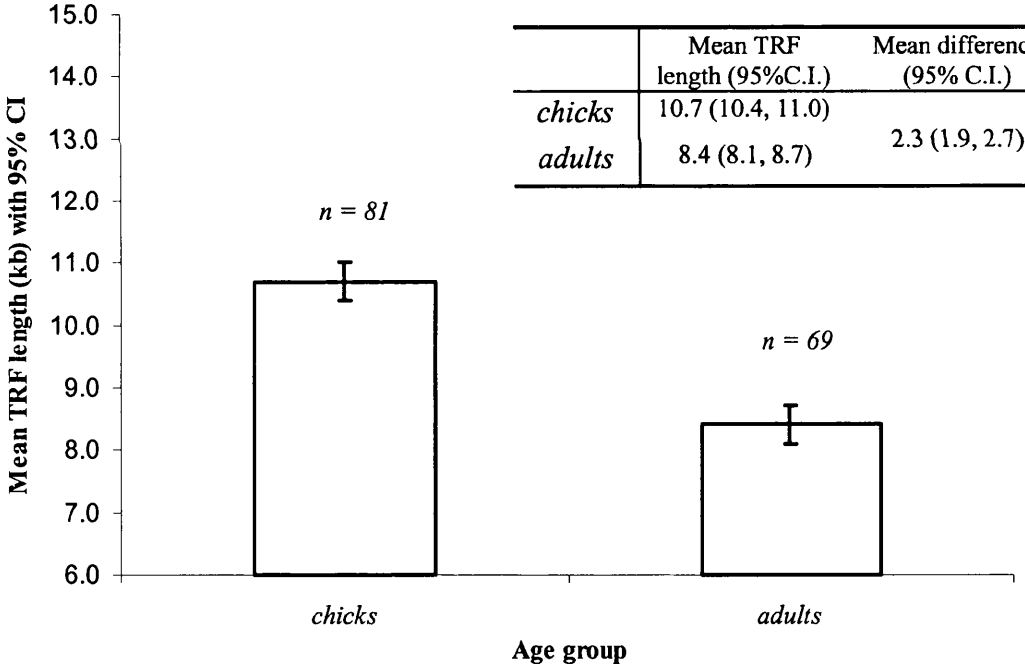



**Figure 6.2** There was a significant positive correlation between the telomere length of parents and offspring. Four pairs of chicks from the same nest have been given different matching nest-specific symbols in the figure. The correlation was significant whether these siblings were included or if only 1 chick per nest was used in the analysis.

*(a)*




*(b)*




| | Mean TRF length (95%C.I.) | Mean difference (95% C.I.) |
|---|---|---|
| *chicks* | 10.7 (10.4, 11.0) | 2.3 (1.9, 2.7) |
| *adults* | 8.4 (8.1, 8.7) | |

**Figure 6.3** *(a)* Mean telomere length of known age shags ($n$ = 144, cross-sectional comparison). The age range of birds under 1 year old is 1 - 39 days. *(b)* The only significant difference in mean TRF length was between chicks and breeding adults ($F_{1,149} = 123.24, p < 0.001$). Means and the mean difference are shown in kilobases.

*(c)*

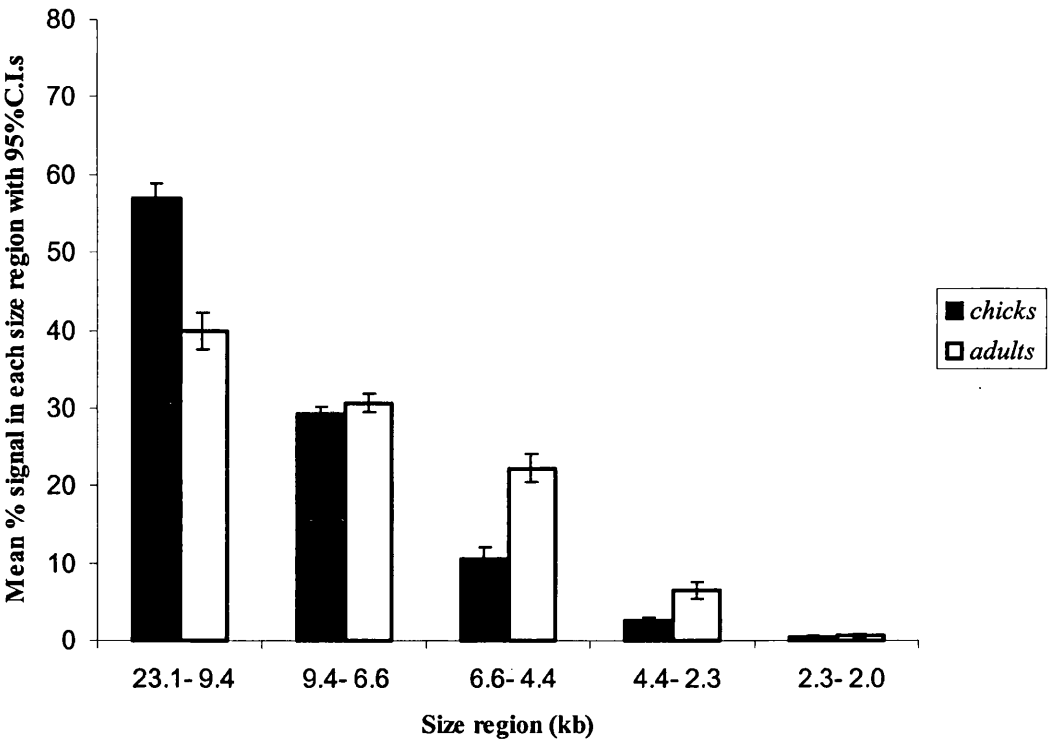
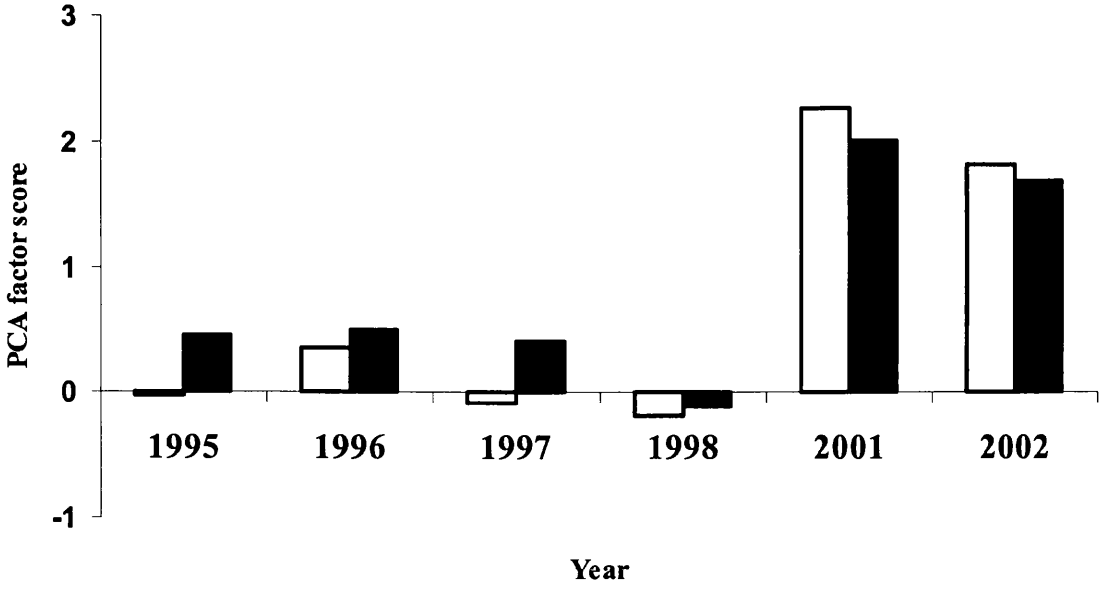



**Figure 6.3** *(c)* The percentage signal intensity in each of 5 size regions defined by the molecular weight standard (after Jennings *et al.* 1999, Cherif *et al.* 2003), for nestling shags (under 1 year of age) compared to adults (2-20 years of age).

| Year hatched | Year sampled | | | | | | | | | | | | Total per hatching year |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1997 | | 1998 | | 1999 | | 2001 | | 2002 | | 2003 | | |
| | age | no. | age | no. | age | no. | age | no. | age | no. | age | no. | |
| 1981 | 16 | | 17 | | 18 | | 20 | **1** | 21 | | 22 | | 1 |
| 1982 | 15 | | 16 | | 17 | **1** | 19 | | 20 | | 21 | | 1 |
| 1983 | 14 | | 15 | | 16 | | 18 | | 19 | **1** | 20 | | 1 |
| 1984 | 13 | | 14 | | 15 | **1** | 17 | **5** | 18 | **1** | 19 | | 7 |
| 1985 | 12 | | 13 | | 14 | | 16 | **1** | 17 | | 18 | | 1 |
| 1986 | 11 | | 12 | | 13 | | 15 | **3** | 16 | | 17 | | 3 |
| 1987 | 10 | | 11 | | 12 | | 14 | **4** | 15 | | 16 | | 4 |
| 1988 | 9 | | 10 | | 11 | | 13 | | 14 | **2** | 15 | | 2 |
| 1989 | 8 | | 9 | | 10 | | 12 | **1** | 13 | **1** | 14 | | 2 |
| 1990 | 7 | | 8 | | 9 | **1** | 11 | | 12 | | 13 | | 1 |
| 1991 | 6 | | 7 | | 8 | | 10 | **1** | 11 | | 12 | | 1 |
| 1992 | 5 | | 6 | | 7 | | 9 | **7** | 10 | | 11 | | 7 |
| 1994 | 3 | | 4 | | 5 | | 7 | **9** | 8 | **1** | 9 | | 10 |
| 1995 | 2 | | 3 | | 4 | | 6 | **4** | 7 | | 8 | | 4 |
| 1996 | 1 | | 2 | | 3 | **2** | 5 | | 6 | **5** | 7 | | 7 |
| 1997 | < 1 | **12** | 1 | | 2 | **1** | 4 | | 5 | **1** | 6 | **7** | 21 |
| 1998 | — | — | < 1 | **15** | 1 | | 3 | | 4 | | 5 | **1** | 16 |
| 2001 | — | — | — | — | — | — | < 1 | **35** | 1 | | 2 | **1** | 36 |
| 2002 | — | — | — | — | — | — | — | — | < 1 | **19** | 1 | | 19 |
| Totals | 12 | | 15 | | 6 | | 71 | | 31 | | 9 | | 144 |

**Table 6.2** The distribution in relation to year of hatching and sampling year of known-age birds in the cross-sectional telomere length data. The dataset contained 19 hatching years (cohorts) and blood samples were taken in 6 different years. The table illustrates how age at sampling depends upon hatching year and sampling year. All ages in the table are given in years, and shags sampled as chicks are shown as age < 1.

*(a)*




□ factor 1 from PCA of mean plot success, no. young ringed, brood size

■ factor 1 from PCA of mean plot success, no. young ringed, brood size and median ringing date

*(b)*

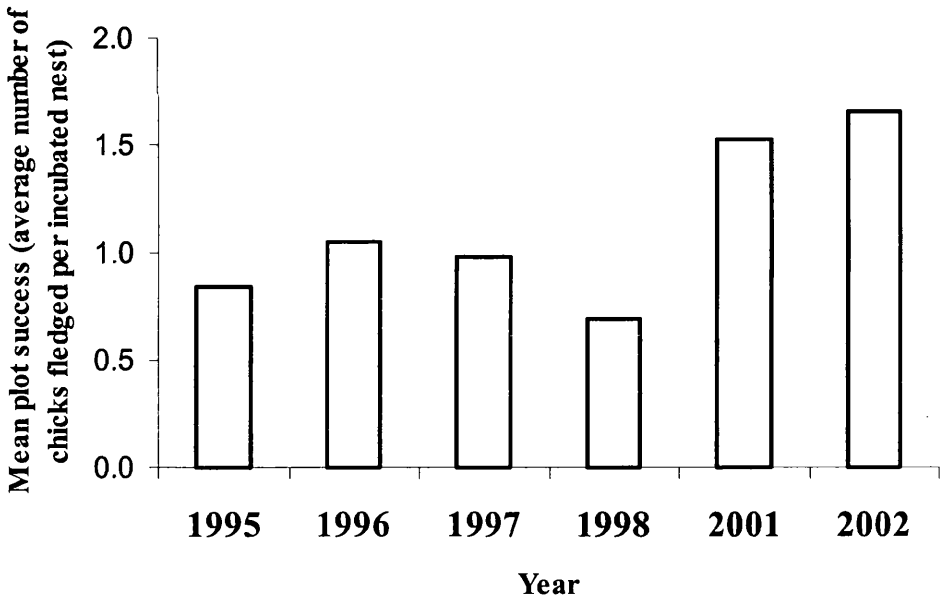
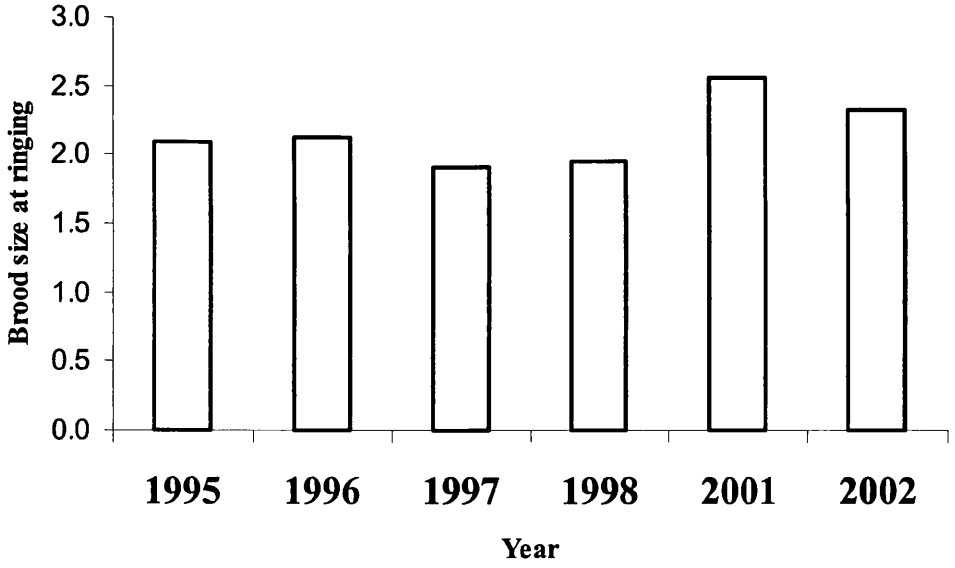



**Figure 6.4** For the 6 different natal years of chicks and 6 year olds in the cross-sectional dataset, *(a)* factor 1 scores from 2 principal component analyses of measures of breeding success, cohort size and the timing of breeding, and *(b)* mean plot success (number of chicks fledged per incubated nest, averaged across monitoring plots).

*(c)*




*(d)*

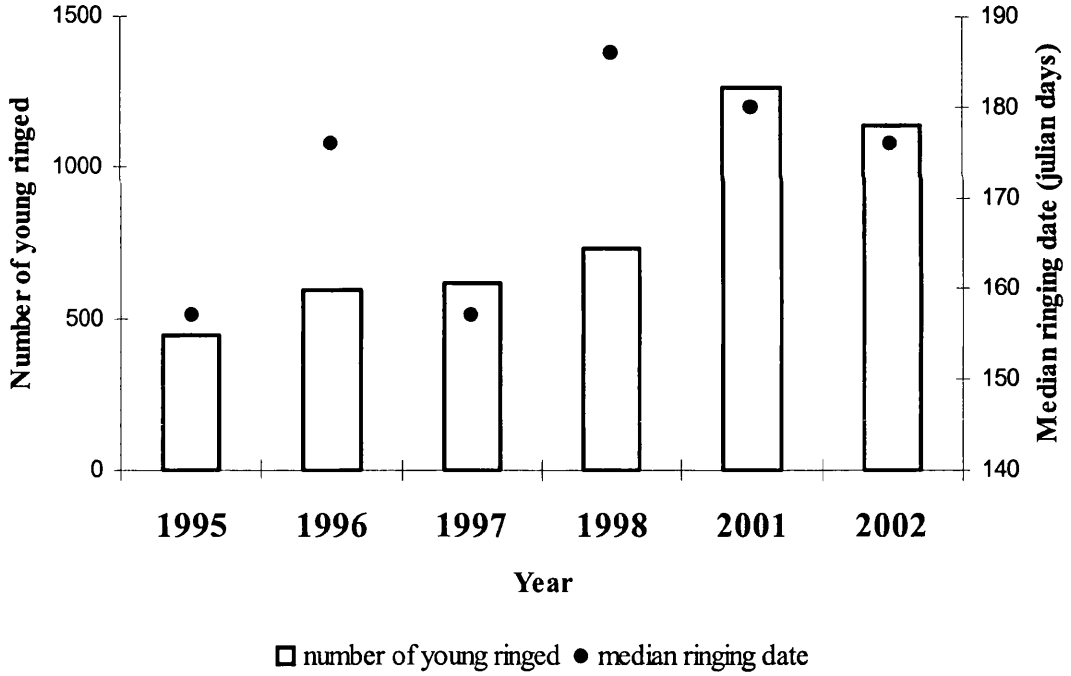
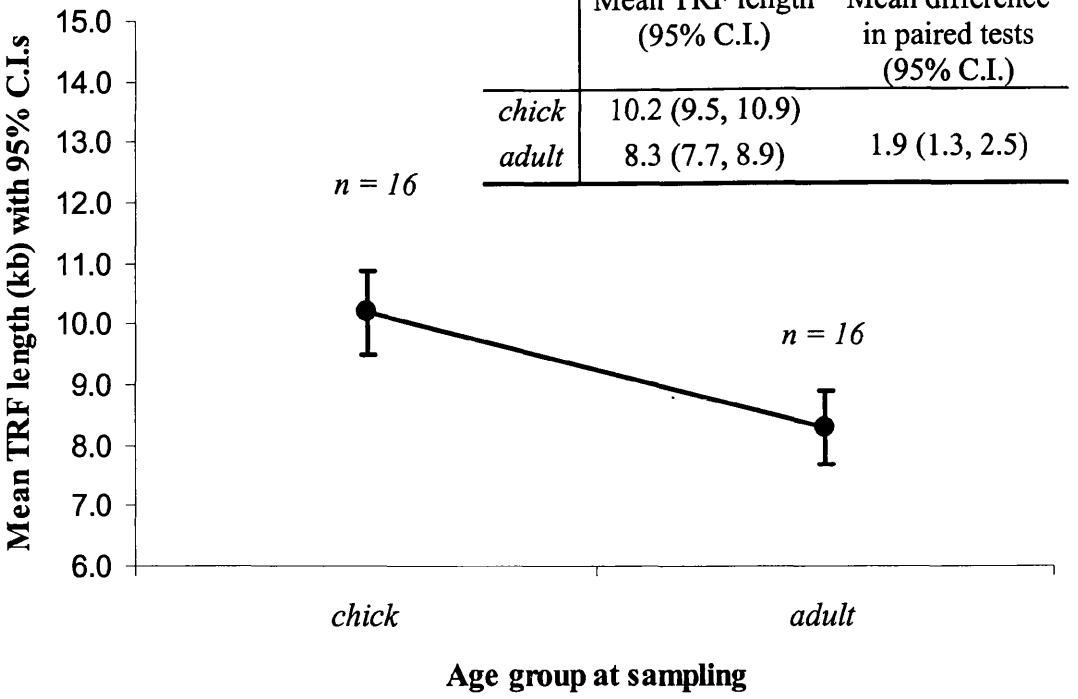



**Figure 6.4** For the 6 different natal years of chicks and 6 year olds in the cross-sectional dataset, *(c)* brood size at ringing, and *(d)* the number of young ringed and the median ringing date each year. Julian day 140 corresponds to May 20th, and julian day 190 is July 9th.

*(a)*

| | Mean TRF length (95% C.I.) | Mean difference in paired tests (95% C.I.) |
|---|---|---|
| *chick* | 10.2 (9.5, 10.9) | |
| *adult* | 8.3 (7.7, 8.9) | 1.9 (1.3, 2.5) |




*(b)*

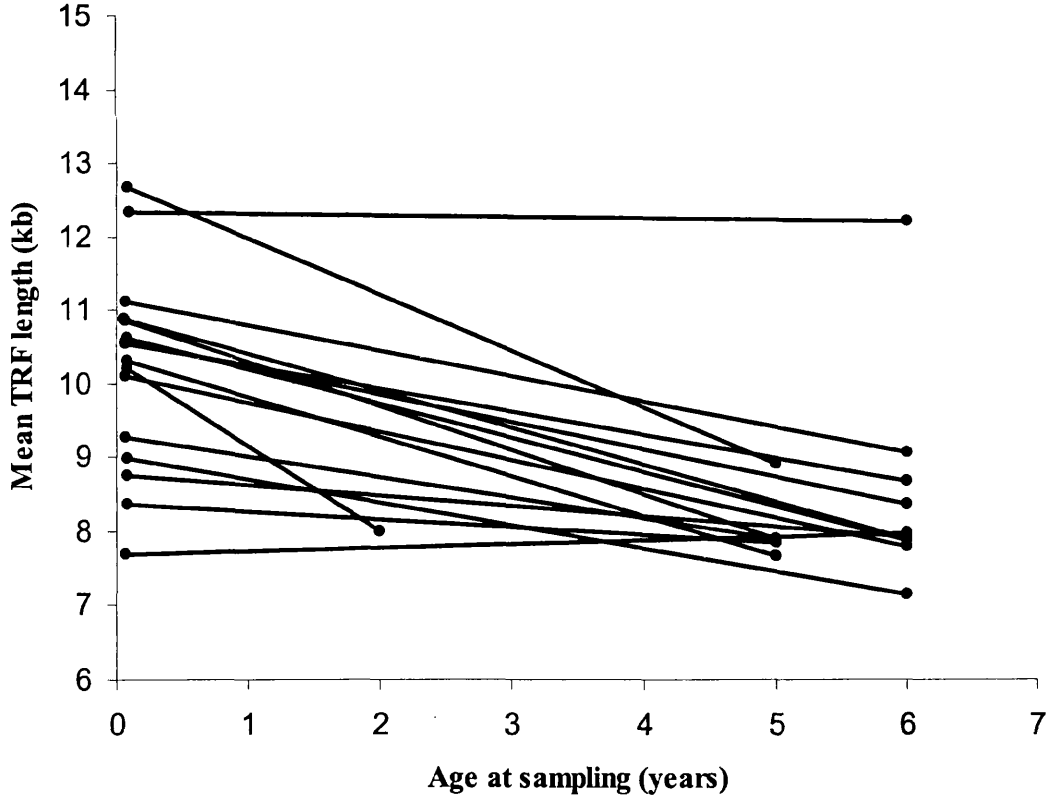



**Figure 6.5** *(a)* Longitudinal comparisons of mean telomere length, for individuals sampled as chicks and again as adults, and *(b)* the rate of change in telomere length varies between individuals.

(c) Longitudinal comparisons

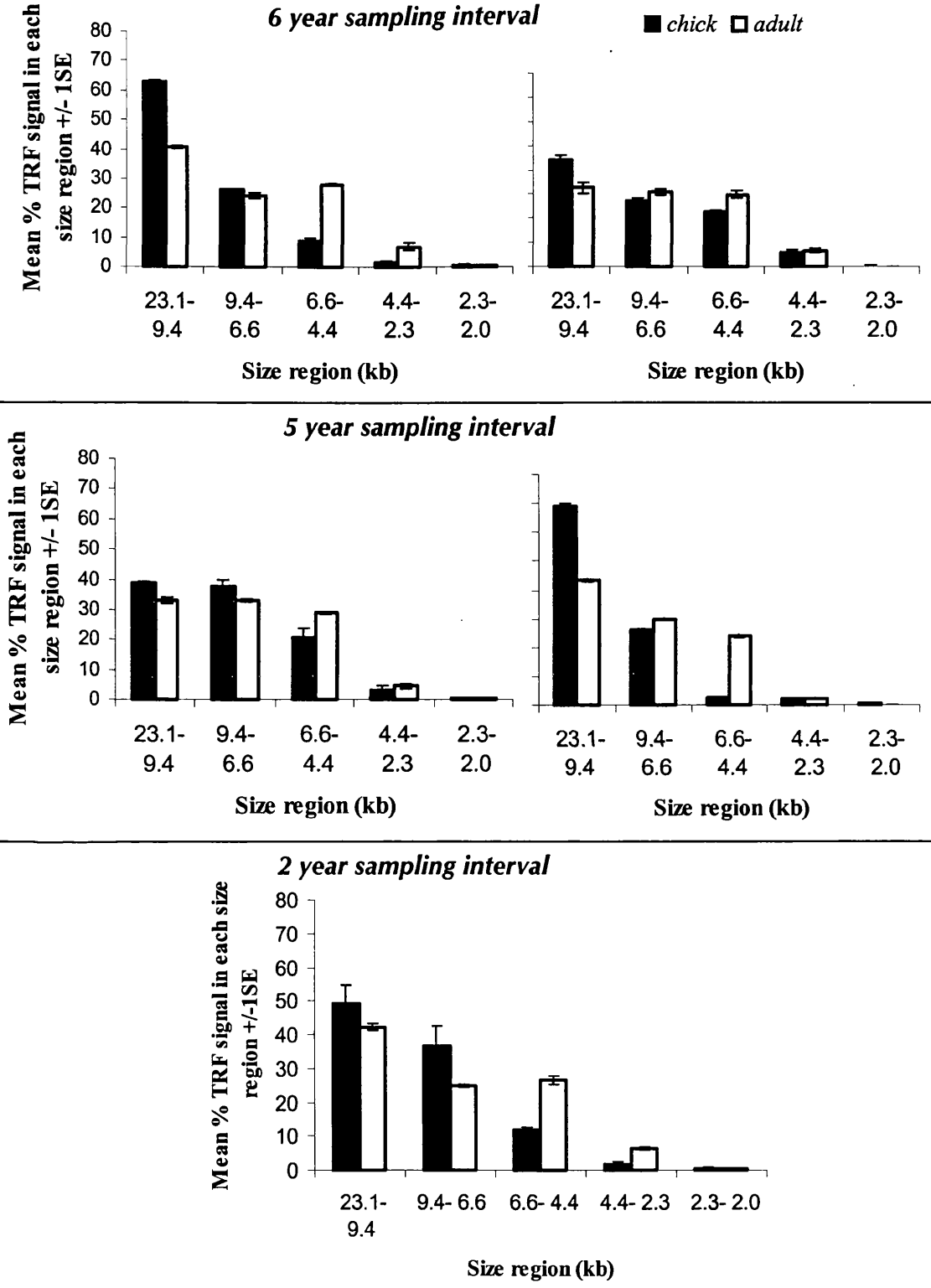
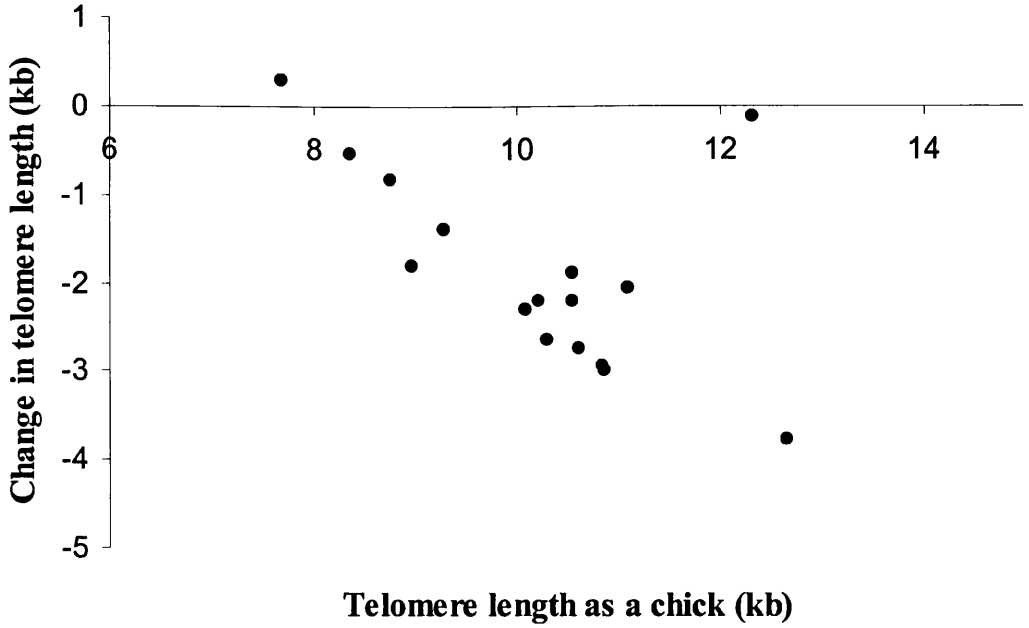



**Figure 6.5** *(c)* Percentage TRF signal in each of the 5 size regions defined by the molecular weight standard, for individual birds as chicks and as adults. 5 of the total 16 individuals are shown here, with at least 1 example for each sampling interval (2, 5, or 6 years). The legend shown top right refers to all charts.

| Bird | Age when sampled as a chick (days) | Age when sampled as an adult (years) | Telomere length as a chick (kb) | Telomere length as an adult (kb) | Difference in telomere length, adult-chick (bp) | Rate of telomere change, adult-chick (bp $yr^{-1}$) |
|---|---|---|---|---|---|---|
| 1 | 29 | 6 | 7.69 | 7.98 | +290 | +49 |
| 2 | 36 | 6 | 12.32 | 12.19 | -130 | -22 |
| 3 | 33 | 5 | 8.37 | 7.83 | -540 | -110 |
| 4 | 30 | 6 | 8.76 | 7.92 | -840 | -142 |
| 5 | 29 | 6 | 9.28 | 7.89 | -1390 | -282 |
| 6 | 34 | 6 | 8.97 | 7.15 | -1820 | -308 |
| 7 | 25 | 6 | 10.55 | 8.67 | -1880 | -317 |
| 8 | 28 | 6 | 11.11 | 9.06 | -2050 | -346 |
| 9 | 29 | 6 | 10.56 | 8.36 | -2200 | -372 |
| 10 | 33 | 2 | 10.21 | 8.00 | -2210 | -1157 |
| 11 | 29 | 6 | 10.09 | 7.78 | -2310 | -390 |
| 12 | 31 | 5 | 10.31 | 7.65 | -2660 | -541 |
| 13 | 33 | 6 | 10.62 | 7.88 | -2740 | -464 |
| 14 | 28 | 5 | 10.85 | 7.90 | -2950 | -599 |
| 15 | 23 | 6 | 10.87 | 7.89 | -2980 | -502 |
| 16 | 31 | 5 | 12.67 | 8.90 | -3770 | -767 |

**Table 6.3** Summary of the telomere changes within the 16 shags in the longitudinal data, arranged by order of increasing telomere loss in terms of absolute difference.

*(d)*




*(e)*

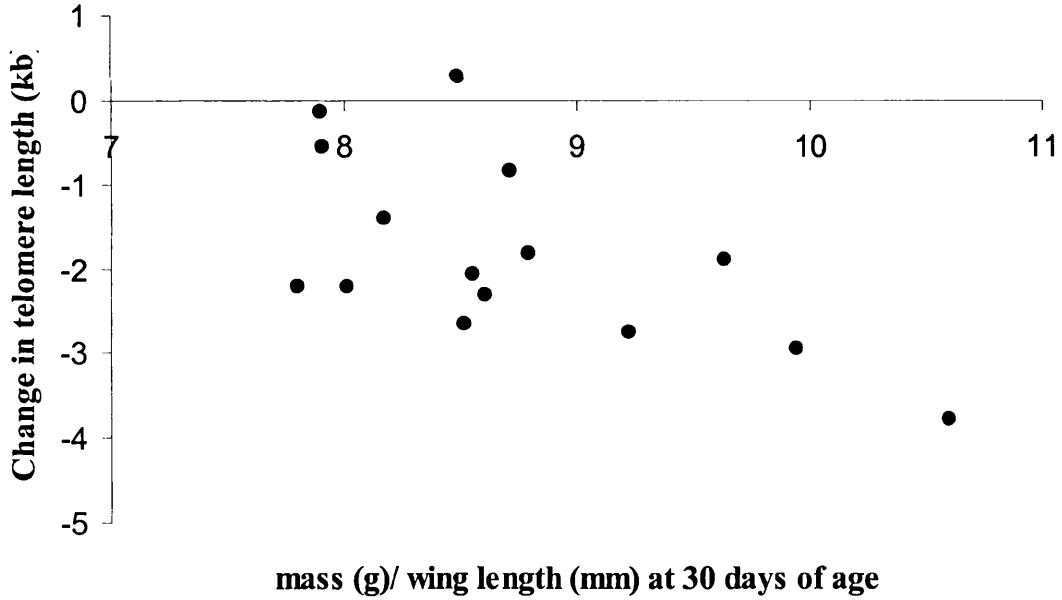



**Figure 6.5** *(d)* Those birds that had larger mean TRF values as chicks showed greater loss of telomeric repeats, and *(e)* chicks that were heavier for their size at 30 days of age underwent greater telomere loss than lighter chicks.

*(f)*

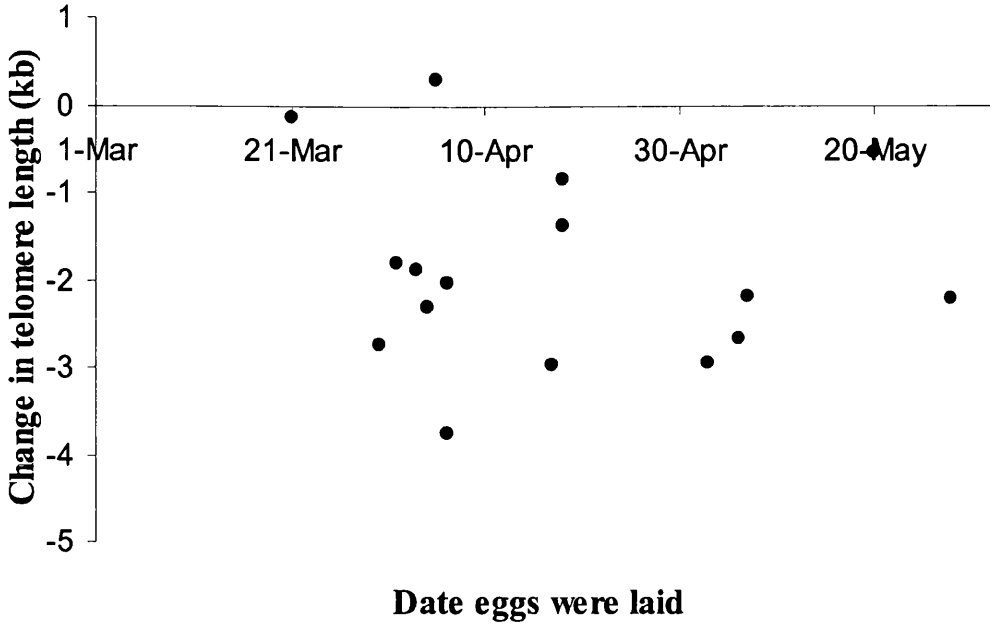
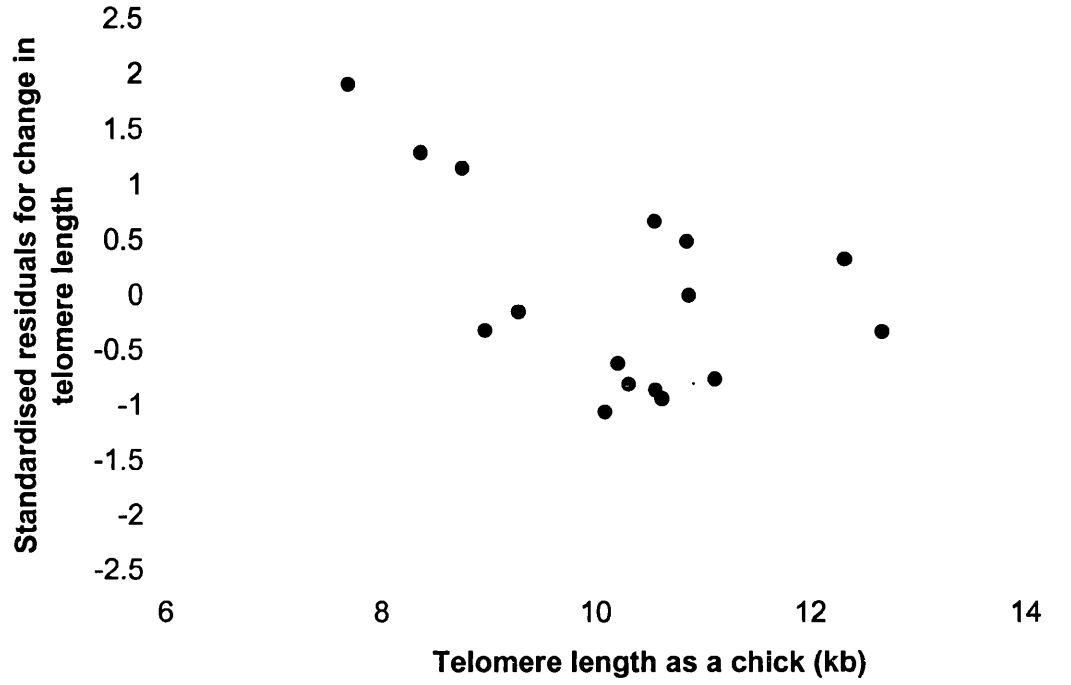



**Figure 6.5** *(f)* Individuals that hatched from eggs laid late in the season showed more subsequent telomere attrition than birds that hatched from earlier-laid eggs.

*(a)*




*(b)*

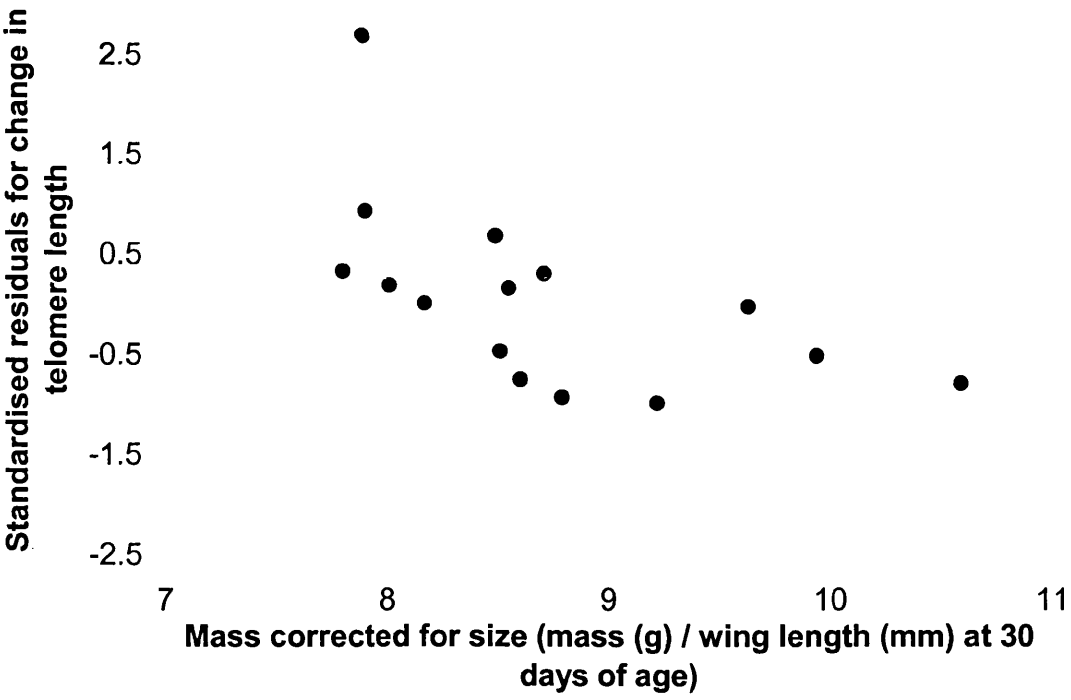



**Figure 6.6** Telomere change within individuals between chick and adulthood, with residuals shown on the y-axes having corrected for the other significant effects, in relation to *(a)* telomere length as a chick, and *(b)* Mass at 30 days of age corrected for size.

*(c)*

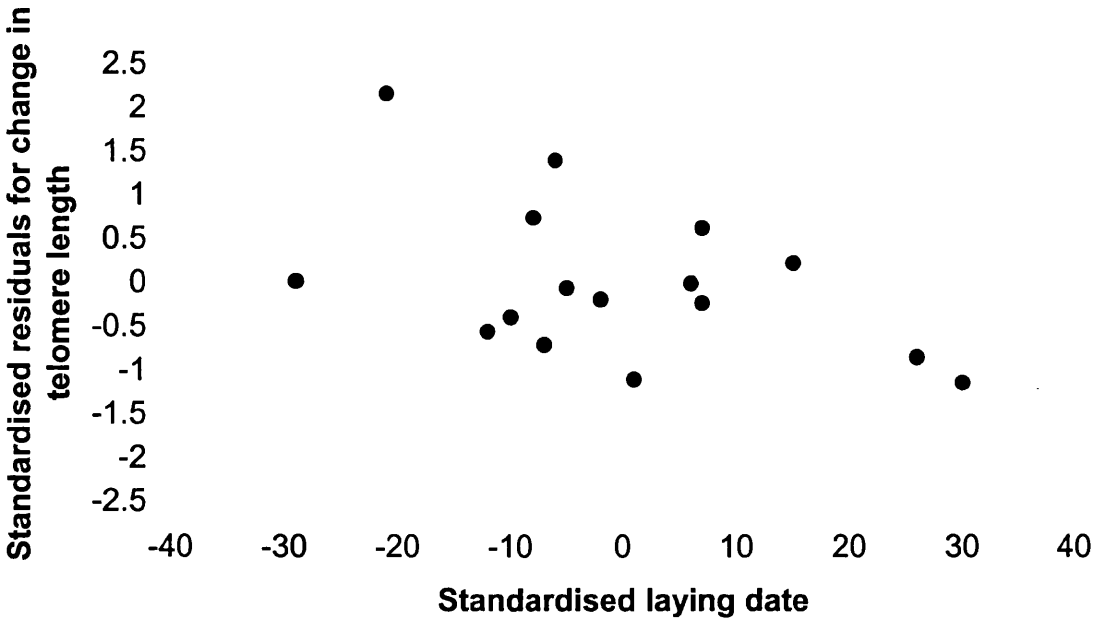



**Figure 6.6** Telomere change within individuals between chick and adulthood, with residuals shown on the y-axes having corrected for the other significant effects, in relation to *(c)* the date on which eggs were laid, standardised for the median laying date in each respective year.

| Species | Minimum value of mean TRF length (kb) | Maximum value of mean TRF length (kb) | Authors |
|---|---|---|---|
| European shag, *Phalacrocorax aristotelis* | 6.1 | 14.1 | this study |
| Wandering albatross, *Diomedea exulans* | 6.6 | 14.2 | Hall *et al.* 2004 |
| Leach's storm petrel, *Oceanodroma leucorhoa* | 16.6 | 19.8 | Haussmann *et al.* 2003 |
| Common tern, *Sterna hirundo* | 7.6 | 9.8 | Haussmann *et al.* 2003 |
| Adélie penguin, *Pygoscelis adeliae* | 5.6 | 9.3 | Haussmann *et al.* 2003 |
| tree swallow, *Tachycineta bicolor* | 12.1 | 17.3 | Haussmann *et al.* 2003 |
| Zebra finch, *Taeniopygia guttata* | 7.4 | 9.3 | Haussmann *et al.* 2002 |
| Chicken, *Gallus domesticus* | 8.0 | 20.0 | Venkatesan & Price, 1998 |

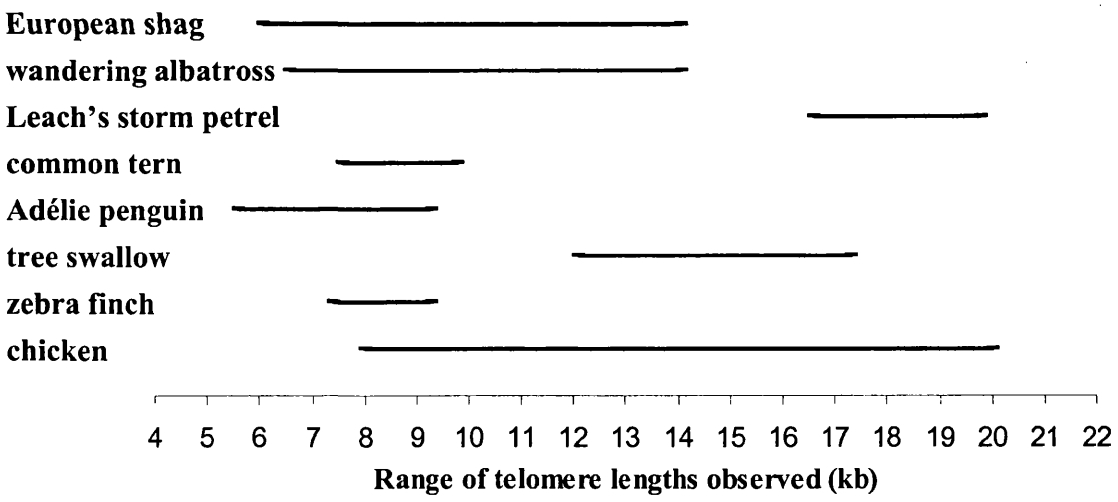



**Figure 6.7** Telomere length range for the 8 avian species in which it has been measured to date. Maximum and minimum TRF length values for the species studied by Haussmann *et al.* were extracted from figure 1 *(a)* - *(e)* in their 2003 publication. With the exception of the chicken, in which several different tissues and transformed cell lines were assessed, these telomere measurements were all made in blood cells.

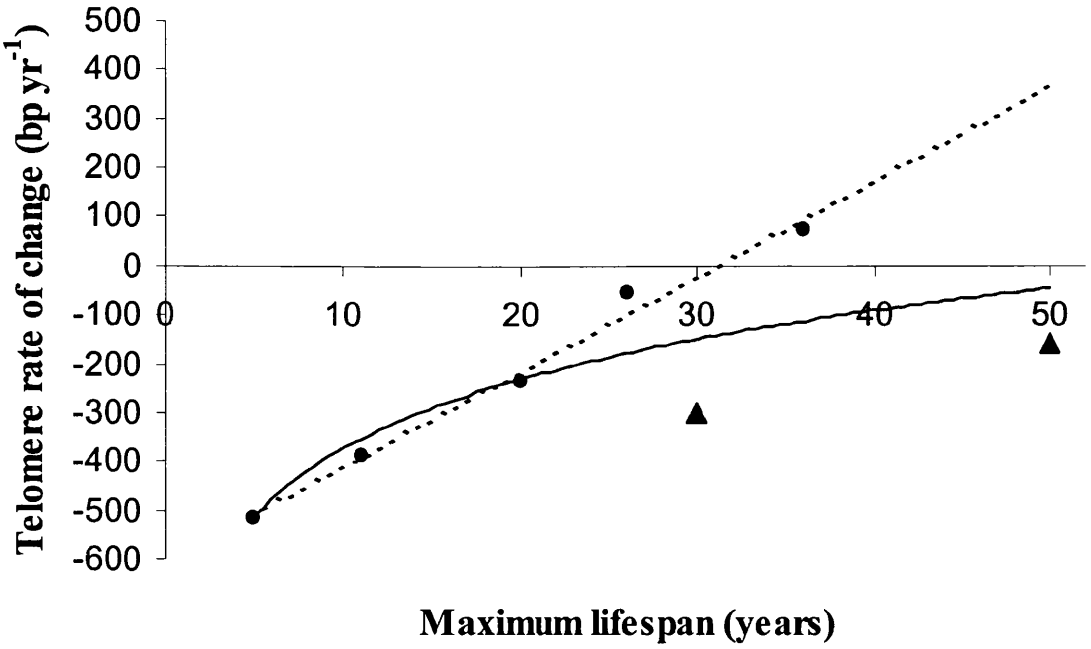



**Figure 6.8** The relationship between telomere rate of change (TROC) and maximum lifespan for the 5 avian species measured by Haussmann *et al.* (2003) shown as circles, and the values of TROC I recorded for the European shag and wandering albatross shown as triangles. In order to be in line with Haussmann *et al.*'s measures, values of the slopes from our cross-sectional regressions in each species are plotted. The dotted line indicates Haussmann *et al.*'s regression equation, extended to show the prediction for TROC in albatrosses. The solid line is a logarithmic curve fitted to all the data (TROC (bp $yr^{-1}$) = -840.9 + (203.4 * ln (max. lifespan)), $F_{1,5} = 8.77$, $p = 0.03$, *adj.* $r^2 = 0.56$). The linear regression was not significant when our data was included ($F_{1,5} = 4.96$, $p = 0.08$).

# General discussion

### *Why study senescence?*

There are numerous reasons for investigating senescence. These include the potential impact on demography of changes in survival or productivity in old age. Ignoring relationships between parental age and offspring survival or fitness could lead to inaccurate estimates of the demographic consequences of changes in age structure. The influence of senescence on demography will be greatest if the population is small, or strongly biased towards old individuals. For instance, shifts in the age structure of populations towards older animals have been suggested as a potential cause of population cycles in voles and lemmings (Boonstra 1994, Tkadlec and Zejda 1998, but see Janova *et al.* 2003). Some knowledge of senescence-related patterns of mortality and reproduction may also be important in conserving endangered populations or in re-introduction programmes, when only a few breeding individuals remain. For example, in 1995 less than 50 individuals remained of the endangered kakapo *Strigops habroptilus*, and 87% of these were over 14 years old (Clout and Craig, 1995).

As has already been implied, studies of senescence can also contribute to our understanding of evolutionary processes, and their interplay with life-history and ecological variation. This includes addressing why and how the lifespan varies among organisms, and the mechanisms of ageing and longevity determination. New applications of longevity research are still emerging: life-history trade-offs in longevity have recently been investigated in relation to their effects on species assemblage diversity (in rockfish *Sebastes*, Bonsall and Mangel 2004). Unravelling the mechanisms that contribute to ageing might eventually contribute to methods of slowing the human ageing process, or increasing the period of active, healthy old age, free from disability.

### *Why focus on senescence in a long-lived, wild bird?*

Birds are exceptionally long-lived for their size (compared to mammals), despite characteristics that would be expected to decrease lifespan such as high metabolic rates, high body temperatures and high blood glucose levels (reviews Holmes and Austad, 1995*a*, *b*). Explanations for this exceptional longevity have been concerned

with either the level of extrinsic mortality (as already discussed) or internal, molecular mechanisms that could protect against the ageing process. The latter include lower levels of oxidative damage as a result of the lower production of free radicals, particularly at mitochondria, a high rate of DNA repair, and low levels of lipid peroxidation (reviews Perez-Campo *et al.* 1998, Barja 2002). Telomere shortening, which is accelerated by oxidative stress, is a potential link between free radical attack and senescence. The investigation of telomere length in long-lived birds has revealed differences in this aspect of ageing, in relation to more commonly studied model organisms, such as mice and humans (see chapter 6).

Telomere shortening is implicated in cellular senescence, and thereby linked to organismal ageing, in most eukaryotes. Thus this common molecular mechanism is relevant to the ageing of individuals, and so to maximum lifespan within species. On an individual level, telomere shortening prior to breeding age in the shag relates to environmental conditions as a chick. In addition, a lack of measurable change in telomere length among breeding adults suggests there may be active telomere maintenance in these long-lived animals. Telomere length in several long-lived bird species is maintained without, it appears, incurring higher risks of tumour development through cell immortalization (chapter 6, Haussmann *et al.* 2003, Hall *et al.* 2004).

By focussing on the European shag, the pattern of senescence in this long-lived species of bird can also be compared to that of shorter-lived birds. Seabirds such as the shag have moderately long lifespans with respect to other avian groups, and extreme life-histories, with slow development, delayed maturity and low reproductive rates. The results obtained from the study of senescence and reproductive performance in the shag (table 7.2) should be considered in this context. Studying these birds in the wild means all natural causes of mortality are included, and not only intrinsic causes, as might be the case for captive bird populations. Although this complicates the identification of maximum longevity, reduces the sample size of old individuals, and creates challenges in assuring an unbiased sample of birds at each age, its advantage is that the actual set of circumstances on which natural selection acts is investigated. Biases and peculiarities specific to captive populations are avoided.

### *The distribution and causes of senescence*

The causes of ageing can be addressed at the level of proximate mechanisms that lead to lower survival and reproductive performance, or the ultimate, evolutionary explanations for senescence. This thesis has dealt firstly with whether reproductive senescence occurs in European shags, and has also sought to identify proximate causes of decline. From an evolutionary perspective, the cause of ageing has been attributed to the falling intensity of natural selection with respect to age, after the onset of reproduction (Medawar 1946, 1952). Though this idea has been developed and modified by subsequent evolutionary theories (see chapter 1), it remains the basic explanatory framework for why senescence occurs.

Senescence is one aspect of age-related variation in reproductive activity and survival probability, which although less often studied and more difficult to identify than improvements early in life, does appear to be widespread. Ageing processes include deteriorating health and condition, declining survival in old age and reproductive declines, each of which can be compared among taxa. Senescent decline in survival and reproductive performance is nearly universal in animals (Williams, 1957). Though it seems intuitively likely, it is currently unknown whether somatic and reproductive senescence are functionally inter-linked, but they may be maintained by common selective factors (Ricklefs, Scheuerlein and Cohen, 2003). In terms of survival, it is accepted that all terrestrial vertebrates and many fish senesce (Rose, 1991). Senescence has also been measured in invertebrates, and in this group it is especially well studied in insects (e.g. *Drosophila*, Partridge 1988, 2001). In plants, when a distinction can be made between soma and germline, and single organisms can be defined, ageing processes have also been identified (e.g. in long-lived tree species, Heath 1957, Noodén 1988). It appears that all species that have a clear distinction between soma and germline show somatic senescence (Bell, 1984). The proximate mechanisms that cause ageing, especially at the fundamental level of the cell (e.g. replicative potential limited by telomere shortening), may be the same in all these organisms. However, the rate and form of senescence is linked to ecological and life-history differences. Williams (1957) formulated a number of predictions with respect to comparative patterns of ageing (table 7.1). The exceptional longevity of birds and bats, in relation to mammals of the same size (Lindstedt and Calder 1976, Holmes and Austad 1995*a*), and the gradual senescence

of some birds, such as seabirds, are consistent with his first prediction. Flight is thought to lead to a reduced chance of predation and lower levels of extrinsic mortality (see chapter 1). A similar contrast can be made between thick-shelled bivalves, which are on average more long-lived than all other mollusc species, with their thinner or non-existent shells (Rose, 1991).

In fish, reptiles and amphibians, positive relationships between female age and various measures of reproductive performance have been interpreted as evidence of increased reproductive effort with age (e.g. Tinkle 1969, Pianka and Parker 1975). However, this interpretation is complicated by increases in body size with age in some species (e.g. in fish: Reznick, Ghalambor and Nunney 2002, in the long-lived painted turtle *Chrysemys picta*: Congdon *et al.* 2003). In mammals and birds, after improvements across the first few breeding attempts, reproductive performance generally remains constant or declines in old age (Clutton-Brock, 1984). For instance, both Antarctic fur seals (*Arctocephalus gazella*) and wandering albatrosses (*Diomedea exulans*) show increases in breeding success over the first 4-7 breeding attempts followed by a decline in older or more experienced females (Lunn, Boyd and Croxall 1994, Weimerskirch 1992, respectively). Among invertebrates, *Drosophila* has been extensively studied in relation to senescence, and maternal age in this insect also correlates negatively with hatching success and the viability of larvae (Hercus and Hoffmann 2000, Kern *et al.* 2001). Similarly, in the cockroach *Nauphoeta cinerea* fertility is lower in old females (Moore and Moore, 2001), although there is no effect of female age on offspring fitness (Moore and Harris, 2003). So, although there is variation in the specific details, such as which stage of reproduction is affected, declines in breeding performance in old age are a common pattern across a wide range of organisms.

Seabirds and primates are both long-lived, but exhibit different patterns of ageing. Both show senescence-related declines in survival at old age, but only in primates does this occur during post-reproductive life. Modern humans represent an extreme example of this, with a doubling time of adult mortality rate of 7-8 years, in contrast to shorter doubling times of between 3.5 and 3.8 years in wild female baboons (*Papio hamadryas*) and 4.8 years in a captive population (Bronikowski *et al.* 2002). For comparison, two bird species, the indigo bunting *Passerina cyanea* and the pied flycatcher *Ficedula hypoleuca*, have mortality rate doubling times of

between 13 and 14 years (Holmes and Austad 1995*a*). Non-human primates live beyond reproductive age even in the wild (e.g. wild chimpanzees, Nishida *et al.* 2003), whereas in birds this has only been observed for short-lived species in captivity, such as the Galliformes, as well as in some captive female raptors (Holmes *et al.* 2003). The reproductive potential of old female mammals typically decline because of the loss of oocytes (Fitzgerald, Zimon and Jones, 1998). However, whether oocyte numbers become depleted in ageing birds is not known (Ottinger, Nisbet and Finch, 1995). The cessation of egg production has not been observed in most birds, despite long breeding lives (albeit at a slow rate, Holmes *et al.* 2003). It is not known whether old wild birds cease to breed before the end of their lives, because they can generally be observed and measured only when they are breeding. In addition, physical and physiological signs of ageing seem to be generally lacking in wild birds, in contrast to mammals, which show a host of changes in appearance and function in old age, as a consequence of age-related deterioration. It should be kept in mind though that changes in birds may be more difficult to identify, either because they are not as familiar to us as the age-related changes that occur in mammals, or because they are concealed (e.g. skin is concealed beneath feathers).

### *Do European shags show senescence-related declines in reproductive performance and condition?*

The reproductive performance (chapters 2 – 4) and condition (chapter 5) of old European shags on the Isle of May was investigated in relation to that of younger breeding birds. This was possible because a large proportion of the birds at this site are known age, as a consequence of long-term ringing effort. The study was motivated by previous observations of significantly lower survival in old shags compared to middle-aged birds (in poor years: Potts 1969, Aebischer 1986, Harris, Wanless and Elston 1998, over a 24 year period: Harris *et al.* 1994*b*), which suggested senescence. Telomere length is related to cellular senescence and organismal ageing (see chapter 6), and was also explored in relation to age in the shag, and to environmental conditions. Where possible, age-related change was tested by longitudinal comparisons within individuals, as well as at the population level by cross-sectional analysis. The main focus of this study has been the potential effects of old age on reproductive success.

Although there was an influence of old age on several features of the breeding biology of shags, senescence effects were conspicuously lacking from other areas of reproductive performance, and from adult state (table 7.2). Thus, old females laid smaller eggs than middle-aged females, but this did not translate into a significant difference in breeding success. Offspring were not, however, monitored postfledging, and there may be differences in offspring quality that were not apparent at the nestling stage. A similar pattern has previously been found in Antarctic blue-eyed shags (*Phalacrocorax atriceps*), in which old females lay smaller eggs, but do not differ in the number of chicks hatched or fledged (Shaw, 1986). In several studies of age-related reproductive performance, declines have been found in some aspects of breeding but increases in others. For instance, while clutch and egg sizes are greatest in middle-aged Arctic terns (*Sterna paradisaea*), breeding success is highest in the oldest age group (Coulson and Horobin, 1976). This is also the case in common terns (*Sterna hirundo*, Nisbet, Winchell and Heise 1984, Nisbet, Apanius and Friar 2002) and white-tailed ptarmigans (*Lagopus leucurus*, Wiebe and Martin 1998). In relation to the latter, Wiebe and Martin suggest parental experience is compensating for a reduced physical ability to produce eggs in old age. This trend is not limited to long-lived birds. In the Rhum population of red deer (*Cervus elaphus*), reproductive performance after birth (duration of suckling bouts, calf body condition, calf survival) increases with parental age, although performance before birth (probability of producing a calf, calf birth weight, and neonatal calf survival) declines (Clutton-Brock, 1984). Moreover, in old red deer this is despite declines in maternal body weight and condition. In another species of deer, the roe deer *Capreolus capreolus,* implantation failure follows a quadratic relationship with female age, with the highest chance of failure in the oldest females, and then in yearlings (Hewison and Gaillard, 2001). Fecundity may be under tight physiological constraints, which prevent increased effort at early stages of reproduction. Alternatively, increased effort may only be worthwhile, and selected for, once the chance of offspring survival crosses a certain threshold. Older female feral horses (*Equus caballus*) are more successful at raising foals because they are more protective of offspring during the first 20 days of life, a critical period for foal survival (Cameron *et al.* 2000). Thus, old mares better target their investment in offspring, perhaps as a result of experience, though they do not invest more overall

than younger mothers. Perhaps older shags are also more competent at rearing chicks than younger birds, and thereby can compensate for their smaller eggs. The breeding experience of individual shags was not examined in this study, but could be looked at in the future by monitoring specific individuals every year.

The lack of appreciable declines in hatching and fledging success, the number of chicks fledged and in adult condition, mirror findings in several other long-lived seabirds, such as the common tern (Nisbet, Apanius and Friar 2002). The life-history characteristics of low annual production and the maintenance of individual condition do not appear to change in old age in these birds. As hatching, fledging and breeding success were compared among birds of differing ages, and not within individuals as they grew older, it is also possible that the selective survival of high quality individuals may have offset any senescent decline in these parameters.

The age difference in pairs increased with female age, and the oldest females were paired with much younger males (chapter 2). Therefore in these pairs old female age is confounded with young male age, and with the recent establishment of the pair bond (see chapter 4 discussion). Pairs of shags made up of an old female and young male more often fail completely in their breeding attempts, but it was not possible to identify whether this is due to the age of either partner or their lack of experience as a pair. In future, it may be worthwhile to specifically investigate the effects of pair duration on reproductive performance in shags, which could also partly explain the lower breeding success of young birds. There is no effect of mate change on breeding performance in Antarctic blue-eyed shags (Shaw, 1986). However, mate change is also unaffected by the age of either partner in blue-eyed shags and is also more frequent (77% mate change from year to year, Shaw 1986) than in European shags (40% in pairs with a male over 6 years of age, Potts 1966). Given the evidence from other birds of paternal effects on egg traits (see chapter 3 introduction), the decline in egg size in old females may also to some extent reflect the age of her partner. However, there is no courtship feeding in shags and males do not defend foraging territories, so a direct paternal effect on egg size is unlikely. Although female shags may adjust egg size in relation to the age or condition of their mate (see chapter 3 introduction), there is currently no evidence for this in these birds.

Old shags were not in poorer condition than younger individuals. However, the possibility of bias in the sample of birds that were monitored should be kept in mind. Only birds at the breeding colony that established a nest site and produced chicks could be included in the sample, due to difficulties in measuring adults at other times (see chapter 5). As productivity was high throughout the three years of this study, results are also unavoidably biased towards years of favourable environmental conditions, when all birds may be able to maintain good body condition. It has previously been shown that in poor years old shags are more likely to skip breeding than younger birds, and old individuals that do breed have lower survival over the following winter (Harris, Wanless and Elston, 1998). The adoption of a non-breeding strategy by old shags in poor years suggests these birds do not make a "last ditch" reproductive effort towards the end of life. This contradicts theoretical predictions that breeding effort will increase with falling residual reproductive value (Fisher 1930, Williams, 1966*b*). Ricklefs (1998, 2000) has suggested wild birds may retain a high level of physical fitness into old age, before succumbing rapidly to intrinsic diseases that kill them over short periods of time. This would reduce the chance of observing lower condition in old birds, because condition would only be low for a brief amount of time just prior to death.

### *Future directions for the study of senescence*

One theme that has emerged from this thesis and from reviewing theories for senescence is the division between evolutionary explanations and those based on the proximate mechanisms of ageing. However, proximate and ultimate mechanisms are inextricably linked (Hochachka and Eadie, 1994). Although collaboration among ecologists, gerontologists, evolutionary and molecular biologists working on ageing seems to be increasing, there is still scope for greater integration of evolutionary, physiological and molecular research. This includes genetic aspects of longevity and senescence, which have so far mainly been studied in yeast, worms, flies and mice. In these model organisms, a few genes and gene pathways have been identified which, on mutation, can lead to dramatic increases in longevity (Lithgow *et al.* 1995, Kenyon 2001, Hekimi and Guarente 2003, Tatar, Bartke and Antebi 2003). There is now genetic and molecular support for antagonistic pleiotropy as an evolutionary explanation of senescence. Genes with antagonistic pleiotropic effect have been

identified (e.g. Longo and Finch 2003). In subsets of yeast proteins, those associated with senescence show the greatest degree of pleiotropy and have significantly higher connectivity with other proteins than expected by chance (Promislow, 2004): this recent study is a clear example of how evolutionary and molecular models of senescence can be integrated and tested.

| Predictions for variation in the pattern of ageing (Williams 1957, Rose 1991) |
|---|
| 1. Low adult mortality rates should be associated with low rates of ageing, and high adult death rates with high rates of ageing. |
| 2. Ageing should be more rapid in organisms that do not increase markedly in fecundity after maturity than those that do show such an increase. |
| 3. Where there is a sex difference, the sex with the higher mortality rate and lesser rate of increase in fecundity should undergo more rapid ageing. |
| 4. Early reproduction should be correlated with early ageing, and conversely longer-lived organisms should show lower early reproductive rates. |

**Table 7.1** Predictions for patterns of ageing among species or taxa, based on Williams (1957), and as re-formulated by Rose (1991).

| In relation to middle-aged shags | |
|---|---|
| **Significant difference in old age** | **No significant difference in old age** |
| • Age of the breeding partner | • Nest site quality |
| • Egg size | • Laying date |
| • Chance of complete breeding failure, prior to hatching or during chick rearing | • Hatching success |
| • Chick growth | • Fledging success |
| • Number and size of red blood cells | • Number of chicks fledged |
| | • Proportion of male chicks produced |
| | • Body size, weight or body condition |
| | • Cell-mediated immune response |

**Table 7.2** Aspects of the breeding biology and adult state of European shags that did, or did not, differ in old age.

# A clutch exchange experiment: the effect of age and egg quality on reproductive performance

In 2002 an experiment was performed designed to distinguish between effects on reproductive performance mediated by variation in egg quality and by variation in the rearing ability of females of differing ages. Clutch exchanges were performed, in which clutches of three laid at approximately the same time in the season were swapped between old and middle-aged females. However, despite the quadratic relationship that exists between egg size and female age, whereby old females lay smaller eggs than middle-aged birds (chapter 3), egg size in the clutches in this manipulation did not differ significantly between the samples of the two age groups available for this experiment. This may be due to the comparatively small sample sizes involved, which were limited by the scarcity of old females and the requirement to match nests for laying date. Alternatively, it may reflect the favourable environmental conditions for shags on the Isle of May that year, as indicated by their overall high productivity, which could mask age-related constraints. A brief résumé of the protocol and the findings of this experiment are provided here.

## METHODS

The clutch exchange protocol (figure A.1) involved swapping clutches of three eggs among quartets of breeding pairs that included two middle-aged (5-8 years old) and two old females (13-19 years old). These exchanges resulted in middle-aged females receiving a clutch laid by an old bird, and old females receiving a clutch laid by a middle-aged bird (experimental groups). To control for effects of the clutch exchange procedure *per se*, in each quartet one middle-aged female also received a clutch from a female in the same age group, and one old female received a clutch from another old bird (control groups). The reproductive success of the experimental groups could then be compared to that of controls that had undergone the same manipulation. Females were matched for laying date in each quartet and, whenever possible, also for the area of the island in which the nests occurred.

The difference in laying date within quartets ranged from 1-8 days, with an average of 3 ± 0.7 days. All clutches were exchanged between 2 and 10 days after clutch completion (mean 6 ± 0.3 days), which corresponds to 5 to 13 days into

incubation (full incubation commences after the second egg is laid in shags, and eggs are laid every third day, Snow 1960). As the period from laying of the first egg to hatching on average lasts 35 days (± 0.3 days, 38 nests monitored in 2001), all clutches were therefore exchanged between 14 and 37% of the way through this period.

After removing each clutch from the natal nest, the length and breadth of the eggs were measured with callipers (to 0.1 mm), before they were exchanged at the next nest. Egg volume was calculated from the linear dimensions of the eggs (see chapter 3 methods). False plaster eggs were placed in the first nest in each quartet at the time of clutch removal, until the swaps at the other three nests had been carried out. The false eggs in nest one could then be replaced with the clutch taken from the fourth nest. Exchanges were carried out in 11 quartets, thus involving a total of 22 middle-aged and 22 old females. All the exchanges were performed between May 4$^{th}$ and May 20$^{th}$. The reproductive success of exchange nests was followed to fledging, and the linear growth rates of chicks at these sites were also measured (see chapter 4 methods).

The clutch exchange protocol sought to match females for laying date, and to ensure that both age groups incubated the clutch for the same length of time. To check this had been achieved, laying date, the length of incubation and the time after clutch completion at which eggs were exchanged were compared between the middle-aged and old female age groups at exchange nests. Clutch volume and, in a repeated measures ANOVA the volume of the A, B and C egg, were also compared between these two age classes. Reproductive performance was measured as the number of eggs that hatched, fledging success (proportion of hatched chicks that fledged), and breeding success (number of chicks that fledged from each nest). Differences in these measures of reproductive success among the experimental categories and control categories were analysed using generalised linear models (error distributions and link functions as for analyses in chapter 4). Chick linear growth rates were compared among exchange categories at the level of the brood. Male ages where known, and the age differences between members of known age pairs, were taken into account in these analyses of breeding performance.

The potential impact of disturbance caused by swapping clutches at the nest was also tested separately in pairs of middle-aged females that laid clutches of three at the

same time in the season. In these pairs, one nest was subjected to the same level of disturbance as an exchange nest, by removing the clutch for a short period. During removal false plaster eggs were placed in the nest, and the sizes of eggs in the removed clutch were measured. The original clutch was then returned. The other nest in each disturbance control pair was not manipulated in any way. Breeding success was then compared between nests that were subjected to disturbance and those that were not disturbed. A total of 8 pairs of disturbance control nests were tested. Within these pairs, the difference in laying date of the females ranged from 1 to 3 days, and all the tests were carried out between May 9$^{th}$ and May 20$^{th}$, hence over the same period as the clutch exchanges. As old females were scarce, and I wanted to maximise the number of clutch exchanges performed, disturbance controls were only tested among middle-aged females. To compare the number of eggs that hatched and the number of chicks fledged between nests at which disturbance was imposed and those where there was no disturbance, a generalised linear model with poisson error distribution and log link function was used.

## RESULTS

The removal of clutches for measuring and their subsequent replacement did not lead to reduced breeding success in comparison to nests at which there was no disturbance (effect of disturbance on the number of eggs that hatched: $F_{1,14} = 0.62$, $p = 0.44$, on the number of chicks fledged: $F_{1,14} = 1.98$, $p = 0.18$). Breeding performance also did not differ significantly between these groups when laying date was taken into account, and there was no interaction between disturbance group and laying date (in all cases $p > 0.10$).

At clutch exchange nests, laying date did not differ between middle-aged and old females ($t_{42} = 1.05$, $p = 0.30$). Therefore, nests occupied by these two age classes were successfully matched for laying date in this experiment. Overall, the median laying date among exchange nests was April 26$^{th}$. This is slightly earlier than the median laying date recorded for a larger sample of nests ($n = 172$) in 2002, which was April 29$^{th}$. There was no difference in the length of the laying-to-hatching period of middle-aged and old females at exchange nests ($t_{42} = 1.87$, $p = 0.07$, average overall = 32 ± 0.40 days). The time after clutch completion at which clutches were exchanged was significantly different between middle-aged and old

females ($t_{42}$ = 3.66, $p$ = 0.001). However, the magnitude of this difference was not great (mean number of days after clutch completion at which clutch was exchanged, in middle-aged females: 7 ± 0.3 days, old females: 5 ± 0.3 days). Thus, the proportion of the total incubation period spent incubating foster eggs was very slightly greater for old females than middle-aged birds (middle-aged females: 69 ± 0.9 %, old females: 72 ± 0.9 %, after arcsine transformation $t_{42}$ = 2.69, $p$ = 0.010).

The clutch volume laid by middle-aged and old females was not significantly different (clutch volume laid by middle-aged females: 137.9 ± 2.2 $cm^3$, by old females: 135.6 ± 2.4 $cm^3$, $t_{42}$ = 0.71, $p$ = 0.48), even within exchange quartets (quartet identity specified as a random factor, female age class: $F_{1,32}$ = 0.56, $p$ = 0.46, quartet: $F_{10, 33}$ = 1.43, $p$ = 0.21, interaction term: $F_{10,22}$ = 1.72, $p$ = 0.14). However, this analysis ignores the differences in volume among eggs within a clutch (see chapter 3). A repeated measures ANOVA with the within-subjects factor egg laying order (A, B or C egg) and the between-subjects factors female age class and exchange quartet was used to examine differences in egg volume between middle-aged and old females in more detail. As expected (see chapter 3), there is a significant effect of laying order on egg volume ($F_{2,44}$ = 13.61, $p$ < 0.001). The effect of laying order does not vary with female age class (laying order × female age class: $F_{2,44}$ = 0.41, $p$ = 0.67) or among exchange quartets (laying order × quartet: $F_{20,44}$ = 0.46, $p$ = 0.97). Although all three eggs of old females were on average smaller than those laid by middle-aged birds (figure A.2), these differences were not significant ($F_{1,22}$ = 0.68, $p$ = 0.42). Egg volume did not differ among exchange quartets, and there was no significant interaction between female age class and quartet identity (quartet: $F_{10, 22}$ = 1.73, $p$ = 0.14, female age class × quartet: $F_{10, 22}$ = 1.71, $p$ = 0.14).

The mean number of eggs hatched, fledging and breeding success at experimental and control nests are shown in figure A.3. Male age, which was known for 26 / 44 pairs and ranged from 4 - 17 years, did not differ among the four exchange categories ($F_{3,22}$ = 1.46, $p$ = 0.25). There was a significant difference in the absolute age gap within known age pairs between middle-aged and old females (mean age gap for middle-aged females: 2 ± 0.9 years, for old females: 7 ± 1 years, $z$ = 3.17, $n$ = 24, $p$ = 0.002). Therefore, the age difference within pairs was included as a covariate in comparisons of the number of eggs hatched, fledging success and

breeding success among exchange groups. There was no significant difference among the four exchange categories in the number of eggs that hatched ($F_{3,40} = 0.20$, $p = 0.90$), in fledging success ($F_{3,38} = 0.63, p = 0.60$), or the number of chicks fledged ($F_{3,40} = 0.36$, $p = 0.78$). For each of these breeding parameters, the interaction between exchange category and the age difference in pairs was also not significant (in all cases $p > 0.05$).

Chick growth during the linear phase was recorded for chicks in all except four of the 2002 clutch exchange nests (each from a different exchange quartet). The four nests at which chick growth was not measured included two nests where no eggs hatched and two nests where, although at least one egg hatched, no chicks fledged. Therefore the linear growth rate was known for a total of 93 chicks, from 40 broods. Among these, there were two broods in which chick mortality occurred during the measurement period, and these cases were excluded prior to analysis. In all remaining broods all the chicks fledged (90 chicks from 38 broods). The overall mean linear growth rate of these chicks was $57.1 \pm 0.7$ g day$^{-1}$.

Chick growth was compared at the level of the brood among exchange categories, using the average growth rate of each brood. There was no significant difference in the average brood growth rate of broods of one, two or three chicks (mean brood growth rate in broods of one: $59.3 \pm 7.1$ g day$^{-1}$, broods of two: $57.0 \pm 1.0$ g day$^{-1}$, broods of three: $55.8 \pm 1.0$ g day$^{-1}$, $\chi^2_2 = 1.92, p = 0.38$). Brood size also did not differ among the exchange categories ($\chi^2_3 = 3.01, p = 0.39$). Average brood growth rate did not differ significantly among the four categories of exchange nest when tested alone ($F_{3,34} = 1.44, p = 0.25$). Male age and the age difference in known age pairs were next included as covariates in this analysis. Male age was not significant ($F_{1,17} = 1.43$, $p = 0.25$), but there was a significant difference among exchange groups when the absolute age difference in pairs was included as a covariate (exchange group: $F_{3,18} = 3.07$, $p = 0.054$, age difference in pairs: $F_{1,18} = 5.89, p = 0.026$). None of the interactions were significant (in all cases $p > 0.05$). In old females there was a significant negative correlation between the absolute age difference in pairs and the mean growth rate of the brood ($r_s = -0.86$, $n = 11$, $p = 0.001$, figure A.4*a*). These variables were not correlated in middle-aged females ($r_s = -0.06$, $n = 12$, $p = 0.84$), which may be due to the restricted range of the age difference in those pairs. Figure A.4*b* shows the average brood growth rate of each

exchange category in relation to the age difference between members of the pair raising that brood.

## CONCLUSIONS

Removing clutches from the nest for measurement did not adversely affect breeding performance. Middle-aged and old females were successfully matched for laying date in the clutch exchange experiment, so there is clearly sufficient overlap in the laying dates of these two age classes to make this type of field manipulation plausible. However, the number of old females at the breeding colony limited sample sizes. This is likely to always be a problem in studies of senescence. Egg volume did not differ significantly between middle-aged and old females in this experiment, although as expected the mean size of all three eggs was lower in the old age group. The productivity of shags on the Isle of May was extremely high in 2002, suggesting conditions were favourable that year. Averaged across plots, 1.66 (± 0.17) young were produced per incubated nest, well above the 95% confidence interval for the 1986 - 2001 average. In addition, 42% of successful pairs ($n$ = 103) raised three young (Wilson *et al.* 2002). Age-related differences may be more pronounced in years of poor environmental conditions, when individuals pay a higher cost for reproduction. Reproductive performance did not differ significantly among the four manipulated groups in this experiment. The percentage of the nests in the experiment that fledged all three chicks was high, at 39% (and 43% fledging two).

There was a significant difference among exchange groups in the average growth rate of broods, when the age difference in pairs was taken into account. This results from the significantly greater age gap between breeding partners in the old female age class, which correlates negatively with the growth rate of the chicks reared at those nests (this experiment and chapter 4). Potential mechanisms by which the age difference in pairs may affect reproductive performance were discussed in chapter 4. Interestingly, for those old females in the clutch exchange experiment that were paired with a male of similar age, average brood growth rates were higher than those of broods raised by middle-aged parents (see figure A.4*b*). This may be due to positive effects of being raised by an old or experienced male (see chapter 4). It also suggests that the old age of the female alone does not cause poor chick growth.

## FIGURES

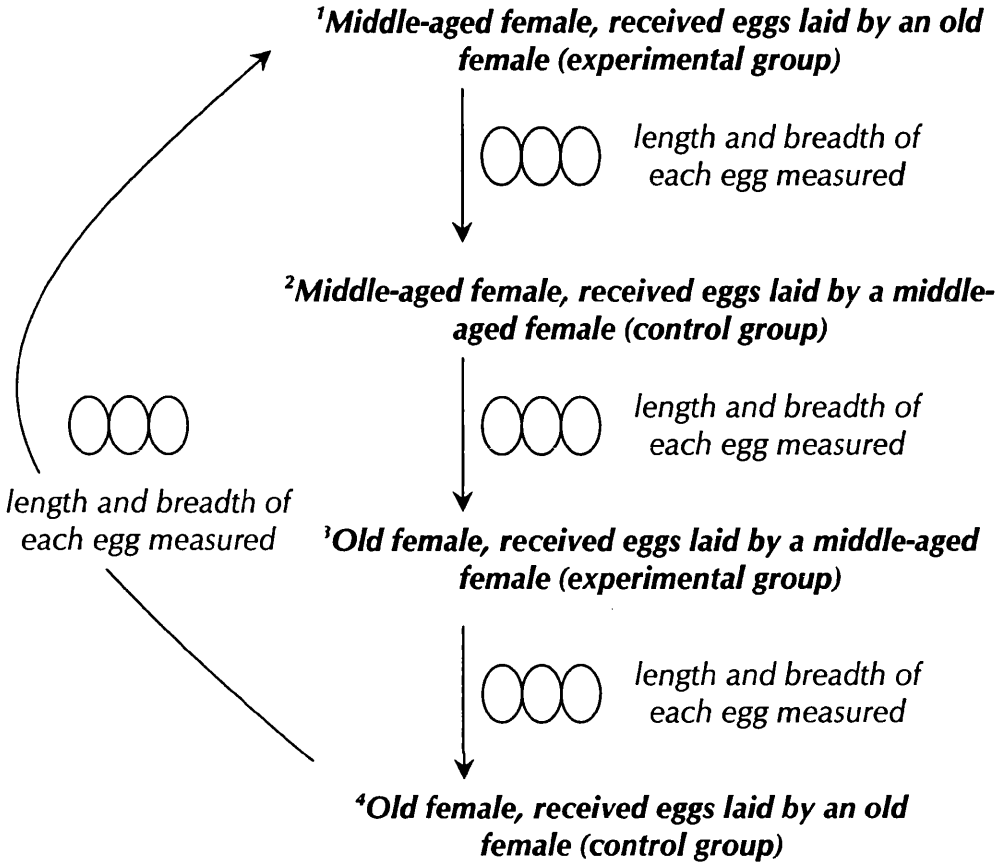



**Figure A.1** The clutch exchange protocol followed in 2002. Clutches of three were exchanged between middle-aged (5-8 year old) and old (13-19 year old) females, matched for laying date, in quartets as shown. Three false, plaster eggs replaced the clutch removed from the first nest in each quartet, until the clutch from the fourth nest could be substituted for these. The length and breadth of the eggs in each clutch were measured before they were placed in a foster nest, and from these measurements egg volume was later calculated. The first nest in each quartet was always visited twice, and the other three nests only once. To control for this, middle-aged and old females were alternated as the first nest in exchange quartets.


**Figure A.2** The volume of A, B and C eggs laid by old females did not differ significantly from those laid by middle-aged females in the clutch exchange nests.

*(a)*

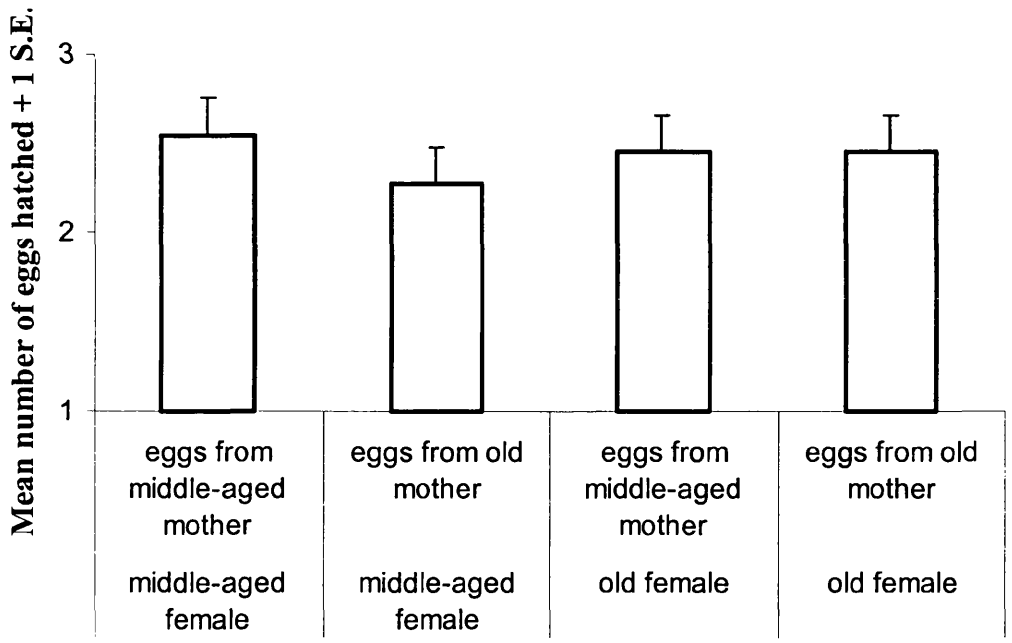
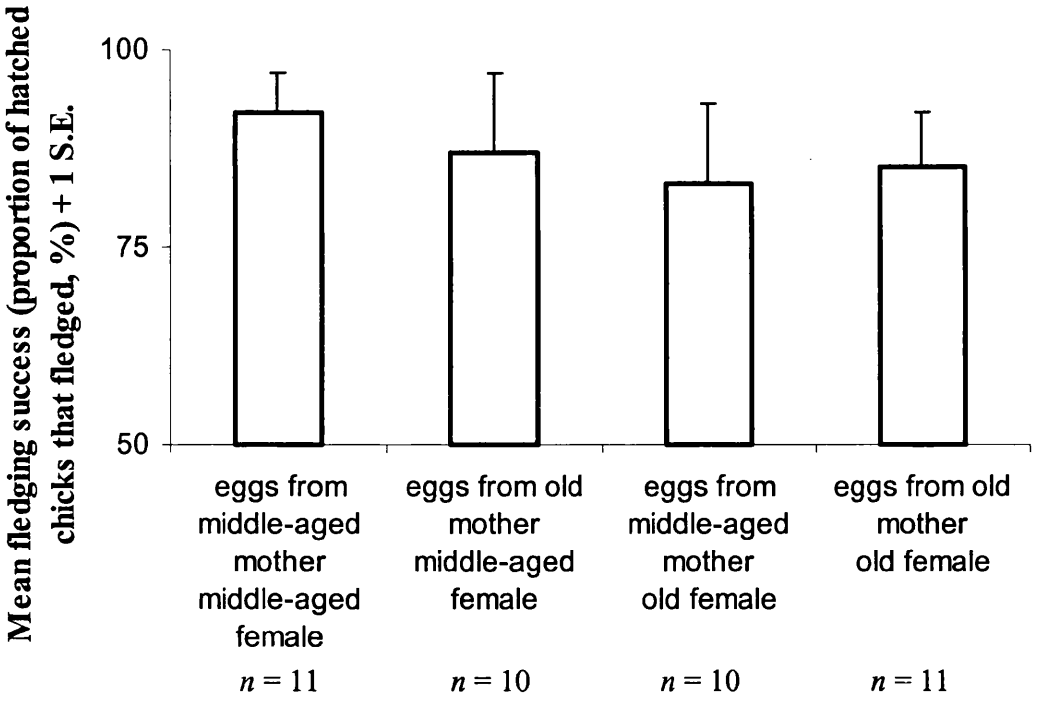



**Figure A.3** *(a)* The mean number of eggs that hatched in each category of egg exchange nest. The sample size in each category is 11 nests.

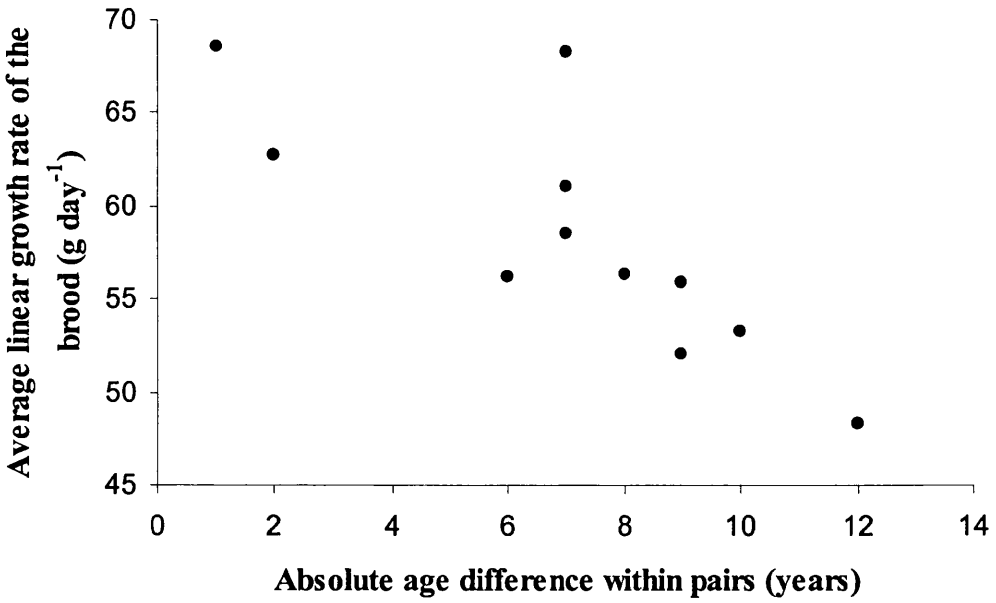
*(b)*




*(c)*


**Figure A.3** *(b)* Mean fledging success, and *(c)* breeding success, in each category of egg exchange nest. For breeding success the sample size in each category is 11 nests. Fledging success was not applicable to two nests where no eggs hatched (sample sizes shown on the graph).

*(a)*




*(b)*

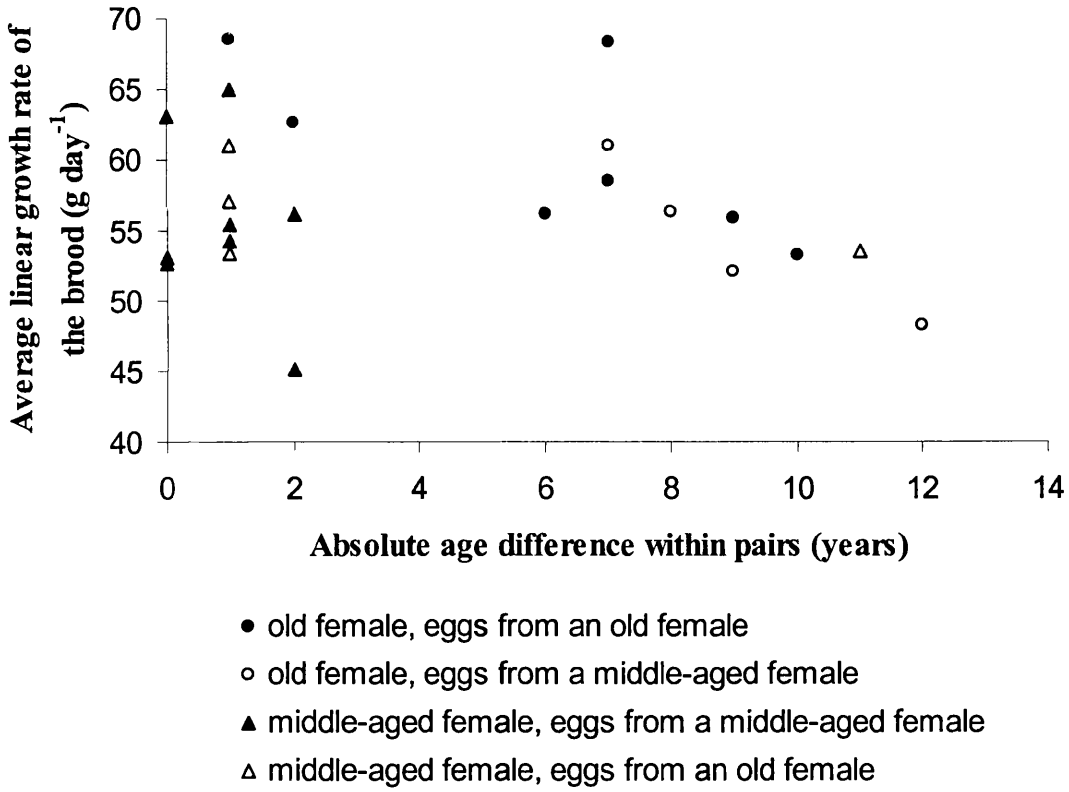



**Figure A.4** *(a)* There was a negative correlation between the difference in age within pairs and the average linear growth rate of broods raised by old females in 2002. *(b)* The average growth rate of broods in each exchange category in the 2002 clutch exchange experiment, plotted in relation to the age difference between pair members raising that brood.

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