REPRODUCTIVE SUCCESS AND MALE TRAITS IN THE SPOTLESS STARLING, *STURNUS UNICOLOR*

Patricia Celis

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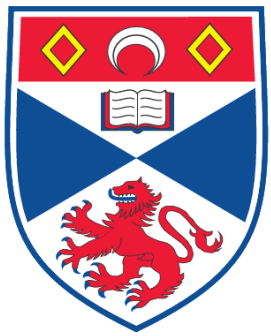
University of St. Andrews



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Reproductive Success and Male Traits in the Spotless Starling, *Sturnus unicolor*

Patricia Celis

A thesis submitted to the University of St. Andrews in application for the degree of Doctor in Philosophy

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Declaration

I, Patricia Celis hereby certify that this thesis, which is approximately 38,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in September, 2003 and as a candidate for the degree of PhD in September, 2004; the higher study for which this is a record was carried out in the University of St Andrews between 2003 and 2008.

Date .....Signature of candidate .....

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Contents

Abstract

Selection operates when the variability among individuals in heritable traits translates to differences in the number of offspring that survive to breed, which is a close estimate of fitness. Consequently, the outcome of sexual-selection should be higher reproductive success for individuals with a greater expression of the selected traits. In this thesis, the relationship between some male spotless starling (*Sturnus unicolor*) traits and reproductive success was assessed. A particular focus was given to the role of throat feathers (TF) as sexually selected trait. The study was conducted in a wild population using a correlative approach in 2004, while in 2005 and 2006 the TF of males were experimentally shortened. The genetic parentage of the offspring was required for determining the reproductive success of males. Nine highly polymorphic microsatellites (with 11.7± 3.2 alleles per locus) were developed and optimised for this species. Parentage analyses were conducted in NEWPAT XL and CERVUS 3.0.3 and confirmed using observational data. Eighty-five percent of the offspring had at least one parent assigned. The levels of intra-specific brood parasitism, extra-pair paternity and quasi-parasitism were 7%, 7% and 1% of the offspring, respectively. Polygamy levels decreased with year, as the study population matured.

The correlative study showed that males with longer TF and with better condition had a higher probability of reproducing and sired more offspring, but their offspring were not of higher quality as measured by their weight and immune response to phytohaemagglutinin. Polygynous males were also in better condition.

In the experimental study, males in better condition had a higher chance of reproducing and sired more fledglings. Conversely, males with reduced TF sired significantly fewer eggs and lighter fledglings than control males. Body condition and TF length are shown to be good predictors of reproductive success and TF length is shown to be under sexual selection.

Chapter 1. Reproductive Success and Male Traits in the Spotless Starling (*S. unicolor*): An Introduction

Sexual Selection

The concept of sexual selection was introduced by Darwin (1859) in order to explain the existence of conspicuous male traits that were likely to reduce survival. Natural and sexual selection work fundamentally in the same way, requiring variability among individuals in hereditable traits that translate to different numbers of offspring (Alcock 1997). In fact, sexual selection is often seen as a subset of natural selection. Sexual selection (SS) is distinguished from the rest of natural selection (NS) in that it favours traits that relate exclusively to reproduction rather than traits that enhance viability and survival (Andersson 1994; Darwin 1871; Møller 1998). Furthermore, sexual selection is based on competition that inherently only exists between conspecifics, unlike other modes of natural selection. In many cases it is not easy to distinguish between NS and SS. Moreover, traits that simultaneously improve survival and mating or reproductive success, such as parasite and pathogen resistance, are under both selective pressures (Andersson 1994; Kokko et al. 2003). In such cases, NS and SS can operate in the same direction or in opposition.

It is widely accepted that inequalities in the potential reproductive rate (PRr) of males and females determine which sex will compete for the other. Increased parental investment (the time, energy and risk invested in each offspring) reduces the chance of having future offspring and, therefore, PRr is inversely correlated with parental investment. Parental investment is generally lower in males so there is a strong association between PRr and sex. Males produce many small gametes while females produce few expensive gametes. Therefore females must be more selective than males in their choice of mate

since they invest more per gamete. This explains the preponderance of species in which males are the “chosen sex” and females are the “choosy sex” (Alcock 1997; Andersson 1994). Females generally give more parental care than males, increasing parental investment and lowering their PRr. However, differences in parental care are also an effect of sexual selection (Kokko et al. 2003). A bias towards female parental care originated by sexual selection and this bias lowers females’ PRr. Therefore, we should be careful with this argument, remembering that the causality of the process depends on the evolutionary stage considered. There are some species in which the “choosy” and “chosen” roles are reversed, since some aspects of their biology (fundamentally related to parental investment) result in females having greater PRr than males (e.g. some species of pipefish and seahorse (Vincent et al. 1992) and Mormon crickets, (Gwynne 1981; Gwynne 1993)).

Two modes of sexual selection are often considered: 1) Intrasexual selection, in which males (or the sex with greater PRr) compete with individuals of the same sex for access to the females. This may favour traits that convey an advantage in fighting, such as weaponry (e.g. antlers, horns and spurs), increased body size and communicatory organs to threaten opponents (Alcock 1997; Harvey & Bradbury 1991); as well as less obvious features like spatial memory, sensory and locomotive organs which, for example, improve the chances of finding females first (Andersson 1994) and behaviours such as mate-guarding. 2) Intersexual selection, in which females (or the sex with lower PRr) choose certain mates. This process accounts for the evolution of conspicuous traits such as brightly coloured feathers of great length, complex song repertoires and elaborate courtship behaviour (Harvey & Bradbury 1991; Alcock 1997). Some authors, like Andersson (1994), avoid making a distinction between intra and intersexual selection, arguing that males will compete with each other through female choice (and sperm competition) even though rivals may never meet.

To be ‘choosy’ is only adaptive for females if there are differences in the benefits that each male represents for them. Direct benefits occur when being selective increases female lifetime reproductive output (direct fitness). Direct benefits are gained when a chosen male is more fertile, offers better

resources (territory, parental care, etc.), provides more protection or diminishes female reproductive costs (Kokko et al. 2003; McNamara et al. 2003). On the other hand, females can gain indirect benefits when the selection of particular male traits enhances the fitness of her offspring in other ways that do not involve providing resources. Therefore, the base of indirect benefits is the inheritance of male genes (Alcock 1997; McNamara et al. 2003). Furthermore, when a male trait that gives direct benefits is heritable, indirect benefits are also conveyed (Kokko et al. 2003). Male offspring will achieve a greater reproductive success due to selection by females using the same direct benefits criteria as their mothers. Therefore, it is important to consider both direct and indirect benefits as factors of female choice.

Sexual selection has been seen mainly as the struggle for mate acquisition, but recently the importance of other processes that take place during different stages in reproduction has been stressed. Post-copulatory selection occurs, for example, when females favour a certain male's sperm, or offspring (cryptic choice) (Eberhard 2000; Eberhard & Cordero 1995; Kokko et al. 2003; Møller et al. 1998). Therefore a measure of male reproductive success determined simply from mate acquisition can differ from a measure incorporating the actual number of offspring sired (including sired extra-pair offspring).

Selection can act in a direct or indirect way. A trait is under direct selection when it confers an increase in fitness. Indirect selection occurs when a trait is genetically correlated with another that is under direct selection. Consequently, the strength of indirect selection depends on the intensity of direct selection acting on the associated trait, the heritability of the associated trait and the magnitude of the correlation. Female preferences can evolve by indirect selection of certain traits via the direct selection of characteristics that confer higher fitness (Kokko et al. 2003).

There has been a tendency to subdivide indirect benefits into those that enhance offspring attractiveness and those that increase their viability and survival. Accordingly, two different models were also established: the "sexy son" model (sometimes referred to as the Fisherian runaway model) and the "good genes" model (Alcock 1997; Andersson 1994). However, Kokko *et al.* (2001; 2002) demonstrated that these benefits and models are not exclusive,

and furthermore, that they are just the two extremes of a sexual selection continuum. In most cases there will be a cost of being choosy (searching and discriminating among males) in terms of a reduction in fecundity or longevity. However, at equilibrium this cost can be extremely low (Kokko et al. 2002). If costs are low, any small benefit from being choosy could compensate for these costs and exaggerated male traits would evolve. Once a strong preference for a trait has evolved, the benefits of being attractive may override the benefits of longer survival or other fitness components. Thus, males may exploit the tactic of displaying their higher quality even to the extreme at which their likelihood of survival is lower than that of lower quality males. So, the trade-off between male attractiveness and other fitness components is mainly determined by the costs of being choosy for the female. A female can still gain from having male offspring that allocate more resources to mate acquisition and not to survival (Kokko et al. 2002; Kokko & Jennions 2003).

Sexually Selected Traits

There is a huge variety of sexually selected traits known. Some are physical traits, like long or colourful feathers, antlers, horns and spurs (Alcock 1997; Harvey & Bradbury 1991); whilst others are behavioural, such as mate-guarding, singing and the performance of courtship displays. There are several traits that seem to be under sexual selection in the spotless starling (*Sturnus unicolor*), according to observations and studies in this species and the closely related species, the European starling (*S. vulgaris*). These traits include physical characteristics such as coloration (including UV reflectance) and feather length; and behaviours such as singing and the provision of green materials.

Conspicuous Colouration

Since Darwin (1859; 1871) suggested the evolution of conspicuous male traits by means of sexual selection, special attention has been given to conspicuous colourations. Darwin (1871) explained those colourations mainly as a result of female choice, but since then some other hypotheses have been put forward

(Andersson 1994; Butcher & Rohwer 1988). Currently, there are four main hypotheses. These suggest that bright colours are used: a) for species recognition; b) to advertise prey unprofitability; c) to threaten competitors in intrasexual contests (intrasexual selection); d) as cues for female choice (intersexual selection).

There are two ways by which species recognition could influence the evolution of colourful signals (Andersson 1994; Butcher & Rohwer 1988). Colour differences between species may evolve in order to avoid mistaken interspecific aggression (Lorenz 1962 & 1966 cited in Butcher & Rohwer 1988). Alternatively, colours could act as a pre-mating isolation barrier, advertising species identity to potential mates and so avoiding interbreeding. This process has been found in some butterflies (Wiernasz & Kingsolver 1992). Thus, species recognition may play a part in the evolution of some species' colourations. However, the general relevance of this factor in the evolution of conspicuous colourations in birds does not seem to be as important as the role of sexual selection, as it will be shown later. Nevertheless, species recognition could account for the initiation of some female preferences.

Bright colours can advertise to predators that a potential prey is poisonous, has weapons, is alert or anything else that makes it unprofitable for the predator to pursue. This aposematic colouring is common in some animal groups such as the amphibia, exemplified by the numerous species of poison-dart frogs (Myers & Daly 1983), and the lepidoptera, such as the butterfly *Eumaeus childrenae* (Contreras-Medina et al. 2003), but it does not seem to be the case in birds (but see Dumbacher *et al.* (1992) for *Pitohui*, a passerine genus, as an example of chemical defence in birds). The strongest evidence refuting this aposematic hypothesis comes from observations showing that these colourful traits are not displayed to predators, but instead to conspecifics, especially in the context of mating and intrasexual competition (Andersson 1994; Butcher & Rohwer 1988). Moreover, if birds use bright colouration to advertise unprofitability, we would expect them to use it preferentially when there is easier alternative prey. Contrary to this, birds tend to moult to their colourful plumage at the beginning of the mating season

(spring) and not in summer when young and, therefore, more vulnerable birds are around (Andersson 1994; Butcher & Rohwer 1988).

The third and fourth hypotheses propose the evolution of conspicuous colourations by means of sexual selection. In birds, these hypotheses are supported by the predominance of certain patterns in the expression of these signals. For example: traits are displayed to potential mates and rivals (see above), traits are acquired at sexual maturity, males are more colourful than females and males are more colourful during the breeding season (Andersson 1994; Darwin 1871). Moreover, when some of these patterns are not exhibited or are even reversed peculiarities of the reproductive cycle or parental roles seem to be responsible. An example of this is evident in ruddy ducks (*Oxyura jamaicensis*); most ducks form pairs in autumn or winter and, accordingly, they change to their colourful plumage in autumn. However, *O. jamaicensis* moults to colourful plumage in spring, which is when they form pairs (Andersson 1994). In some species, such as the painted snipe (*Rostratula benghalensis*; Darwin (1871)), the Wilson's phalarope (*Phalaropus tricolor; C*olwell & Oring (1988)), the red phalarope (*P. fulicaria*) and the red-necked phalarope (*P. lobatus*; Shamel & Tracy (1991)), the courtship and parental roles are sex-reversed and the females are more colourful than males. .

In particular the third hypothesis stipulates that bright colouration evolved as a result of intrasexual selection. Bright colours may have evolved to show, at a distance, that some resources (including mates) are occupied and that the owner is prepared to defend them. This may apply particularly when resources are more valuable to the owner than to the intruder; the "priority hypothesis" (Andersson 1994; Butcher & Rohwer 1988). Evidence for this has been found from the red-winged blackbird, *Agelaius phoeniceus* (Beletsky & Orians 1989) and the European robin, *Erithacus rubecula* (Tobias 1997). Another way in which colouration may play a role in intrasexual conflicts is by honestly advertising the quality of males, so that good fighters are recognizable and contests do not escalate into fights, when the outcome is predictable. Evans & Hatchwell (1992a) experimentally modified the scarlet pectoral tufts of scarlet-tufted malachite sunbirds, *Nectarinia johnstoni*. The tufts which are displayed in contests were either reduced or enlarged in the

study. Males with reduced tufts were involved in more aggressive interactions and lost territory area, while enlarged-tuft males gained area. Similarly, Pryke & Andersson (2003) found that males of the territorial red-shouldered widowbird, *Euplectes axillaris,* have larger and redder carotenoid-based epaulettes (lesser wing coverts) than floater males; that males with larger and redder epaulettes monopolized feeders in captivity; and that more aggressive interactions occurred when epaulettes of competitors were experimentally modified to be similar. Thus, intrasexual selection seems to play a role in the evolution of bright colour-traits. Nevertheless, female choice may also play an important part in the evolution of these traits, provided females can increase offspring fitness by choosing males with attributes that reflect dominance.

Finally, there is the hypothesis of female choice as the force that gives rise to colourful traits. There is some direct evidence of female preference of colourful traits in some species. For example, Hill (1990; 1991) demonstrated in house finches (*Carpodacus mexicanus*) that: captive females preferred more colourful males; in the field, paired males were more colourful than unpaired males; and colour was a reliable indicator of parental care and possibly of survival. Likewise, evidence of female preference for redder beaks (and red marking-rings) was found in zebra finches (Burley & Coopersmith 1987). Burley (1986) also found that preferences for red-ringed zebra finch males result in a bias towards males in the sex ratio of the offspring. However, Rutstein *et al.* (2004; 2005) did not find this effect on the primary sex ratio when controlling for female quality. Gil *et al*. (1999) found differential investment in eggs according to these colour preferences. Females mated with more attractive males deposited more testosterone and 5α-dihydrotestosterone in their eggs. However this effect was not found in a study by Rutstein et al. (2004).

UV Reflectance

In the last few years it has been stated that in addition to colours within the human visual spectrum, UV-reflectance may play an important role in bird sexual communication. It has been found that birds are able to detect UV light using special cone-cells (additional to the three types of cone-cells shared

with humans) and oil droplets (Bennett & Cuthill 1994). Since mammals do not see UV, exploiting these wavelengths for ornamentation by birds does not make them more conspicuous to mammalian predators (Hausmann et al. 2003). Furthermore, UV does not penetrate as far through air as longer wavelengths, so it may be useful in short distance communication without attracting more distant predators, even other birds (Bennett & Cuthill 1994; Hausmann et al. 2003; Hunt et al. 2001).

Hausmann *et al.* (2003) found that among 108 Australian bird species, 72% had one or more plumage regions that reflect UV. Similarly, Eaton and Lanyon (2003) sampled feather patches of species from 142 families of birds and found that UV reflectance was widespread. Even when white plumage was excluded, 140 families and 95.2% of the species examined presented plumage with UV reflectance.

But, is UV reflectance a sexually selected trait? Some evidence is available that supports the hypothesis that UV reflectance evolved as a result of SS. Sexual dimorphism in UV colouring has been found in some species, such as starlings, *Sturnus vulgaris* (Cuthill et al. 1999) and blue tits, *Parus caeruleus* (Andersson et al. 1998; Hunt et al. 1998), typically with brighter, larger or more numerous UV-reflective patches in the males. A second line of evidence comes from a study by Hausmann *et al*. (2003) who found a strong positive correlation between UV colouration and courtship displays in Australian birds. Thirdly, female preference for males with high UV reflectance in plumage has been found in several species. Siitari *et al.* (2002) experimentally altered the UV reflective patches in pairs of otherwise similar male pied flycatchers (*Ficedula hypoleuca*) males. The UV reflectance of the crown, mantle and back of one male of the pair was reduced and was slightly increased in the other male. The experiment showed a clear female preference for males with the increased UV reflectance, when territory quality was controlled for. Similarly, blue tit males with highly UV-reflective crown plumage experience less paternity loss than males with lower UV-reflective crowns, although the latter sired more extra-pair offspring (Delhey et al. 2003). Additionally, blue tit males with brighter UV colouration sired more male than female offspring (Griffith et al. 2003; Korsten et al. 2006; Sheldon et al. 1999) and experienced

greater survival (Griffith et al. 2003). In starlings, females have been found to rank males in order of the UV reflectance of their throat feathers (Bennett et al. 1997). However, in the absence of ambient UV, the females still consistently ranked the males but in a different order, suggesting that other cues may also be used. Similarly, the removal of UV reflectance has been found to greatly reduce zebra finch females' preference for males (Bensch 1996; Hunt et al. 1997). Nevertheless, Hunt *et al.* (2001) found that the removal of medium wavelengths (500-600 nm) and long wavelengths (600-700 nm) of visible light had a stronger impact on female preference than the removal of UV (300-400 nm) and short visible wavelengths (400-500 nm).

In summary, there is evidence supporting the evolution of certain UV reflectance traits in birds as a result of sexual selection (principally involving female choice), although the relative importance of these cues is not known.

Long Feathers

In addition to colouration (including UV reflectance), there are other visual traits under sexual selection. The most studied of these traits in birds are long feathers, particularly long tails. Some classical examples are found in widowbirds, swallows and peacocks.

Andersson (1982) tested female widowbirds' preference for males with enlarged tails, shortened tails and control males. The results indicate a higher reproductive success for males with elongated tails than for males with shortened tails or controls. Since then, other studies have found supporting evidence for this (Pryke & Andersson 2002; Pryke & Andersson 2005; Pryke & Andersson 2008; Pryke et al. 2001; Savalli 1995), while others have failed to (Craig & Villet 1998; Savalli 1994).

Similarly Møller (1988) found that male swallows (*Hirundo rustica*) with elongated marginal tail feathers obtain mates in less time than control males or males with shortened tails. Male swallows with elongated tail feathers also experienced higher reproductive success and were preferred by females for extra-pair copulations (females paired to other males solicited or accepted copulations from these males more often). Paradoxically, the lengths of tail

streamers of males of a North American subspecies of swallow (*Hirundo rustica erythrogaster)* were found not to correlate with male reproductive success, or extra-pair paternity (Neuman et al. 2007). Furthermore, Neuman *et al.* did not find a correlation between the proportion of paternity loss suffered by males (number of offspring in their nests produced by extra-pair copulations) and their tail-streamer lengths. This brought them to conclude that the function of sexual signals varies geographically in this species.

Another famous example is that of the train of peacocks. Petrie *et al.* (1991), found in an observational study, that the number of eye-spots in the train of peacocks (*Pavo cristatus*) correlated with their mating success. Subsequently, Petrie & Halliday (1994) experimentally reduced the number of eye-spots on the trains of a group of peacocks and found that males of this negative treatment experienced a decrease in their mating success. Other studies have been done on this species; some of them found additional evidence supporting the role of male trains as a sexually selected trait (Loyau et al. 2005b; Loyau et al. 2007; Møller & Petrie 2002; Petrie et al. 2009), while others did not (Hasegawa 1995; Takahashi et al. 2008).

Besides tails, longer crests appear to be a trait subject to female selection in some species. For example, in the monogamous crested auklet (*Aethia cristatella*) there is a preference both for females and males with longer crests (Jones & Hunter 1993).

Similarly, the length of the throat feathers seems to be under sexual selection in the spotless starling, *S. unicolor*. This assumption is based on the fact that this trait is sexually dimorphic, but no experimental studies have been done (see spotless starling section).

Song

Song appears to be a sexually selected trait in many animals. Some of the most common examples are orthopterans (e.g. grasshopper *Chorthippus biguttulus*, Klappert & Reinhold (2003)), frogs (e.g. gray tree frog, *Hyla versicolor*; Gerhardt *et al.* (2000)) and birds. Until the 1970's attention was focused in the function of song in male contests, but since then its importance

for female choice has been emphasized (Andersson 1994). Furthermore, it has been found that some birds (and frogs) seem to use different kinds of song depending on the context; mate attraction or male contest (e.g. American warblers, *Parulinae*, Lemon *et al* (1987); European starlings, *Sturnus vulgaris*, Adret-Hausberger & Jenkins (1988), but see Eeens *et al.* (1993)). In birds, two aspects of song seem to have some importance in female choice: 1) length of song bout and 2) repertoire size.

The European starling (*S. vulgaris*) is one of the most-studied species in this area, and its song structure seems to be very similar to that of the very little-studied spotless starling (*S. unicolor*, Gil D. pers. comm.). Male starling song often begins with whistles, followed by a series of different song types (including heterospecific imitations) and often ending with high-frequency song types (Eens et al. 1991a). Eens *et al*. (1991a) found that first year males have smaller repertoires and sing shorter song bouts. They also reported a positive correlation between average song bout-length and repertoire size. These two variables inversely correlated with pairing date, but the study did not control for age and arrival date; variables that also correlate with pairing date. Bout length and repertoire size positively correlated with the degree of polygyny and the number of fledglings. However, only the correlation between the number of fledglings and song bout length remained significant when yearlings were excluded.

Adrethausberger & Jenkins (1988) , reported, from qualitative observations, that starlings used two types of song in two different contexts (the “whistles” in contests and the “warbling song” for mate attraction). However, Eens *et al.* (1993) found no support for this. Conversely, they found that the two song types tended to be more common in the opposite contexts. In another experiment, Eens *et al.* concluded that male starlings’ songs are used principally for mate attraction and not for male contests, since males spent more time singing and used larger repertoires when a female was introduced in an already occupied aviary than when a male was introduced. Additionally, Gentner and Hulse (2000) found that females prefer longer song bouts, while males do not make a distinction between them. Even when controlling for nest-site preference, Mountjoy and Lemon (1996) found a positive correlation

between a male's repertoire size and female preference. Although repertoire size correlates with condition, condition was found to be a less important factor for female choice.

In starlings the benefits obtained by female choice for longer song bouts and larger repertoires seem to be mainly indirect, since they do not defend a territory, only the nest-site itself. There did not appear to be a correlation between males' song quality and the likelihood of occupying preferred nest sites (Mountjoy & Lemon 1996). Furthermore, Mountjoy and Lemon (1997) found that males with larger repertoires did not help more with incubation or in feeding the chicks, than did males with smaller repertoires. In addition, some evidence points to the existence of indirect benefits. Buchanan *et al.* (2003) found that early developmental stress is reflected in a delay to start singing and that as adults they sing for less time and with fewer, shorter song bouts. Therefore, male song quality and quantity may be considered as traits that reflect male response to early developmental stress. Additionally, Duffy & Ball (2002) found a positive correlation between song bout-length and humoral immunity, while song rate positively correlated with cell-mediated immunity.

Green Nesting Material

The addition of fresh plants (especially aromatic plants) to nests has been observed in some bird species (see Wimberger (1984)) including the European starling (Clark & Mason 1985; Fauth et al. 1991; Gwinner 1997; Gwinner et al. 2000) and the spotless starling (Veiga et al. 2006). Two non-exclusive hypotheses have been suggested to explain this behaviour. According to the nest protection hypothesis, fresh plants reduce the number of ectoparasites in the nest (Wimberger 1984); while the courtship hypothesis proposes that fresh plants are used for mate attraction (Fauth et al. 1991).

There is some controversy about the function of green nesting material in the European starling, since different studies have had contradictory results. In laboratory and field experiments, Clark & Mason (1985) found that certain plants preferred by European starlings had a detrimental effect on the development of northern fowl mites (*Ornithonissus silviarum*) and bacteria,

providing support for the nest protection hypothesis. Contrary to this, Fauth *et al.* (1991) did not find any effect of herbs on mites in a different starling population.

Eens *et al.* (1993) presented evidence that supports the use of green nesting material in intra-specific nest-site defence and courtship. They found that males in aviaries increased the number of times they collected and introduced green material into their nest-boxes (and not any other material) when presented with another male or female. Moreover, there was a greater increase in this behaviour during female introductions than during male introductions. Gwinner (1997) found further evidence for the use of green nesting material in courtship. She observed that male starlings collected green plants (and also large feathers, flowers, lichens and some human artefacts) and displayed them in their beaks while singing or approaching a female. Occasionally, these items were left in front of the female and were subsequently incorporated into the nest. Conversely, females rarely gathered green-plants and, when they did, they did not conspicuously display them. Gwinner also found that the amount of green material incorporated positively correlated with the duration of courtship; there was a greater amount of greenery in secondary nests (maybe as a result of longer advertising periods); and that nests used for second or replacement broods contained fewer green plants than newly occupied nest-boxes. These findings are contrary to the expectations of the nest protection hypothesis. However, some species of plants were incorporated as green material into nests more often than would be expected from their abundance. Most of these preferred species were rich in volatiles that may work as insecticides (evidence supporting the nest protection hypothesis). Moreover, Gwinner *et al.* (2000) found that nestlings in nests with green nesting material weighed more, had higher haematocrit levels (particularly close to the fledging date), had more basophils and fewer lymphocytes and exhibited a higher return rate as yearlings (reflecting higher survival rate) than nestlings in control nests. Thus, green plants seem to improve nestling condition, possibly by stimulating the immune system although no difference was found between the mite loads of nests with and without greenery.

In summary, there is evidence supporting both the nest protection and the courtship hypotheses in the European starling. Therefore, the collection and incorporation of green nesting materials into nests seems to be a behaviour selected by females because of direct and indirect benefits. This could be the case also for spotless starlings. However, in spotless starlings, the addition of green material has been considered exclusively as a courtship behaviour that indicates indirect benefits (Polo et al. 2004; Veiga et al. 2008; Veiga et al. 2006), even though no experimental studies have been done to test the possible existence of direct benefits from the addition of green materials (see the spotless starling section).

Multiple Sexually Selected Traits

The presence of multiple sexually selected traits appears to be common in birds. Some birds have plumage ornaments combined with mating displays. A classic, well-studied example is the zebra finch, *Taeniopygia guttata*, in which species the female prefers males with redder beaks, greater UV reflectance in their feathers and that sing at higher rates (Bennett et al. 1996; Birkhead et al. 1998; Burley & Coopersmith 1987; Hunt et al. 1997). Models have been created to try to understand the evolution of multiple sexually selected traits and multiple sexual preferences. Pomiankowski and Iwasa (1993) showed that multiple female preferences for Fisherian traits may evolve and be maintained if the cost to females in choosing mates does not increase greatly. As the cost of choosing increases, the preference for just one trait predominates and the others disappear. Similarly, using another model they showed that multiple handicap traits can only evolve when the cost of choice by females does not increase greatly. However, if the cost increases substantially, the first preference to evolve will block the evolution of others even when those traits are better indicators of male condition (Iwasa & Pomiankowski 1994). But even in the cases when just one preference for a handicap trait evolves, multiple preferences for Fisherian traits are likely to evolve alongside (Iwasa & Pomiankowski 1994; Møller et al. 1998). Additionally, Johnstone (1995) found that multiple condition dependent traits

that signal different aspects of the quality of a male can evolve to be evolutionarily stable and honest signals.

In addition to the models, several hypotheses have been suggested to explain the evolution of multiple signals or sexually selected traits (Møller & Pomiankowski 1993). A) The tactics used by males to compete for mates may change according to factors such as their age, their social status and their condition. B) Different ornaments may provide different information or reflect different aspects of the male quality. C) Intra sexual selection and inter sexual selection may favour different traits. Explicitly, competition between males may be based on different traits than female choice. D) Similarly, different traits may function in different contexts. E) Mate choice may be based on a combination of traits that are assessed simultaneously by the females. F) Multiple traits may be maintained because they are not costly for males and the female choice is weak and not costly. However, in this case these traits would not be reliable signals of male condition or attractiveness. G) Multiple sexually selected traits may evolve if female preferences are flexible, i.e. if females change their preferences according to changes in the environment (e.g. changes in the abundance of food) or individual social and physical condition (Marchetti 1998; Møller & Pomiankowski 1993).

It is unlikely that just one mechanism is responsible for the evolution of multiple traits. Different mechanisms may be acting in different species or even in the same species. In peacocks, *Pavo cristatus*, it has been shown that different sexually selected traits correlate with different aspects of the male quality (Loyau et al. 2005b; Møller & Petrie 2002). Møller *et al.* (1998) showed that female swallows (*Hirundo rustica*) chose mates based on two male traits and that the importance given to a phenotypic plastic trait (song rate) depended on the level of expression of a stable morphological signal (the length of the tail). An interesting case was reported by Marchetti (1998); in females of the yellow-browed leaf warbler, *Phylloscopus inornatus;* females assessed male quality through many traits and, thus, when one of the traits was experimentally manipulated they were still able to assess the real (before manipulation) quality of the males using the other signals. Another study in lark buntings, *Calamospiza melanocorys* (Chaine & Lyon 2008) showed that

females of this species seem to select for multiple traits, but the intensity and even the direction of selection vary from year to year, probably in relation to changes in the ecological and social environment. The possible existence of multiple traits and of female flexibility in choice makes the study of sexual selection more complicated, requiring more precise and long-term studies. When assessing the importance of a characteristic as a sexually selected trait, other traits that appear to be under sexual selection should be taken into account and controlled for, if possible.

Reproductive Success

The quality of a male in evolutionary terms is defined by his fitness and measured as the number of offspring that survive to breed. It is very difficult (if not impossible) to calculate the total fitness of individuals in the wild, since all their offspring survival (and their offspring in future generations) would have to be considered. Therefore, most studies attempt to partially assess the fitness of individuals calculating their reproductive success as the number of offspring produced per reproductive event, year or lifetime.

Before molecular parentage studies were available, it was thought that most bird species were truly monogamous and that polyandry in birds was extremely rare (Lack 1968). However, using molecular methods for parentage analysis, extra pair paternity (EPP) has been detected in more than 75% of the bird species previously considered to be monogamous (Bennett & Owens 2002; Tang-Martinez & Ryder 2005). EPP occurs when offspring in a brood are sired by males that are not the attending male or 'brood father' (for examples and details see Chapter 3). The existence of cases where an individual had one social partner (attending male or female) but sired offspring with others, resulted in the necessity to make a clear distinction between social and genetic (or true) monogamy.

It is important to consider EPP when measuring male reproductive success, since it represents a loss for males whose females "cheated" and a gain for males that sired offspring in additional nests. Other phenomena that affect reproductive success are intra-specific brood parasitism (IBP) and quasi

parasitism (QP). IBP refers to cases where females lay eggs in the nest of conspecifics (hosts) and do not provide any parental care (Arnold & Owens 2002; Griffith et al. 2004; Zink 2000). QP is similar to IBP in that females lay eggs in the nest of a host-female, but QP eggs are fertilized by the host-male (the social mate of the host-female).

In summary, to calculate the reproductive success of males it is necessary to know which offspring they really sired, including the offspring sired in other nests and excluding offspring sired by other individuals in their nests. Modern molecular genetic methods are well suited to detecting cases of EPP, QP and IBP.

Molecular methods for parentage analysis

The first parentage studies were done in the 1970's using chromosomal polymorphisms; followed shortly by allozyme electrophoresis (Jones & Ardren 2003). In the 1980's, parentage analysis using DNA fingerprinting became possible and by the late 1980's the genotyping of microsatellites using PCR (polymerase chain reaction) had become widespread (Jarne & Lagoda 1996).

There are several characteristics of microsatellites that make them especially useful (for details see Chapter 2). They are highly polymorphic co-dominant markers that obey Mendelian rules of inheritance and each locus and each allele within a locus is independent. The fact that they are short sequences makes them relatively easy to amplify even from degraded samples. Additionally, alleles can now be sized with high precision and can be reliably compared among individuals (Jarne & Lagoda 1996; Queller et al. 1993; Selkoe & Toonen 2006; Sunnucks 2000). All this explains why microsatellite markers are currently the most popular and powerful genetic tool used for studying population processes at a very fine scale, such as parentage analysis (Jarne & Lagoda 1996; Sunnucks 2000).

Most of the programs created for parentage analysis are focused on their use with microsatellite data (Jones & Ardren 2003). Currently, two of the most popular programs for parentage analysis are NEWPAT XL (Summers & Amos

1997), and CERVUS 3.0.3 (Kalinowski et al. 2007). NEWPAT XL uses the exclusion approach to assign paternity. This approach is based on the rules of Mendelian inheritance: each individual inherits one allele from its mother and one from its father. With this assumption, incompatibilities between the offspring genotype and the candidate parents' genotype can be searched for and incompatible candidate parents can be excluded (for details see Chapter 3). If there are sufficient markers and they are sufficiently variable, only the genetic parents will remain (Jones & Ardren 2003). CERVUS, uses the likelihood approach (Kalinowski et al. 2007; Marshall et al. 1998), based on the calculation of the ratio of the likelihood that individuals (or pairs) are the true parents of a given offspring against the likelihood that they are not. Subsequently, CERVUS uses simulations to estimate the statistical confidence of this likelihood (for details see Chapter 3).

The Spotless Starling, *Sturnus unicolor* Temminck

The spotless starling is a hole-nesting, facultative polygynous species, mainly confined to the Iberian Peninsula (Aparicio et al. 2001; Cordero et al. 2001). Although this species can nest in dense colonies when nest-boxes are provided, they also occupy natural holes in trees at much lower density (Aparicio et al. 2001). In high density nesting colonies, males defend as many nest-sites as they can, which counters the recruitment of other individuals to the colony (Cordero et al. 2001). Feeding territories are communal and only nest-sites are defended against conspecifics (Veiga et al. 2001). These birds exhibit high levels of philopatry and are relatively long-lived (80% were still breeding after 5 years of observations; Cordero *et al.* (2001)).

The spotless starling's great abundance and propensity to nest in nest-boxes make this species a popular model for studies in the wild. A high proportion of the studies on spotless starlings include hormone manipulation. The levels of androgens in eggs were increased by Wendt *et al.* (2007) to see their effect on the development and survival of the chicks. Hormone manipulation has been done to adult males to see the effect on parental care and reproductive success (Moreno et al. 1999) and to assess possible costs of polygyny

(Cordero et al. 2003). The level of testosterone has been manipulated in females to see how this affected their social rank, the sex ratio of their offspring (Veiga et al. 2004), the females' lifetime fitness (Veiga & Polo 2008) and the number of extra pair offspring in their broods (García-Vigón et al. 2008). Additionally, chicks of spotless starlings have been used as models concerning the function of parent-absent begging (Bulmer et al. 2008); the links between hormone levels (testosterone and corsticosterone) and sibling competition; the developmental plasticity (differential investment in organs used in sibling competition) of chicks (Gil et al. 2008); and the relationship between juvenile colouration, condition and immune response (Soler et al. 2007).

In the area around Madrid, the spotless starlings' breeding season starts in late March and ends in late July (Veiga et al. 2001). Females lay around 5 eggs per clutch and can breed once or twice in a season (first and second broods), sometimes laying a replacement clutch when the first is lost. Magpie (*Pica pica*) predation rates of the whole brood have been reported between 15 and 48% (Moreno et al. 2002). They prey upon begging chicks that have grown large enough to move to the entrance of the nest (Moreno et al. 1999; Moreno et al. 2002).

Spotless starlings reproduce synchronously with their neighbours. They tend to have two consecutive reproductive events per year (referred to as events in this thesis). Typically, the clutch for the first event is laid between mid April and the beginning of May and the second clutch is laid during June, but the dates vary from year to year. When an event is not completed, due to the loss of the eggs or chicks (as a result of predation, intra-specific sabotage, etc), the nest tends to be re-occupied by the same or different birds and this replacement event will be out of synchrony with the two main reproductive events. Therefore events can be classified in first, intermediate (or replacement) and second events, according to the date they were started in respect to the general starting date of events in the population. Appendix 10 and Appendix 16 show the distribution and classification of the reproductive events in the study population during 2004, 2005 and 2006.

Intra-specific nest parasitism occurs among spotless starling broods and is much more common in first clutches (≥35%) than in intermediate and second clutches (≤20%) (Calvo et al. 2000). Extra-pair paternity also seems to be common in this species. Cordero *et al*. (2003) reported between 10 and 20% extra-pair fertilizations. Moreover, an increase in the loss of paternity seems to be one of the costs suffered by polygynous males, as was shown in an experiment that controlled for male quality in the degree of polygyny (Cordero et al. 2003).

A significant difference in egg provisioning (the eggs containing female embryos were heavier), as well as a seasonal variation in sex ratio (shifting from a bias towards daughters to one towards sons as the season advances) were found in this species (Cordero et al. 2001). In this way females may increase their reproductive success, since the probability of recruitment as yearlings into the breeding population is linked to laying date in females and to weight at fledging in males. Female reproductive success in first broods seems to be related to female maternal quality (Moreno et al. 2002). Nevertheless, Moreno *et al*. (2002) found that the probability of laying a second brood depends on mating status (at least in one of the two years studied) and that additional broods were more likely for primary than for secondary females. They also found that the clutch size and hatching brood size were larger for primary than for secondary females, whereas monogamous females had intermediate values, although not significantly different.

The spotless starling is a sexually dimorphic species (Figure 1); males are brighter, exhibiting more purple iridescence in their plumage (possibly UV reflective) and bearing longer throat feathers than females (around 41% longer, Aparicio *et al.* (2001)). There is also a slight size dimorphism, males being around 6% heavier and 3% longer than females (Cordero et al. 2001). The shape of throat feathers is also sexually dimorphic, since males' throat feathers narrow abruptly from the middle region towards the tip, while female feathers narrow gradually (Hiraldo & Herrera 1974; Lezana et al. 2000). The colour of the base of the beak of adult males also differs from that of females:

the base of the female's beak is pink, while that of the male is blue-grey in colour.

Figure 1. Male and female spotless starlings (*Sturnus unicolor*) showing sexual dimorphism in throat feather length (longer in males), plumage (darker and more iridescent in males) and beak's base colouring (blue/grey in the male and pink in the female).

The length of throat feathers (TF) is thought to have an effect on mate attraction. It may also be correlated with increased heterozygosity, however the sample studied was small and the results were contradictory (see Aparicio *et al*. (2001)). Aparicio *et al.* (2001) reported a positive correlation between mating success and average length of TF, but male reproductive success (observed or genetically verified) was not assessed. There is a lack of studies that test the hypothesis that any of these dimorphic physical traits are sexually selected.

Singing and the addition of green material to the nest are behaviours exhibited by this species that are suspected to be under sexual selection. The song structure of male spotless starlings seems to be very similar to that of the European starling (D. Gil pers. comm.). But while the song of European starlings has been the focus of many studies (see above), there have been no studies in the spotless starling. The addition of green nesting-materials (see above) to the nest is thought to be a courtship display used by males to stimulate females to breed (Veiga et al. 2006). Two studies have experimentally added green materials to nests to try to increase the quality of males as perceived by the females. One study found that females increased

the sex ratio of their offspring (Polo et al. 2004) as predicted by the sex allocation theory (Trivers & Willard 1973). However, in the second study only young and medium aged females increased the brood sex-ratio, while older females decreased it (Veiga et al. 2008).

Since paternal care in spotless starlings is reduced or absent in secondary nests (Moreno et al. 2002), secondary females experience a cost from mating with polygynous males. Theory predicts that some compensatory benefits should arise from female choice when this choice is costly (Harvey & Bradbury 1991). Since direct benefits do not seem to play an important role in female choice in this species (especially in the case of secondary females), the benefits of choosiness are likely to be related to the production of more viable or more attractive offspring. Therefore, males should be chosen according to traits that indicate their condition, genetic quality or attractiveness. Males that are more attractive should attain a higher reproductive success.

Aims

The key aim of this study is to determine the relationship between certain male characteristics (body condition, size and TF length), and male reproductive success, with a particular focus on the length of the throat feathers as a sexually selected trait. Male reproductive success is defined, in this study, as the number of offspring sired in one reproductive season. Thus, reproductive success will depend on the degree of polygyny, the avoidance of paternity loss and the number of extra-pair offspring sired. Therefore, to assess the real reproductive success of males, the paternity of the chicks needed to be determined using genetic markers.

The first stage of this study, presented in Chapter 2, is the acquisition of microsatellites via cross-species amplification and direct isolation. The 9 microsatellites described are molecular tools that can be used not only in the spotless starling, but also in closely related species such as the European starling.

The effectiveness of these microsatellites for parentage analysis becomes clear in Chapter 3, where the parentage analyses of offspring (from more than one thousand chicks and embryos) from three reproductive seasons (2004 to 2006) are presented. The levels of polygyny, extra-pair paternity, intra-specific nest parasitism and quasi-parasitism, detected using this molecular determination of parentage, are also reported.

Chapter 4 presents a correlative approach aimed at finding a link between three male characteristics (body condition, size and throat feather length) and male reproductive success. Factors such as the level of polygyny, the level of paternity loss, the quality and quantity of offspring produced are considered.

Chapter 5 presents an experimental study of throat feathers as a sexually selected trait, using experimentally shortened throat feathers vs. unmanipulated control males. Finally, the findings of these investigations are collectively discussed in Chapter 6, presenting the overall conclusions of this thesis and suggesting further work in this field.

Chapter 2. Acquiring Microsatellites via Cross-species Amplification and Direct Isolation from Spotless Starlings (*Sturnus unicolor*).

Introduction

Microsatellites have become a very important tool for biologists. When multiple microsatellite loci are scored, the resulting genotypic arrays are one of the most sensitive genetic markers. They are especially useful to study population processes at their finest scale, e.g. to determine parentage and relatedness (Sunnucks 2000). Microsatellites are tandem repeats of series of up to 6 base pairs. Usually these loci are composed of between five and 40 repeats (Selkoe & Toonen 2006). There are several characteristics of microsatellites that make them especially useful: a) They exhibit a high mutation rate (between 10^{-2} to 10^{-6} mutations per locus per generation (Goldstein & Pollock 1997; Sunnucks 2000), due to slippage and proof-reading errors during replication. As a consequence they are highly polymorphic (Jarne & Lagoda 1996; Selkoe & Toonen 2006; Sunnucks 2000; Van Oosterhout et al. 2004). b) Only a very small tissue sample is needed (for extraction of DNA) to successfully amplify them. They can be amplified even when some DNA degradation occurs since they are very short sequences (Selkoe & Toonen 2006). c) Each marker locus is independent (unless linkage is detected). Therefore, combining the data from the different loci gives more precise information that helps to reduce the effects of sampling error (Selkoe & Toonen 2006; Sunnucks 2000). d) Currently technology permits alleles to be scored with an accuracy of one base-pair and since microsatellites are codominant, both alleles of a locus can be recorded (Jarne & Lagoda 1996; Selkoe & Toonen 2006; Sunnucks 2000). e) They are considered to be selectively neutral (Schlotterer 2000; Selkoe & Toonen 2006).

Spotless starlings (*Sturnus unicolor*) and European starlings (*Sturnus vulgaris*) are hole-nesting, facultatively polygynous species (Aparicio et al. 2001; Cordero et al. 2001; Loyau et al. 2005a). Although high levels of both intra-specific nest parasitism and extra-pair paternity occur in both species (Cordero et al. 2003; Loyau et al. 2005a), no microsatellite markers have been developed specifically for them. Indirect criteria such as the overall appearance of eggs, the overlapping of two eggs' laying date or the laying of an egg outside of the laying period, have been used to detect nest parasitism (Calvo et al. 2000). Multi-locus DNA fingerprinting has been used to assess extra-pair paternity in spotless starlings (Cordero et al. 2003). Recently, Loyau *et al.*(2005a) used five microsatellites developed for other species to evaluate extra-pair paternity and intra-specific nest parasitism in European starlings. And García-Vigón *et al.* (2008) used 6 microsatellites from other species (including two presented in this chapter) to assess extra-pair paternity in the spotless starling. For this study of the spotless starling we wanted to use a more powerful approach to assess paternity by using a larger number of polymorphic microsatellites, and ideally markers that had been developed from this species. Firstly we tried the cross-species amplification of 11 microsatellites developed for other bird species. Then we isolated microsatellites directly from the spotless starling. Finally we optimised PCR conditions and surveyed the variability of these markers in the European starling (*Sturnus vulgaris*)

Materials and Methods

DNA was extracted using the PUREGENE protocol (Gentra Systems) from blood-samples of adult spotless starlings captured in Soto del Real (Spain). For the cross-species amplification of eleven microsatellites isolated from other bird species 20 DNA extractions were chosen. These were used as template for the trials. The samples were chosen based on their abundance of DNA content since it was desirable to try the PCRs of each locus under all conditions with the same template material.

PCRs were performed using 3 different concentrations of $MgCl_2$ and a gradient of annealing temperatures. The temperature gradient applied was defined by the lower melting temperature (LT) of the two primers for that locus. The temperature gradient went from six degrees under LT to one degree above it. For each set of conditions 7 DNA samples and a negative control were used. PCRs were performed in a reaction of 15 µL with: ~20 ng of DNA, 1.5.µL of 10x NH_4-based Reaction Buffer (Bioline), 0.375 U of BIOTAQ DNA polymerase (Bioline), 1.5 pM of each primer, 0.20 nM of each dNTP and either 1.25, 1.5 or 1.75 mM $MgCl_2$. The PCR program used was: a) 94^{o}C for 4 minutes; b) 30 cycles of: 94^{o}C for 20 seconds, the annealing temperature (gradient) for 30 seconds and 72^{o}C for 30 seconds; and c) a final step at 72^{o}C for 5 minutes.

The PCR product was then run in a 2.5 % agarosa gel with ethidium bromide and viewed using a UV trans-illuminator. The success of the amplification (presence of bands) and the conditions in which the amplification seemed to be better (the observed bands were brighter, there was less stutter and less non-specific amplification) were recorded. If the amplification was successful, the PCRs were repeated for 20 samples (10 males and 10 females) using the optimal amplifying conditions (Table 1). In order to determine the clarity of the bands (the ease with which they could be scored) and their degree of polymorphism (number of alleles), PCR products were run in 6% polyacrylamide gels and visualized by silver nitrate staining. A 10bp DNA ladder (Invitrogen) was used to estimate the size of the products.

The number of available markers was increased by direct isolation of microsatellites from spotless starlings. From five adult female and five male spotless starlings DNA extractions, 100 ng of DNA was pooled (1µg in total) and digested overnight at 37ºC with MboI (Promega), 1X Buffer C, 0.1 mg/ml BSA in a 50 µL reaction. 50 ng of this digested DNA was ligated to 840 ng of annealed SauLA and SaulLB linkers (Armour et al. 1994) using 3U of T4 DNA ligase, 1x ligase buffer (Bioline) in a 35 µL volume at 16ºC for 3 hours. This product was amplified by PCR using SauLA as a primer following methods in Muniz *et al.* (2003).

Enrichment was done with $(CA)_{15}$ oligonucleotides cross-linked to 0.7 cm^2 nylon membranes as in Becher *et al.* (2002) and Muniz *et al.*(2003). The enrichment was repeated twice. The enriched DNA was run in a 1.5% agarose gel and fragments selected for sizes between 200 and 1000 base pairs. The selected DNA fragments were excised from the agarosa and purified with a QIA-quick gel extraction kit (QIAGEN). The linkers were removed with MboI (Promega), 1X Buffer C, 0.1 mg/ml BSA in a 50 µL reaction. This reaction was left overnight at 37ºC to remove the linkers; then heated to 65ºC for 12 minutes, to denature the enzyme. The DNA was precipitated with 3M sodium acetate and 96 % ethanol, washed with 70 % ethanol, dried and re-suspended in 30 µL of water following methods in Sambrook, *et. al* (1989).

This enriched library was ligated to a *BamH*1-digested pUC19 vector (Bioline) following the manufacturer's protocol using two independent mixes of 3 and 15 ng of the purified library DNA respectively, with 100 ng of plasmid in each. Supercompetent *E. coli* cells (Stratagene) were heat-shock transformed with these plasmids following manufacturer's protocol. The cells were grown and screened with $(CA)_{24}$ oligonucleotides kinase labelled with ($\gamma^{32}P$) dATP using Ready-To-Go T4 Polinucleotide Kinase Kit (Pharmacia). Positive clones were detected using an X-ray sensitive film. After obtaining positive clones, some of them were sequenced in one direction on an ABI3730 capillary DNA sequencer. Based on the clarity of the sequence, the size of the repeat motifs and the flanking regions, a selection of those were sequenced in both directions. Sequences with long repeat-motifs (at least 7 tandem repeats) and adequate flanking regions were chosen and primers were designed using Primer3 (Rozen & Skaletsky 2000). Then the optimization of the amplification was attempted.

The loci whose amplification worked well (clear bands) were tested for polymorphism using 20 DNA samples from adult spotless starlings from Soto del Real (Spain). PCRs were performed in a reaction of 15 µL with: ≈20 ng of DNA, 1.25mM $MgCl_2$, 1.5.µL of 10x NH_4-based Reaction Buffer (Bioline), 0.375 U of BIOTAQ DNA polymerase (Bioline), 1 µM of each primer and 0.20 nM of each dNTP. The PCR program used was: a) 94^oC for 4 minutes; b) 30

cycles of: 94^{o}C for 20 seconds, annealing temperature (see Table 1) for 30 seconds and 72^{o}C for 30 seconds; and c) a final step at 72^{o}C for 5 minutes. The PCR products were run in 6% polyacrylamide gels and visualized by silver nitrate staining.

From both the cross species and newly isolated microsatellites, the ones that presented high levels of polymorphism and were clear to score (showed less stuttering and/or nonspecific amplification) were selected. One of the primers for each of these selected loci was fluorescently labelled using the WELLRED^{TM} dye system (Beckman Coulter). PCRs where then performed as before, but replacing the appropriate primer with the fluorescent one. The alleles were scored using a Beckman Coulter CEQ^{TM} capillary sequencer and the CEQ 8000^{TM} Genetic Analysis System.

Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested in GENEPOP (Raymond & Rousset 1995), using the genotypes obtained for 184 adults (88 males and 96 females). The allelic richness and the expected (He) and observed heterozygosity (Ho) were also determined. The proportion of null alleles was determined using Cervus 3.0.3 (Marshall et al. 1998) and NEWPAT XL (Wilmer et al. 1999). For one microsatellite that was found to be sex-linked (on the Z chromosome), HWE, He, Ho and the proportion of null alleles were calculated using only the males' data.

Finally the cross-species amplification in the European starling of the newly isolated microsatellites was performed. This is a closely related species to the spotless starling that is a popular model system. The newly isolated loci were tested with 8 samples of adult European starlings (*Sturnus vulgaris*) from Belgium with the same PCR conditions and reaction mix.

Results

From the eleven microsatellites cross-species amplified, one was variable but difficult to score (it exhibited non specific amplification), one was monomorphic and another failed to amplify at all (Table 1). The remaining 8

were polymorphic and clear to score, but only 2 were highly polymorphic (Pca7 and FhU2, Table 1 and Table 3).

Table 1. Microsatellite loci isolated from other bird species and cross-species amplified in *S. unicolor*.

Locus Name	Source Species	Publication	Optimal PCR Conditions	Variability
Pca7	Blue tit (*Cyanistes caeroleus*)	Dawson *et al* (2000)	1.25 mM $MgCl_2$ 58 ºC	Clear to score and polymorphic (see table 3)
Mme12	Song sparrow (*Melospiza melodia*)	Jeffery *et al* (2001)	1.25 mM $MgCl_2$ 56 ºC	2 or 3 alleles. Difficult to score due to stutter.
HrU2	Swallow (*Hirundo rustica*)	Primmer *et al* (1995)	1.25 mM $MgCl_2$ 54 ºC	2 alleles
HrU7	Swallow (*Hirundo rustica*)	Primmer *et al* (1995)	1.5 mM $MgCl_2$ 55 ºC	Monomorphic
Escμ2	Reed bunting (*Emberiza schoeniclus*)	Hanotte *et al* (1994)	None	Did not amplify
FhU2	Pied flycatcher (*Ficedula hypoleuca*)	Primmer *et al* (1996)	1.25 mM $MgCl_2$ 58 ºC	Clear to score and polymorphic (see table 3)
FhU3	Pied flycatcher (*Ficedula hypoleuca*)	Primmer *et al* (1996)	1.5 mM $MgCl_2$ 49 ºC	3 alleles
Ase18	Seychelles warbler (*Acrocephalus sechellensis*)	Richardson *et al* (2000)	1.5 mM $MgCl_2$ 50 ºC	6 to 7 alleles. Difficult to score due to extra bands
Ase19	Seychelles warbler (*Acrocephalus sechellensis*)	Richardson *et al* (2000)	1.5 mM $MgCl_2$ 57 ºC	2 to 3 alleles with some scoring difficulties.
Mjg1	Mexican jay (*Aphelocoma ultramarina*)	Li *et al* (1997)	1.5 mM $MgCl_2$ 60 ºC	3 alleles
Patmp2	Black capped chickadee (*Poecile atricapillas*)	Otter *et al* (1998)	1.5 mM $MgCl_2$ 63º C	2 alleles

From the second approach, the direct isolation of microsatellites from the spotless starlings, 151 positive clones were obtained. Fifty-eight, of the ones showing high levels of radioactivity, were selected and sequenced one way. From those 29 were sequenced in the other direction. Seven of these were discarded for lacking long enough flanking areas or repeat motifs. Primers were designed for the remaining 22 sequences. From these, 14 were successfully optimized while the remaining 8 either did not amplify or exhibited complex stuttering that prevented accurate interpretation of product lengths. From the 14 optimized *S. unicolor* primers, 6 were monomorphic and one dimorphic (Table 2).

The other 7 were highly polymorphic, presenting between 7 and 17 alleles (11.86 ± 3.57 alleles per locus). Additionally, the two highly polymorphic loci obtained in the cross-amplification assays, Pca7 and FhU2, presented 10 and 12 alleles respectively (Table 3). No linkage disequilibrium was found between the nine loci after Bonferroni correction for multiple tests. The expected and observed heterozygosity ranged from 0.53 to 0.90 and 0.33 to 0.89, respectively. Three loci (Sta213, Sta70 and Pca7) were out of Hardy-Weinberg equilibrium after Bonferroni correction.

The proportion of null alleles for these polymorphic loci was also very high (between 10 and 37%; Table 3). The results were very similar when analysed using Cervus 3.0.3 (Marshall et al. 1998) and NEWPAT XL (Wilmer et al. 1999). One of the loci (Sta97) appears to be sex linked. All females (the heterogametic sex ZW) were scored as homozygous, while males (ZZ) presented the expected frequencies of homozygous and heterozygous. Therefore it appears to be in the Z chromosome.

Table 2. Characteristics of 4 monomorphic and 1 dimorphic* *Sturnus unicolor* microsatellite loci.

Locus	Repeat motif	Primer sequence (5'-3')	Ta (^{o}C)	Product size
Sta91	$(CA)_6$ TA $(CA)_4$	GGCACAGAACTGAGGCTAGG GAGTCACCAACAAGCAGCA	59	160
Sta198	$(TG)_8$	CCTTTGGACCTGTCCTGTGT TGTAGAAGCTGGTGGCAA	63	220
Sta233	$(CTGT)_2$ CT $(CA)_8$ CT $(CA)_4$	CAAGTGCCACCAACAAAAGA GATCAATGGTTTCCCCATTC	55	190
Sta245	$(CA)_2$ A_2 $(CA)_9$	AGTCAGCTGCAACCACAATG CCTTTGGACCTGTCCTGTGT	58	170
Sta171	$(CA)_7$ CT $(CA)_3$	GAGGTGTAAGGGGAGGGAAG GGAGTGTCCCAATGTGCTCT	59	245
Sta198	$(GT)_9$ T_2 $(GT)_2$	CCTTTGGACCTGTCCTGTGT TGTAGAAGCTGGTGGCAATG	57	258
Sta271*	$(CA)_{10}$ $(GA)_5$ $(A)_6$ $(TG)_3$	TGTGGCTGGGGAACATTTAT GAAGCAGGTGCAAGTCATCA	63	162-170

Table 3. Characteristics of nine microsatellite loci amplified for 184 adult spotless starlings. Ta, annealing temperature; He, expected heterozygosity; Ho, observed heterozygosity. a Sex-linked Microsatellite. b Results calculated using only the males' data. c Microsatellites isolated in other species and cross amplified in the spotless starling. d The proportion of null alleles calculated, using CERVUS 3.0.3 (Marshall et al. 1998). d The proportion of null alleles calculated, using NEWPAT XL (Wilmer et al. 1999).

Locus	Repeat motif	Primer sequence (5'-3')	T_a (^oC)	No. of alleles	Product size range	H_e	H_0	P value (HWE)	Proportion of null alleles	GenBank Access. No.
Sta308	$(CA)_{25}$	GCTTAAGCACCCCTCACAAC CTGCAATCAGGGTTTGGATT	58	14	130-154	0.90	0.89	0.373	0.01[d] 0.01[e]	DQ860237
Sta269	$(CA)_{15}$	TGGGGATTAATAGGGGTGTG GCAGTGAGAAGAGGGCTTTG	58	14	181-211	0.85	0.84	0.315	0.003[d] 0.003[e]	DQ860238
Sta97	$(CA)_9CT(CA)_7CT$ $(CCTC)_2TCTG(CT)_{15}$	GTCTGGTTGTCCGTTTGCTA GGATCAGACCCAGAGAGAGAGA	60.5	7	237-251	0.72[b]	0.78[b]	0.715[b]	-0.047 [b, d] 0.003 [b,e]	DQ860239
Sta294	$(CA)_3GA(CA)_7$	AGGAACATGGCTGGAGTGAA CACAGTCACATTGGCATTGA	58	9	293-309	0.73	0.78	0.22	-0.042 [d] 0.003[e]	DQ860240
Sta296	$(CA)_{11}$	CGGGGATCAAGCAGGTATATT ATGCTTCCTCTCAGCAGCTC	58	9	314-330	0.74	0.72	0.085	0.011[d] 0.011[e]	DQ860241
Sta213	$(ACCAC)_7$	TTGGCCTTGCTGAACTTCTT GATCAAGTGCACCTTCAGCA	60.5	17	155-228	0.85	0.53	<0.001*	0.224[d] 0.224[e]	DQ860242
Sta70	$(CA)_{14}$	AGGTGTGTGGGAGAGAATGG ATGGACAAAAGAAGGCATGG	60.5	13	224-264	0.72	0.33	<0.001*	0.376[d] 0.376[e]	DQ860243
PCA7[c]	$(TG)_{24}$	TGAGCATCGTAGCCCAGCAG GGTTCAGGACACCTGCACAATG	58	10	90-126	0.53	0.43	<0.001*	0.101[d] 0.101[e]	-
FhU2[c]	-	GTGTTCTTAAAACATGCCTGGAGG GCACAGGTAAATATTTGCTGGGCC	58	12	118-151	0.80	0.81	0.309	-0.009[d] 0.003[e]	-

The seven polymorphic loci isolated from spotless starlings also amplified in the 8 samples of adult European starlings (*Sturnus vulgaris*) from Belgium. They all were polymorphic with between 5 and 10 alleles (7.14 ± 1.95 alleles per locus). The expected and observed heterozygosity of these loci ranged from 0.73 to 0.94 and 0.62 to 1.00, respectively (Table 4).

Table 4. Characteristics of the microsatellite loci cross-amplified in *Sturnus vulgaris.* He, expected heterozygosity and $H_{0,}$ observed heterozygosity for 8 samples.

Locus	No. of alleles	Product size range	He	H_0
Sta70	7	224-248	0.87	0.75
Sta97	6	243-253	0.83	0.62
Sta213	9	155-213	0.92	0.87
Sta269	8	186-204	0.80	0.65
Sta294	5	295-307	0.73	0.75
Sta296	5	315-331	0.79	0.62
Sta308	10	122-154	0.94	1.00

Discussion

In total, 9 highly polymorphic microsatellites were obtained for this study. Two of them were found via cross-species amplification and the other seven through the direct isolation from the spotless starling. One of the loci is sex linked, appearing as monomorphic in females (the heterogametic sex). Three loci are not in HWE. The most likely cause of this is the presence of null alleles. This can represent some limitations for their use.

Both the spotless starling and the European starling are quite popular model species. Their popularity stems from their abundance and accessibility, but also in the occurrence of interesting behaviours. These species present facultative polygyny (Aparicio et al. 2001; Cordero et al. 2001; Loyau et al. 2005a), intra-specific nest parasitism (egg dumping) and extra-pair paternity (Cordero et al. 2003; Loyau et al. 2005a). Hybridisation between the two

species has also been observed. The study of all these phenomena would benefit greatly from the existence of appropriate genetic markers. These nine polymorphic microsatellites could be useful in assessing parentage and intra-specific nest parasitism in these species. Additionally they may be used in studies of the population genetics and of the hybridization between the two species.

Chapter 3. Parentage Assignment: Detecting Levels of Polygyny, Extra-pair Paternity, Intra-specific Nest Parasitism and Quasi-parasitism.

Introduction

Changes in the perception of avian reproductive systems

Before molecular parentage studies were available, it was thought that most birds were truly monogamous and that simultaneous polyandry in birds did not exist, except in a couple of cooperatively breeding species (Lack 1968). Lack reported that 93% of the passerine subfamilies were monogamous with strong pair associations, a scenario which left no room for extra-pair copulations. Perhaps the most important breakthrough that was to come from the early years of parentage analysis is the evidence found for alternative reproductive strategies (Birkhead 1998; Griffith et al. 2004; Jones & Ardren 2003) such as: extra-pair paternity (EPP), intra-specific brood parasitism (IBP) and quasi-parasitism (QP).

Nowadays, the percentage of birds classified as socially monogamous is around 85% (Bennett & Owens 2002; Tang-Martinez & Ryder 2005). This figure is very similar to the one reported by Lack (1968). However, EPP, where offspring of the brood mother are sired by males that are not the brood father, has been detected in more than 75% of the socially monogamous birds (Bennett & Owens 2002; Tang-Martinez & Ryder 2005). Thus, a clear distinction has been made between socially and genetically (or truly) monogamous systems. Moreover, Griffith et al. (2002) estimated that 90% of all the species studied exhibited EPP. A socially monogamous system where

EPP exists can be interpreted as a form of polyandry. Therefore, we can say that 90% of bird species are polyandrous (or polygamous); our vision of bird reproductive strategies has been fundamentally reversed in the last 30 years.

Extra-pair paternity (EPP)

A series of costs and benefits for birds engaging in extra-pair copulations (EPC) has been suggested, and these differ between males and females. Some of the proposed benefits for males are: An increase in fitness due to the increase in number of offspring sired (Alcock 1997; Birkhead 1998; Birkhead & Møller 1996; Petrie & Kempenaers 1998); an increase in the potential to mate in the future; and an insurance against the infertility of their mates (Alcock 1997; Birkhead 1998; Birkhead & Møller 1996; Petrie & Kempenaers 1998). The costs include: sperm depletion; an increase in divorce rate (mate–desertion); an increase in the risk of paternity loss (because of the decrease in male guarding); an increase of the risk of contracting diseases and parasites (Alcock 1997; Birkhead 1998; Birkhead & Møller 1996; Petrie & Kempenaers 1998).

The proposed benefits that females could gain from EPC are: to ensure the fertilization of their eggs; to decrease the risk of genetic defects due to long term sperm storage (Birkhead & Møller 1996); to acquire nutritional benefits (e.g. nuptial gifts); to increase the genetic diversity or quality of their offspring (Alcock 1997; Birkhead 1998; Birkhead & Møller 1996; Griffith et al. 2002; Petrie & Kempenaers 1998); to avoid the costs of rejecting males (e.g. sexual-coercion and infanticide). The costs proposed for females are: a decrease of the genetic quality of their offspring (when the EPC are forced or just tolerated); a decrease in foraging (e.g. if seeking or avoiding EPC is time consuming and/or drives them out of the best foraging territories); an increase of the risk of contracting diseases and parasites (Alcock 1997; Birkhead 1998; Birkhead & Møller 1996; Petrie & Kempenaers 1998); and an increase of the risk of suffering aggression (including infanticide) by the mate of the extra-pair male (Mays & Hopper 2004; Sandell & Smith 1996; Sandell & Smith 1997; Westneat & Stewart 2003).

The proportion of EPP varies greatly, from species exhibiting levels close to zero to others with more than 70% of EPP offspring (Griffith et al. 2002). The ecological basis of this great variation in the amount of EPP is only starting to be understood (Westneat & Stewart 2003). Some studies relate high levels of EPP with high rates of adult mortality and low levels of parental care (Arnold & Owens 2002). However, it is important to note that some of these factors, may be the result and not the cause of EPP, as an alternative strategy in a species (Westneat & Stewart 2003). Moreover, the benefits and costs of EPP seem to vary drastically between species and even populations, sometimes correlating with the breeding density (Petrie & Kempenaers 1998). In some species of birds, females are reported to actively seek extra-pair copulations (EPC), while females of other species avoid them. It has been found that in one population of red-winged blackbirds (*Agelaius phoeniceus)* females start EPC while in another population, it is the male that initiates EPCs (Gray 1996; Westneat 1992). There are few convincing examples of females actively seeking EPC. For example, Double & Cockburn (2000) found that female superb fairy-wrens (*Malurus cyaneus*) left their territory before dawn, to go directly to the extra-pair male territory and return immediately after. However, observational data in many species of waterfowl shows that most of the EPC are resisted (Cheng et al. 1982; McKinney et al. 1983). This suggests that the benefits and costs of EPC for females and males varied between species and even between populations (Petrie & Kempenaers 1998).

Intra-specific brood parasitism (IBP)

IBP refers to the cases where females lay eggs in the nest of conspecifics (hosts) and do not provide any parental care (Arnold & Owens 2002; Griffith et al. 2004; Zink 2000). The study of IBP has been performed, in some cases, using only observational clues (without the use of genetic methods) such as the detection of super-normal clutch sizes, shorter than normal laying intervals, and high variance in egg morphology (size and colour) (Brown 1984; Lyon 1993). However, the use of these clues conceals a high risk of failing to recognise IBP (e.g. if females remove a host egg when laying the IBP egg or if IBP eggs are very similar to the host eggs) and a risk of confusing host eggs with IBP eggs (e.g. when a clutch is unusually big or morphologically varied,

but belongs to a single female) (Gronstol et al. 2006; McRae 1997). Arnold and Owens reported in 2002 that, using molecular techniques, 20 out of the 69 species of birds studied until then presented IBP.

The benefits of engaging in IBP for females lay in the increase in fitness due to the increase in number of offspring reared. Since, in general, IBP eggs are less successful than eggs laid by females in their own nest, females experience a trade-off between the number of eggs laid in their own nest and in the host nest (Maruyama & Seno 1999). IBP has been predicted to occur in species with high fecundity and relatively inexpensive parental care (Arnold & Owens 2002). In other species, like the European starling (*Sturnus vulgaris*) and the Barrow's goldeneye (*Bucephala islandica*), IBP seems to be a tactic used by floaters, females that do not have their own nest (Eadie & Fryxell 1992; Sandell & Diemer 1999). Another factor that could favour IBP is relatedness (kin selection); if one of the host parents is related to the parasite, the cost of rearing the IBP chick would decrease (because of inclusive fitness benefits). For example, according to McRae (1996) for neighbour moorhens (*Gallinula chloropus)* the likelihood of being a first order relative was around 18% and this species is remarkably tolerant to parasitic eggs (females lay up to 6 IBP eggs and females rarely reject them).

Quasi-parasitism (QP)

Quasi-parasitism is similar to IBP in that females lay eggs in the nest of a host-female, but QP eggs are fertilized by the host-male (the social mate of the host-female). Even when females may gain genetic benefits from QP (e.g. increasing genetic variation of her chicks), the main benefits for the females seem to be the increase in the ability to lay parasitic eggs (access to more host-nests) (Griffith et al. 2004). QP has been reported in around 12 species of birds, but at very low frequencies (Griffith et al. 2004). It is not clear if QP really exists in these species as a reproductive strategy or if it is just an artefact of other reproductive tactics (for details see review by Griffith et al., 2004). For QP to be considered a reproductive strategy *per se*, a female needs to gain access to the host nest in exchange for copulations with the host-male.

QP may occur by chance in species with high levels of IBP and EPP; since, just by chance, some IBP eggs may be dumped in the nests of the males that fertilized them. Moreover, the study of species that exhibit rapid mate-switching is likely to result in the detection of QP (Griffith et al. 2004), but this will be just an artefact of the rapid change of mates. In most cases, usurping females eject eggs when taking over a nest. However, any eggs laid by the previous female that survive in the nest will probably produce quasi-parasitic chicks, sharing a father with the chicks of the usurping female.

Molecular methods for parentage analysis

Allozyme electrophoresis was the first technique used for paternity analysis in birds (Griffith et al. 2004; Jones & Ardren 2003). Allozyme genetic analysis exploits the variation in the electrophoretic motility of proteins (typically metabolic enzymes), due to changes in the amino-acid sequence. Unfortunately, the degree of polymorphism in allozymes is very low and few loci can be scored, therefore the probability of detecting mismatching fathers was only between 40 and 70% (Burke 1989).

In the 80's, parentage analysis using DNA fingerprinting became possible. The use of multilocus (Minisatellite) DNA fingerprinting solved many of the problems encountered when using allozymes (Burke 1989; Jeffreys & Wilson 1985). Minisatellites are codominant markers that obey Mendelian rules of inheritance and are more variable that allozymes. The detection of EPP using this method was considered to be highly reliable (Burke 1989). DNA fingerprinting is generally used to confirm genetic paternity of males observed attending the brood. However, the use of multilocus fingerprinting to determine the true extra-pair father is limited, since the mutation rate of minisatellites is high (Burke 1989), there is an unknown error rate when sizing and matching bands, there is a relatively low number of bands scored (less than 25) and there is high variability across individuals (Burke 1989; Queller et al. 1993), all these factors leading to an uncertain assignment of parentage.

By the late 80's the diffusion of PCR techniques allowed the widespread use of microsatellites (Jarne & Lagoda 1996). There are several characteristics of

microsatellites that make them especially useful for paternity analysis (for details see Chapter 2). Firstly, they are highly polymorphic co-dominant markers that obey Mendelian rules of segregation and each locus and each allele within a locus is independent. The fact that they are short sequences makes them easy to amplify even from degraded samples. Additionally, alleles can be sized with high precision and can be reliably compared among individuals (Jarne & Lagoda 1996; Queller et al. 1993; Selkoe & Toonen 2006; Sunnucks 2000). All this explains why microsatellite markers are the most popular and powerful genetic tool used for studying population processes at a very fine scale, such as parentage analysis (Jarne & Lagoda 1996; Sunnucks 2000).

Techniques for parentage data analysis

Alongside the advance of molecular techniques, many techniques of data analysis were also developed. Most of the programs created for parentage analysis are focused on use with microsatellite data, but some can deal with other types of genetic data (Jones & Ardren 2003). These programs are based on one or more of the following approaches: parental reconstruction; exclusion; and likelihood (Jones & Ardren 2003).

Parental reconstruction works by generating the genotype of an unknown parent based on the genotypes of the offspring and a known parent (Jones 2001; Jones & Ardren 2003). This method is computationally demanding and is impractical for datasets with more than six potential parents (Jones & Ardren 2003).

The exclusion approach to paternity assignment is based on the rules of Mendelian Inheritance: each individual inherits one allele from its mother and one from its father. With this assumption, incompatibilities between the offspring genotype and the candidate parents' genotype can be searched for and incompatible candidate parents are rejected. If there are sufficient markers and they are sufficiently variable, only the genetic parents will remain (Jones & Ardren 2003). When one of the parents is known, a variation of the exclusion process can be used. For example, the software NEWPAT

(Summers & Amos 1997) deduces the paternal alleles using the offspring and maternal genotypes. For example, for an offspring that has the genotype AB and a mother AC, the father is expected to have at least one B allele. Then the program searches for matches between the candidate fathers and this inferred genotype, obtaining a shortlist of the potential fathers.

The likelihood approach is based on the calculation of the ratio of the likelihood that individuals (or couples) are the true parents of a given offspring against the likelihood that they are not. Commonly this is expressed as a 'likelihood' score that is the natural logarithm of this ratio; this is known as the LOD score ('logarithm of odds') (Hoffman et al. 2003; Jones & Ardren 2003; Kalinowski et al. 2007; Marshall et al. 1998; Slate et al. 2000). Log-transforming the likelihood ratio produces a likelihood score that is easier to interpret: A positive LOD indicates a higher probability that an individual is the true parent than not.

The most widely used program for paternity analysis is CERVUS, which uses the likelihood approach (Kalinowski et al. 2007; Marshall et al. 1998). CERVUS does not merely calculate the LOD scores; having done this, it uses simulations to establish the lower LOD scores necessary to provide a relaxed and a stringent level of confidence (e.g. 80% and 95% respectively). These simulations take into account the allele frequencies, the number of potential parents, the percentage of potential parents sampled, the estimated typing error of loci and how complete the genotypes are (Kalinowski et al. 2007; Marshall et al. 1998; Slate et al. 2000).

Dealing with null alleles

One of the main difficulties with the use of microsatellites for parentage analysis is the occurrence of null alleles (Jones & Ardren 2003; Pemberton et al. 1995; Soulsbury et al. 2007; Summers & Amos 1997). Null alleles occur as a result of variation in the flanking regions of the microsatellite that coincides with the primer annealing sites, disrupting them and impeding amplification by PCR (Jones & Ardren 2003; Pemberton et al. 1995; Soulsbury et al. 2007). Therefore, individuals with null alleles either appear as homozygous for a non-

null allele (false homozygous) or they do not amplify at all (where the individual possesses two null alleles). This presents a problem for parentage analysis, since falsely homozygous chicks may mismatch their parents. For example, a chick with genotype AN, where N is a null allele, would appear as AA. The parent that provided the null allele to the chick could have genotype BN, which would appear as BB. Therefore, the chick and null-allele-bearing parent would not appear to share any allele.

NEWPAT uses a likelihood approach to allow for null alleles by estimating their frequency in the population. The frequency of null alleles is estimated from the deviation of observed allele frequencies from those expected at Hardy-Weinberg equilibrium. The predicted frequency of null alleles is used to generate a probabilistic allowance of the presence of null alleles (Summers & Amos 1997). Homozygous candidate parents that do not share a visible allele with a homozygous offspring at a locus assessed as having null alleles are thus not immediately excluded. This way, NEWPAT can correctly identify parents that would otherwise be discarded as mismatches due to the presence of a null allele. CERVUS cannot handle null alleles with frequencies greater than 0.05 (Jones & Ardren 2003) and in its manual, the authors recommend the use of NEWPAT where higher frequencies of null alleles are suspected (Kalinowski et al. 2007). A series of other approaches have been suggested to try to solve the problem of null alleles in parentage analysis. For example: the disregarding of loci for which the offspring is homozygous (Pemberton et al. 1995), or allowing a greater level of mismatches (Jones & Ardren 2003; Pemberton et al. 1995). Jones and Ardren (2003) also suggested creating 'dummy' alleles for the loci with higher rates of null alleles and substituting one of the alleles from all the homozygotes for these loci with the dummy. For example, an individual of genotype AA would be treated as AX, where X is the dummy allele. In this way, the mismatches due to null alleles are avoided since parent and offspring will share the dummy allele that represents the null allele.

Parentage studies in the spotless starling

Cordero et al. (2003) used multi-locus DNA fingerprinting to determine parentage in the spotless starling (*Sturnus unicolor*) and found a high level of EPP; depending on the year, between 10 and 20 % of chicks were found to result from extra-pair paternity. They also reported one instance of IBP and one of QP out of the 334 chicks studied. A previous study (Calvo et al. 2000) reported a much higher degree of IBP, with more than 35% of the first clutches and between 12 and 20 % of intermediate and second clutches containing a parasitic chick. However, their study was based solely on observational data (differences in egg colour and size or the appearance of 2 newly-laid eggs on the same day). Therefore, the identification of parasitic eggs was less reliable than via DNA fingerprinting and it was impossible to differentiate between IBP and QP. In the last months, a new study was published (García-Vigón et al. 2008) in which the parentage of spotless starling chicks was confirmed using six microsatellites. One of them (Ase-18) was isolated from the Seychelles warbler (*Acrocephalus sechellensis*) (Richardson et al. 2000) and tried in this study but discarded because of the presence of extra bands (see chapter 2). Another one was isolated from the blue tit (*Cyanistes caeruleus*) (Dawson et al. 2000) and also used in this study. While the othe four were isolated by Rubenstein (2005) from the superb starling (*Lamprotornis superbusand*). Unfortunately, García-Vigón *et al* (2008) did not report the characteristics of these for the spotless starling, but this four microsatellites could be a good addition to the ones presented here, in future studies.

In this study we have used genotypes from nine microsatellite loci together with observational data to identify the parentage of spotless starling offspring, with the aim of detecting polygyny, EPP, IBP and QP. The greater resolution of this type of genetic marker compared with DNA fingerprinting, offers the potential to not only detect the existence and level of EPP, IBP and QP in this species but also to identify the true parents exhibiting these reproductive tactics with high confidence. In addition to the improvement of the technique used to assign parentage, this study's sample size (more than 1200 offspring)

is much greater than those of previous studies and, furthermore, encompasses three consecutive years.

Methods

Site

Field-work was conducted in Soto del Real, central Spain. The study site is an open forest, mainly of ash (*Fraxinus angustifolius*) and oak (*Quercus spp*.), with some areas of pasture.

Between December 2003 and the beginning of March 2004, 78 nest-boxes were installed at the study site. All nest-boxes were made of wood and with the same dimensions (17 x 17 x 35 cm.) and arranged in groups of three (trios). Each box was located 7 to 11 metres from the other two boxes of the same group and groups were at least 65 metres apart. In total, there were 26 groups of three nest-boxes (trios).

These 78 boxes were used for the field season of 2004, but in 2005 and 2006 only 69 boxes were used. The other nine nest-boxes were removed (three complete trios), since they were closer to a neighbouring road and presented a very high predation rate during the 2004 field season.

Captures

During March of each year, nest-boxes were regularly checked to see if there was fresh nesting material in them. We used this as a means to indirectly assess when males began to defend nest-boxes. Once a nest-box was seen to be in use, we attempted to trap the occupying male. Birds were captured in the nest-boxes using a trap mechanism (triggered by a tripwire) that blocks the entrance when the bird enters.

Trapping was performed before the females started to lay eggs and again when the offspring were between 4 and 11 days old, to minimize abandonment resulting from the disturbance. Male starlings are readily

caught by this method prior to egg-laying since they defend and prepare the nest. Once the eggs have hatched, brooding females will visit the nest-box more frequently than the males. Thus the two complementary periods of trapping allowed optimal sampling of the breeding population.

Additional nest-boxes were deployed to capture males that were difficult to capture in their own box. These additional boxes were set close to trios of established nest-boxes and left for one or two days to allow birds to become familiarized with them. Traps were then set, catching any exploring or defending adults that entered the boxes.

During captures a series of measurements (Chapters 4 and 5) were recorded, adults were tagged using a unique combination of plastic coloured leg-rings and a numbered aluminium leg-ring; and a blood sample was taken. In total, 165 male and 204 female adult birds were captured (see Appendix 1 for details) and of these blood samples were obtained from 154 males and 188 females. In 2005 and 2006 males that were captured before egg laying started were subjected to a treatment to alter the length of their throat feathers (see Chapter 5 for details).

Offspring sampling

When the chicks were 6 days old they were ringed, measured and a blood sample was taken. A tissue sample was taken from embryos of un-hatched eggs and from any chick that died before the seventh day. During these three seasons a study was performed that required taking a biopsy of the yolk from one of the eggs of each clutch (this study is not reported in this thesis). In 2005 and 2006 the biopsied egg was taken in the fourth day of incubation while in 2004 only un-hatched eggs were removed. A tissue sample was taken if an embryo was present in these eggs. In total, samples were collected from 1244 offspring during these three years (see Appendix 2 for details).

All the blood and tissue samples used for the paternity analysis (microsatellite genotyping) and for sexing, were kept in approximately 0.5 ml of buffer (100mM TRIS, pH = 8.5; 100mM EDTA, pH = 8; 2 % SDS). These samples were stored for up to 6 months at room temperature or in a -20ºC freezer then

transported from Spain to the UK at room temperature and ultimately stored at -20 ºC.

Nest observations

After hatching, observations were made to record which adults visited the nest-boxes, thus being the probable social parents of broods within. In 2004, 43 nests boxes were observed between the 5th and the 7th day after hatching. Nests were observed through a telescope for 30 minutes during the morning from temporary hides located about 20 metres away. The identity of all adults that were observed going inside or close to the focal nest was determined from their unique combination of colour rings.

During 2005 all the nest-boxes that contained chicks were observed at least once, and most were observed with video cameras twice; first between the 4th and the 8th day, and then between the 10th and the 14th day after hatching. Recordings were done during the morning for an hour. This was used not only to identify the parents, but also to determine feeding rates and the male's contribution to parental care.

In 2006, observations were also performed using video cameras. In this year, only nest-boxes that contained chicks in the first reproductive events were recorded and solely for the identification of the adults. Therefore, these recordings were shorter (around 30 minutes) and were not repeated once the adults were identified. No observations were made of subsequent reproductive events at nest-boxes after they had been observed once.

Other recorded data

Where an adult male or female was not observed at a nest-box, the most likely social parents could often be distinguished from the other genetic parents by considering which adults were originally trapped in the box. Nest-boxes were checked regularly to determine laying-date, clutch size and hatching date. To avoid additional disturbance to incubating birds or adults with young chicks, we made noise when approaching a trio prior to checking, giving the adults time to leave. Even so, during these revisions it was not

uncommon to find adults incubating or lying on top of young chicks and even males fighting inside the boxes. During these encounters the colour ring combination was recorded (when visible) and used to complement the other observational data in identifying the social parents associated with a nest-box. We never lifted or touched adults with eggs or young chicks.

Laboratory work

DNA was extracted from blood samples of adults and chicks using the PUREGENE protocol (Gentra Systems). The sex of the offspring was determined molecularly by amplifying two conserved CHD (Chromo-helicase-DNA-binding) genes located on the avian sex chromosomes. The genes were amplified using published primers P2 and P8 (Griffiths et al. 1998). Using this single set of primers, homologous sections of the CHD-W and CHD-Z genes are amplified. These genes contain different introns such that the PCR products differ in length. On an agarose gel, males have just one visible band (ZZ) and females have two (WZ). It was necessary to obtain the sex of the offspring for the paternity analysis in order to interpret one of the microsatellite loci, which was found to be sex-linked.

Genotypes were obtained for nine highly polymorphic microsatellite loci (see Chapter 2) from each adult and chick. In the cases were there were two samples of an adult in different years, only one was genotyped. There were 19 instances were an individual was sampled as a chick and subsequently as an adult. In these cases (13 females and 6 males) both samples were genotyped to confirm the identity. One of the adult samples had been mislabelled and therefore had a different genotype to the chick correctly labelled with the same number. These data were also used to estimate the error rate of genotyping. There were 3 instances where an allele varied between the two independent genotypes obtained for the 18 multiply-sampled individuals. With a comparison using 512 alleles, these 3 instances constitute a genotyping error rate of 0.59%.

Genotypes were obtained for 317 adults (171 females and 146 males) and 1225 offspring. The mean number of amplified loci was 8.4 ± 2.0 per individual

for the adults (8.3 ±2.2 and 8.4 ± 1.8 for females and males respectively) and 7.9 ± 1.8 per individual for the offspring (see Appendix 3 for details). Non-amplification occurred in all loci, at a rate between 3.9% and 26% of the samples (on average 18.11% ± 8.01 non-amplifications per locus).

Paternity analysis

Identity checking

Data were checked for duplicate entries (Identity checking) using NEWPAT XL (Wilmer et al. 1999) and CERVUS 3.0.3 (Kalinowski et al. 2007; Marshall et al. 1998), to ensure that none of the individuals were entered twice. Both programs detected 2 cases in which a pair of siblings had the same genotype (one in 2004 and the other in 2006) and a case where a pair of siblings had identical genotypes except for one allele (in 2005). This low number of identical (or very similar) genotypes is expected given the high quantity of chicks analysed and their relatedness. There was no instance of 2 adults sharing the same genotype.

Microsatellite characteristics

The characteristics of these microsatellites, such as allelic richness, linkage disequilibrium, concordance with Hardy Weinberg equilibrium (HWE) and the presence of null alleles, were assessed and have already been reported (Table 3). Three of the loci were not in HWE. The analysis in both NEWPAT and CERVUS, revealed high levels of null alleles for these three loci (from 10 to 37%, Table 3). In addition, another locus is sex linked (in the Z chromosome).

Confirmation of mothers

Data were checked for mismatches between the observed or captured mothers and the chicks, using NEWPAT XL. When a mismatch was detected, genotypes were double-checked manually and any scoring errors were corrected. It was also determined whether the mismatch could be due to null alleles. If the mismatch was genuine, there were 2 possible interpretations: a) that it was a parasitic chick, indicated by there being other chicks in the nest and the rest or most of them matching the potential mother; b) that the

observed or captured female, was not the mother of those chicks in which case the female would not match any of the chicks in the brood. In the first case, the chick was analysed without a known mother separately from the other chicks of the nest. In the second case, the whole brood was analysed for paternity without a known mother.

Processing the sex linked locus

Neither CERVUS 3.0.3 nor NEWPAT XL are able to deal with sex-linked loci (a previous version of NEWPAT did). Since one of the microsatellite loci is sex–linked, values of this locus were deleted for all the females (chicks and adults). Therefore all females were presented as un-scored for that locus, and only males' values were used. There were a few cases in which a chick was re-sampled as an adult and was also a possible parent. To avoid mistakes (i.e. using chicks as parents) the parentage analysis was done one year at the time.

Parentage analysis using NEWPAT XL

Parentage was assessed, first using NEWPAT XL (Summers & Amos 1997), and then using CERVUS 3.0.3 (Kalinowski et al. 2007). This approach was taken since there were three loci with high levels of null alleles, which NEWPAT XL can handle but CERVUS cannot.

Since NEWPAT XL was designed to obtain paternities, to find both parents, data had to be analysed at least twice: once for the father and again for the mother. A dataset was created, containing a list of all the chicks' genotypes. Each set of siblings was preceded by the female that was observed or captured in that nest, provided that no mother-chick mismatch had been found previously. For the broods where no females were observed, the parasitic chicks were pooled together preceded by a 'dummy' female with an un-scored genotype. In this way NEWPAT uses the exclusion process to assign parentage with or without a known parent.

NEWPAT requires values for several parameters. The maximum number of mismatching loci allowed was set at 1, to allow for some errors in the scoring. The minimum acceptable probability for a match containing null alleles was

set at 0.05; since an increase in that probability to 0.1 resulted in a much higher number of candidate fathers per offspring and a lower probability precluded almost every match with null alleles. The number of un-scored loci allowed for paternity (number of loci lacking scores for the candidate father and/or the chick) was first set to two, in the first run of analysis; and then to four, in the second one. All females presented at least one un-scored locus since the scores of the sex-linked loci were removed. Therefore, two un-scored loci were chosen as the most stringent level of this criterion, allowing females to have just one extra un-scored locus. The first, more stringent analysis returned fewer possible fathers allowing faster and easier interpretation, but it did not identify fathers for all offspring where genotyping was incomplete. The second analysis complemented the first in allowing the inclusion of individuals with more incomplete genotypes (both chicks and adults). Only paternity not assigned in the first analysis needed to be considered when interpreting the results of the second.

Criteria for assigning parentage

The first stage of the assignment process was acquiring a list of candidate fathers based on analysis of the genetic data by NEWPAT. Having acquired this list, assignment was confirmed using observational data.

The list of candidates comprised those males that were not excluded by the analysis in NEWPAT (less than 1 mismatch, more than 5 genotyped loci, etc.). For each of the candidate males NEWPAT reports the relatedness (offspring-father), the number of mismatched loci and null alleles between the male genotype and the inferred paternal genotype and a randomization number, which indicates the probability of a match by chance in a database of similar size (Summers & Amos 1997).

The lists of candidate males for all offspring in a brood were searched for the identified social father (male observed feeding or incubating the brood). Where the social father matched the paternal genotype it was accepted as the true father and the remaining candidate fathers were not checked. In all other cases, all candidate males reported by NEWPAT were assessed based on observational data.

For offspring that could not immediately be assigned to the social father of the brood, the genotypes of candidate males that had been observed or captured in the area were checked. Paternity was then assigned to the candidate fathers that were observed in the area. There was just one case where a chick presented two candidate fathers that were both observed in the area of the nest. In this case, the father was not assigned.

Additionally, the true father could be assigned if it genetically matched 3 or more chicks in the brood, given that it is highly improbable that this would have occurred by chance. The father can be assigned in this way regardless of it having being observed or captured nearby.

Where the candidate list of a chick may have excluded the candidate father shared by most of the chicks in the brood due to a high number of un-scored loci (incomplete offspring genotype), the male was accepted as the true father if it was consistent with the inferred paternal genotype at the loci available.

In summary, paternity was assigned to males that were not excluded by the analysis in NEWPAT and that were observed or captured in that trio of nest-boxes or matched 3 or more of the chicks in that nest. Even when NEWPAT found only one potential father, the real father may have been un-sampled. Therefore, applying these rules minimised the likelihood of incorrectly assigning a father.

Once paternity was assigned, NEWPAT XL was run again to assign maternity. This time the dataset contained the known fathers (if available) and it was used to identify the mothers. The same parameters were used for running the program and the same criteria were used in interpreting the output (see above). If a mother was found for chicks where no female had been observed at the nest, the paternity assignment was run again using the relaxed parameters (4 un-scored loci) and with the maternal genotype in place of the ‘dummy’.

Parentage analysis using CERVUS 3.0.3

The results obtained with NEWPAT were checked using CERVUS. Since CERVUS cannot handle null allele frequencies higher than 5%, the data were modified to improve the way CERVUS dealt with the null alleles. The approach chosen was that suggested by Jones and Ardren (2003), the 'dummy-allele' approach. A 'dummy' allele was created for the 3 loci with higher rates of null alleles, and then all the homozygotes for these loci were converted to heterozygotes, substituting one of the homozygous alleles with the dummy.

The parentage analysis in CERVUS was done following the same procedure as in NEWPAT. First the paternity was analysed with known mothers (if available) followed by the maternity analysis (with known fathers). If any new mother or father was found, the analysis was repeated for those offspring. This process was performed for the offspring of each year separately. All parameters established in CERVUS were set as follows and conserved in the different analyses. The proportion of parents sampled was set to 80%; the proportion of mistyped loci to 1% (slightly higher than the one calculated by us); the minimum number of typed loci to 5; and the confidence parameters to 80% (relaxed) and to 95% (strict).

Results

Parentage analysis allowed us to identify at least one of the parents for 85% of the offspring. This made it possible to determine the incidence of polygamy (36 out of 181 males (20%) were polygynous) and the identification of IBP, EPP, QP offspring (7.35%, 7% and 1% of the offspring, respectively; affecting 21%, 19% and 3% of broods, respectively).

Parentage results obtained using CERVUS and NEWPAT were quite similar. From the 781 mothers assigned by NEWPAT, just 4.5% were not found using CERVUS. Similarly, from the 771 fathers, 5.2% were not found using CERVUS (Table 5 and Table 6). In most of these instances the substitution of true alleles by dummy ones seemed to be causing false mismatches. Therefore

the LOD scores of these true parents decreased to the point of being negative.

Maternity was assigned in 73.5% of the cases to the females with the maximum LOD score of all the sampled females. Mothers assigned with more than 80% confidence account for 54.1% of the cases, while 35.7% of the mothers were assigned with a confidence greater than 95% (Table 7)

Table 5. Mothers assigned using NEWPAT and CERVUS. The total number of assigned mothers; the mothers that CERVUS was not able to detect (Negative LOD); and the ones detected (using CERVUS) at different confidence levels are shown.

Year	Assigned mother by NEWPAT	Negative LOD	Positive LOD	Max LOD (Most likely mother)	>80%	>95%
2004	270	15 (5.55%)	255 (94.44%)	201 (74.44%)	159 (58.89%)	101 (37.41%)
2005	291	10 (3.44%)	281 (96.56%)	213 (73.2%)	191 (65.64%)	132 (45.36%)
2006	220	10 (4.54%)	210 (95.45%)	160 (72.73%)	125 (56.82%)	80 (36.36%)
Total	781	35 (4.48%)	746 (95.52)	574 (73.5%)	475 (60.82%)	313 (40.08%)

Table 6. Fathers assigned using NEWPAT and CERVUS. The total number of assigned fathers; the fathers that CERVUS was not able to detect (Negative LOD); and the ones detected (using CERVUS) at different confidence levels are shown.

Year	Assigned father by NEWPAT	Not in CERVUS	Positive LOD	Max LOD (Most likely father)	>80%	>95%
2004	260	18 (6.92%)	242 (93.08%)	201 (77.31)	145 (55.77%)	84 (32.31%)
2005	299	11 (3.68%)	288 (96.32%)	244 (81.61%)	220 (73.58%)	140 (46.83%)
2006	212	11 (5.19%)	201 (94.81%)	161 (75.94%)	133 (62.74%)	78 (36.79%)
Total	771	40 (5.19%)	731 (94.81)	606 (78.60%)	498 (64.59%)	302 (39.17%)

Paternity was assigned in 78.6% of the cases to the individual with the maximum LOD score of all sampled males. Fathers assigned with more than 80% confidence account for 64.6% of the cases, while 39.2% of the fathers were assigned with a confidence greater than 95% (Table 8).

Not all the parents assigned by NEWPAT and CERVUS were accepted as such. To be accepted, they had to be observed or captured in the vicinity of the nest and/or their genetic parentage assignment needed to be shared by most of the offspring in the nest. From the 674 mothers assigned by CERVUS with more than 80% of confidence, 29.5% were not confirmed. This is also the case for 10.6% of the mothers assigned by CERVUS with more than 95% of confidence (Table 7).

Table 7. Mothers assigned by CERVUS with 80% and 95% of confidence, and accepted (confirmed) or rejected (not confirmed) as the true mother using the established decision process.

	2004		**2005**		**2006**		**Total**	
	95%	80%	95%	80%	95%	80%	95%	80%
Mothers in CERVUS	109	215	160	285	81	174	350	674
Confirmed	101 (92.66%)	159 (73.95%)	132 (82.50%)	191 (67.02%)	80 (98.77%)	125 (71.84%)	313 (89.43%)	475 (70.48%)
Not confirmed	8 (7.34%)	56 (26.05%)	28 (17.5%)	94 (32.98%)	1 (1.23%)	49 (28.16%)	37 (10.57%)	199 (29.52%)

From the 644 fathers assigned by CERVUS with more than 80% of confidence, 22.7% were not confirmed. This was also the case for 11.4% of the fathers assigned with a confidence level higher than 95% (Table 8).

From a total of 1225 genotyped offspring from the 3 field seasons at least one parent was assigned to 1042 (85%) of them. Both parents were assigned to 690 (56.32%). For 188 (15.35%) offspring just the mother was found and for 164 (13.39%) just the father. The percentage of parents assigned was higher for 2005 with 94.0% of the chicks with at least one parent assigned, while in 2006 it was only 75.8% (Table 9).

Table 8. Fathers assigned by CERVUS with 80% and 95% of confidence, and accepted (confirmed) or rejected (not confirmed) as the true father using the established decision process.

	2004		2005		2006		Total	
	95%	**80%**	**95%**	**80%**	**95%**	**80%**	**95%**	**80%**
Fathers in CERVUS	91	190	151	282	99	172	341	644
Confirmed	84 (92.31%)	145 (76.32%)	140 (92.72%)	220 (78.01%)	78 (78.79%)	133 (77.33%)	302 (88.66%)	498 (77.33%)
Not confirmed	7 (7.69%)	45 (23.68%)	11 (7.28%)	62 (21.99%)	21 (21.21%)	39 (22.67%)	39 (11.44%)	146 (22.67%)

Table 9. Number (and percentage) of offspring for which both parents or just a mother or a father was assigned during each of the three years of the study and in total.

Year	Both parents assigned	Just mother assigned	Just father assigned	Total
2004	233 (53.93%)	75 (17.36%)	60 (13.89%)	368 (85.18%)
2005	246 (61.35%)	67 (16.71%)	64 (15.96%)	377 (94.01%)
2006	211 (53.83%)	46 (11.73%)	40 (10.20%)	297 (75.76%)
Total	690 (56.32%)	188 (15.35%)	164 (13.39%)	1042 (85.06%)

From the offspring with assigned mothers, 11.1% had less than 5 scored loci. For these individuals the main mother of the nest (the female assigned as the mother of most of the other chicks in the brood) was accepted as the mother, when all the available loci were consistent with this. The same was true for 9.7% of the chicks with assigned fathers.

From the 341 events with genotyped offspring, both parents were assigned to at least one offspring for 214 events (62.8%); just the mother was assigned for 39 events (11.4%) and just the father for 57 events (16.7%). The percentage of events and nests with allocated parents was higher in 2005, but for 2004 and 2006 these percentages were quite similar (Table 10).

Table 10. Number (and percentage) of Nests and Events with: genotyped offspring; allocated mother; allocated father; and both parents allocated during each of the three years of the study and in total. [a] These total numbers of nests contain the same nests during different years.

Year	Type	Genotyped offspring	Mothers identified	Fathers identified	Both parents identified
2004	Nests	77	64 (83.11%)	64 (83.11%)	54 (70.13%)
	Events	126	90 (71.43%)	96 (76.19%)	73 (57.94%)
2005	Nests	68	59 (86.76%)	63 (92.65%)	53 (77.94%)
	Events	110	89 (80.91%)	92 (83.64%)	73 (66.36%)
2006	Nests	69	53 (76.81%)	57 (82.61%)	49 (71.01%)
	Events	105	74 (70.45%)	83 (79.05%)	68 (64.76%)
Total	Nests	214[a]	176[a] (82.24%)	184[a] (85.98%)	156[a] (72.9%)
	Events	341	253 (74.19%)	271 (79.47%)	214 (62.76%)

There were 66 broods (19.4%) with one or more extra pair paternity (EPP) offspring, a total of 86 EPP offspring (7.0%). The father of 46 of them (53.5%) was identified (Table 11). EPP seem to be slightly more common in 2005 (8.0% vs. 6.7% and 6.4% in 2004 and 2006, respectively) but this was not statistically significant ($\chi^2 = 0.88$, df = 2, P = 0.65, for the number EPP offspring; and $\chi^2 = 0.185$, df = 2, P = 0.912, for the number of broods with EPP).

There were 90 cases (7.4% of the chicks) of intra-specific brood parasitism (IBP) detected in 72 Events (21.1%). The proportion of IBP offspring varied greatly between years ($\chi^2 = 12.1$, df = 2, P = 0.002). In 2004, it corresponded to just 3.9% of the genotyped offspring in 11.1% of the events; while in 2005 and 2006, it comprised 10.0% and 8.4% of the offspring in 25.5% and 28.6% of the events, respectively. Both parents were assigned to 27.8% of the parasitic chicks, while just the mother or the father was found for an additional 20.0% of them (Table 11).

Table 11. Occurrence of IBP, QP and EPP detected in each of the studied years and in total. This table shows the total number of chicks and broods with this kind of chick and the number (and proportion) of these that had assigned parents.

Year	No. Chicks No. Broods	Intra-specific Brood Parasitism (IBP)				Quasi Parasitism (QP)		Extra-Pair Paternity (EPP)	
		Total	Known mother	Known father	Both parents known	Total	Know mother	Total	Known father
2004	**432**	17 (3.9%)	4 (23.5%)	2 (11.8%)	2 (11.8%)	1 (0.2%)	0	29 (6.7%)	8 (27.6%)
	126	14 (11.1%)	3 (21.4%)	2 (14.3%)	2 (14.3%)	1 (0.8%)	0	23 (18.3%)	8 (34.8%)
2005	**401**	40 (10.0%)	5 (12.5%)	4 (10.0%)	15 (37.5%)	10 (2.5%)	4 (40.0%)	32 (8.0%)	24 (75.0%)
	110	28 (25.5%)	3 (10.7%)	3 (10.7%)	13 (46.4)	8 (7.3%)	4 (50.0%)	23 (20.9%)	19 (83.0%)
2006	**392**	33 (8.4%)	0	3 (9.1%)	8 (24.2%)	1 (0.3%)	1 (100.0%)	25 (6.4%)	14 (56.0%)
	105	30 (28.6%)	0	3 (10.0%)	8 (26.7%)	1 (1.0%)	1 (100.0%)	20 (19.1%)	13 (65.0%)
Total	**1225**	90 (7.4%)	9 (10.0%)	9 (10.0%)	25 (27.8%)	12 (1.0%)	5 (41.7%)	86 (7.0%)	46 (53.5%)
	341	72 (21.1)	6 (8.3%)	8 (11.1%)	23 (31.9%)	10 (2.9%)	5 (50.0%)	66 (19.4%)	40 (60.6%)

In addition to the detection of IBP chicks, cases of quasi-parasitism (QP) were also detected (offspring that shared the father but not the mother with their brood mates). Twelve (0.98%) offspring were found in 10 (2.93%) events to be quasi-parasites (Table 11). The number of QP offspring varied between years (χ^2=14.1, df= 2, P=0.001); ten occurred in 2005 while, in each of 2004 and 2006, just one was found.

The paternity was assigned to 54, 69 and 58 males in each of the three years of study respectively. Of these 181 males (some of which were the same male repeated across different years), 14 (7.7%) were solely fathers of IBP chicks and 21 (11.6%) were identified exclusively as the father of EPP offspring. The remaining 146 (80.7%) were assigned as the main father of one or more reproductive events (Table 12).

There were 110 (60.8%) socially monogamous males. Nine of them sired at least one IBP chick, in addition to the offspring in their nest. Ten sired at least one additional EPP offspring (one of these males sired both, EPP and IBP offspring). There were 36 (19.9%) cases of polygyny (males assigned as the main father of more than one nest). Seven of these polygynous males also sired at least one IBP chick and another two sired EPP offspring (Table 12). The variation between years of the proportion of polygynous versus monogamous males was marginally significant (χ^2=5.5, df= 2, P=0.064). The incidence of polygyny was higher in 2004 with 16 polygynous males (29.6%), decreasing in 2005 and 2006 with 13 (18.4%) and 7 (12.15) polygynous males, respectively. The level of polygyny was also higher in 2004, with males having up to 4 nests; in 2005 the maximum number of nests per male was 3, while in 2006 no male had more than 2 nests. Therefore, there was a significant difference between years in the proportion of nests defended by polygynous males (χ^2=11.3, df= 2, P=0.004). Of the nests with identified fathers 37 (56.1%), 27 (39.7%) and 14 (25.9%) were occupied by a polygynous male in 2004, 2005 and 2006, respectively.

Table 12. Males that were assigned as either the main fathers of a reproductive event, or as just having fathered IBP or EPP offspring. The main parents of a nest are divided into social monogamous (main fathers of just one nest) and polygamous (main fathers of more than one nest). Some of these monogamous and polygamous males also sired IBP chicks and EPP chicks in other nests. [a] One of them sired 2 chicks in these conditions and [b] one of them sired a parasitic and an extra-pair chick. [c] 12 males had 2 nests, 3 had 3 and 1 had 4. [d] 12 males had 2 nests, 1 had 3. [e] All of them just with 2 nests. [f] This total is the sum of the number of fathers assigned per year, therefore it contains males repeated in different years.

Year	Males assigned as fathers	Main Father of the Reproductive Event							Just parasitic males	Just Extra-pair fathers
		Total	Socially Monogamous			Socially Polygamous				
			Total	+ Parasitic	+ Extra- pair	Total	+ Parasitic	+ Extra- pair		
2004	**54**	**45 (83.33%)**	29 (53.7%)	0	1	16[c] (29.63%)	2	0	1 (18.52)	8 (14.81%)
2005	**69**	**54 (78.26%)**	41 (59.42%)	6[a]	8[a]	13[d] (18.84%)	4[a]	2	7[a] (10.14%)	8 (11.59%)
2006	**58**	**47 (81.03%)**	40 (68.96%)	3[b]	1[b]	7[e] (12.07%)	1	0	6 (10.34%)	5 (8.62%)
Total	**181[f]**	**146 (80.66%)**	110 (60.77%)	9[a]	10[a]	36 (19.89%)	7[a]	2	14[a] (7.73%)	21 (11.6%)

There were 127 nests with more than one reproductive event recorded in a year. Sufficient data were available from 116 of these nests to determine if the parents in the first event were the same as in the second event. A total of 57 (49%) nests exhibited changes of at least one parent from one event to another (see Appendix 4 for details).

During the three years of the study 234 different adults were assigned as parents (112 males and 125 females; Appendix 5). From those, 112 (50 females and 62 males) produced offspring in more than one year, but just 19 pairs remained together for two years and none stayed together for all three years. Three of the 19 enduring pairs produced just an EPP or a QP chick in one of the years, but produced a complete brood in the other. The remaining sixteen pairs sired whole broods in both years (see Appendix 5 for details).

Discussion

The results obtained using NEWPAT XL were very similar to those obtained with CERVUS 3.0.3 and the 'dummy-allele' approach. Less than 5% of the parents assigned by NEWPAT were not detected with CERVUS. Therefore, we can say that the 'dummy-allele' approach worked well with this data set, even when a third of the loci presented high frequencies of null alleles.

The problem with this approach is that there are two kinds of homozygotes at loci with null alleles: a) The false homozygotes, which appear to be homozygous because one of the alleles (null allele) failed to amplify; b) The true homozygotes, which have two identical alleles, both of which amplified (appearing as one band). The 'dummy allele' approach requires that all homozygotes (true and false) are converted to heterozygotes with a 'dummy' allele. When the parentage of a true homozygote with a 'dummy-allele' is analysed without a known parent, the true parents are not erroneously rejected. However, when one of the parents is known, true homozygotes can present some false mismatches. This problem is illustrated by the following example, where an individual with a known mother of genotype AB is a true homozygote with genotype AA. The offspring genotype is changed to AX, according to this analytical approach, and the paternity analysis infers that the

paternal genotype will contain the dummy allele (X). Heterozygous males with the A allele (the true one) will be falsely rejected as they mismatch the inferred paternal genotype. Nevertheless, this approach should work well when the number of alleles (level of polymorphism of the loci) and the proportion of null alleles is high; that is, when the proportion of true versus false homozygotes is small.

In this data set, one locus with an estimated 10% of null alleles (PCA7) also exhibited a predominant allele with a frequency of over 50%. This meant that many of the homozygotes where actually true homozygotes. The conversion of these true homozygotes to heterozygotes using the dummy allele led to mismatches. For loci like this, where one allele predominates and, thus there are many true homozygotes, the convenience of the 'dummy-allele' approach is not clear. Its efficacy will depend on the degree to which the allele predominates and on the proportion of null alleles at that locus.

Paternity analyses of data sets with high frequencies of null alleles will, in general, benefit from the use of the 'dummy-allele' approach. This is especially the case if the loci containing null alleles are highly polymorphic and if allele frequencies at these loci are not predominated by just a few alleles.

The fact that parentage was assigned in more than 75% of cases to the individual with the highest LOD score, implies that the resolution provided by these microsatellites is high. Moreover, the use of observational data and the presence of more than one offspring with the same parents in a nest make this parental analysis highly reliable. It is important to note that even when a parent was assigned with 95% or 80% of confidence by CERVUS, it was not automatically accepted, but required the support of other data. More than 20% of the parents assigned with 80% confidence were rejected due to the lack of supporting observational data. The same was true for more than 10 % of the parents assigned with 95% confidence. Parentage assigned to chicks or parents with incomplete genotypes, rarely achieved high levels of confidence, but they did achieve positive LOD scores and parents were always shared among the brood. The combination of observational and genetic data for the assignment of parentage gives a very high confidence level to our results.

The assignment of the parentage in this study was conservative. As a result, not every chick had parents assigned. In general, the proportion of parentage assigned was considerably high, with 85% of the offspring with at least one parent assigned. The failures to assign a father and/or mother were sometimes due to adults that were not genotyped. In other cases a parent may have not been assigned because of the lack of supporting observational evidence. This explains why in 2005, when more observations were made, a greater proportion of chicks had parents assigned (94% vs. 85% and 75% in 2004 and 2006, respectively; Table 9). The number of nests and events, where parents were assigned, follows the same tendency (Table 10).

The percentage of EPP offspring detected in this study is lower than the one reported by Cordero et al. (2003). They based their estimate on nests for which the social father (true father of most of the brood) was known. In contrast, the percentage of EPP presented here is based on the data from all genotyped offspring, irrespective of whether a reproductive tactic could be assigned or not. The offspring that could not be assigned to a reproductive tactic counted towards the total number of offspring and may include some cases of EPP, IBP and QP. Therefore, the 7.0% of EPP reported in our study is conservative; it is likely to be an underestimate. Conversely, the level of parasitism found by Cordero et al. (2003), was extremely low compared with the levels found in this study and those reported by Calvo et. al (2000): Cordero et al reported 1% of the broods being parasitized (1 out of 96) versus 21.1% (72 out of 341) in this study and between 12% and 37% of the broods in Calvo et al (2000). This disparity could be due to the accuracy of the technique used. DNA fingerprinting (as used in Cordero's study) is used to accept or reject the observed parent as the genetic parent, but cannot be used to identify EPP fathers and the probability of mistaking the true parent with a genetically similar individual is high. Furthermore, Cordero et al. apparently considered chicks as EPP when they did not match the social father, even when the mother was unknown. This may have artificially increased the number of cases of EPP and reduced the cases of IBP. On the other hand, Calvo et al (2000) used only observational data to detect IBP, which may have inflated their estimate. For example, eggs may have

classified, based on their colour or size, as parasitic whilst they were actually laid by the same female. The use of microsatellite genetic data in this study (with the support of observational data) to determine parentage, allowed the detection and differentiation of EPP and IBP. While, all the cases considered here as EPP and IBP are almost certainly assigned correctly, there may be cases in our study where EPP and IBP were not detected. The latter is very likely in nests where the main parents could not be assigned due to a lack of observational data.

Additionally, the levels of EPP and IBP reported vary within the studied populations between years (Moreno et al. 2003 and this study) and also between populations in the same year (Calvo et al. 2000). Therefore, the differences found in the levels of IBP and EPP (and QP, see bellow) between studies, may be due to true variation between years and sites. Similar patterns have been observed in the European starling (*Sturnus vulgaris*) for which the proportion of IBP chicks changed from 14% to 27% in 25% and 64% of the nests in two consecutive years (Loyau et al. 2005a). The species with the most variation in the proportion of EPP offspring reported is the Willow warbler (*Phylloscopus trochilus*); with a study failing to find a single case of EPP (Gyllensten et al. 1990) while others reported between 23.5% and 33% of EPP offspring in 47% to 58% of broods (Bjornstad & Lifjeld 1997; Fridolfsson et al. 1997; Gil et al. 2007).

The percentage of quasi - parasitism (QP), detected in this study, varied between years (from 0.2% to 2.5% of the chicks). Cordero et al. (2003), reported one QP chick out of 334. To our knowledge, this is the first study that reports a significant number of QP offspring in a bird species, and where the true mother could in some cases be identified. Four of the QP mothers were detected with more than 95% confidence and another with more than 80%. The latter QP female was the true mother of the previous brood in that nest-box, with the same male. Another of the QP females occupied a neighbouring nest-box and produced 6 chicks in two broods with a single social partner, while the QP egg was fertilized by the neighbouring host-male. The other 3 cases involved females that were observed or captured in the vicinity of the host nest, but they did not produce a brood of their own. This low level of QP

probably occurs just by chance (IBP eggs deposited by chance in the nest of genetic fathers) or due to rapid mate-switching. Consequently, it is likely to be an artefact of the reproductive strategies present in the population (EPP and IBP) and not a strategy per se, but this possibility cannot be dismissed.

The number and degree of polygyny varied considerably during the three years of this study. The age of the colony (time since the introduction of nest-boxes in the area) may be part of the reason for this change. The higher levels of polygyny were observed in 2004 with almost 30% of all fathers being polygynous and occupying almost half of the nest-boxes (37 out of 77). Prior to the installation of the nest-boxes in 2004 the availability of nest sites for this species (natural fissures in trees and walls) was considerably lower. The number of available nest sites in a given year may influence the number of individuals visiting the area the following year. Consequently, during 2004 fewer individuals may have arrived in the area and competed for nest sites than in subsequent years. This may explain why more males managed to defend more than one nest in that year. However, there could be other ecological factors affecting the level of competition for nests (winter survival, availability of food in the area in a given year, etc).

By 2005 and 2006 the proportion of polygynous fathers went down to 19% and 12%, respectively (Table 12). Additionally, the degree of polygyny (number of nest-boxes defended simultaneously by a male) also decreased. This could be the result of higher competition for nest-boxes. Interestingly, the decrease in polygyny in 2005 also coincided with an increase of IBP and QP. Birds could be responding to the increase in competition, by adopting alternative reproductive strategies, such IBP and even QP.

The parentage data also allow the calculation of the levels of rapid mate switching (change of social partners within a year) and mate retention (maintaining the same social partner between years). It is very interesting to see that, in the cases where just one of the parents changed between clutches, females were twice as likely as males to change. It is also interesting to see that the level of mate retention is quite low and that no pair remained together for more than 2 years.

Several questions arise from these results. What is special about polygynous males? Which males suffer more paternity loss (EPP)? Which males and females suffer more IBP? Are males that sired EPP chicks of higher quality? Are EPP chicks of better quality? These kinds of questions inspired this study and I attempt to answer some of them in the following chapters.

Chapter 4. Male Traits and Reproductive Success: A Correlative Approach

Introduction

Sexual selection, as a subset of natural selection operates when the variability among individuals in heritable traits translates to a difference in the number of offspring that survive to breed (Alcock 1997). In the case of sexual selection, the forces that result in the differences in reproductive success are not specifically related to viability or survival, but with reproduction *per se* (Andersson 1994; Darwin 1871; Møller 1998). There are two scenarios in which sexual selection operates (see Chapter 1 for details): intra-sexual selection, the competition between individuals of the same sex (generally males; see Chapter 1) for access to individuals of the opposite sex; and inter-sexual selection, the selection of individuals of one sex by individuals of the other (generally females selecting males) (Alcock 1997; Andersson 1994; Harvey & Bradbury 1991). Irrespective of which form of sexual selection is operating, the outcome should be a higher reproductive success for individuals with a higher expression of the traits under selection.

Different models have been proposed that explain the evolution of sexually selected traits. The two most popular models are the indicator mechanism or handicap model (Grafen 1990; Zahavi 1974; Zahavi 1975; Zahavi 1977) and the Fisherian runaway model (see Chapter 1 for details). For the Fisherian model, costs become important once the trait becomes so exaggerated that natural selection acts against it (Fisher 1930; Lande 1981; Pomiankowski & Iwasa 1993; Pomiankowski & Iwasa 1998; Pomiankowski et al. 1991). Conversely, the handicap model proposes that costs drive the evolution of handicap traits from the beginning of the evolutionary process (Iwasa &

Pomiankowski 1991; Pomiankowski 1987a; Pomiankowski 1987b; Zahavi 1974; Zahavi 1975; Zahavi 1977). Irrespective of which model is used, theory predicts that evolved sexually selected traits must have some costs (Andersson 1994; Iwasa & Pomiankowski 1991; Iwasa & Pomiankowski 1999; Møller & Alatalo 1999; Pomiankowski 1987a). Therefore, provided that there is a cost to a given sexually selected trait, the intensity of the expression of that trait should reflect the quality of the sex that bears it, usually the males. The quality of an individual in evolutionary terms is defined by fitness and measured as the number of offspring that survive to reproduce (offspring in future generations). To consider survival or body condition as measurements of fitness can be erroneous. Once a strong preference for a trait has evolved, the benefits of being attractive may override the benefits of survival or other fitness components. Thus, males may exploit the tactic of displaying their higher quality even to the extreme where their survival is less than that of lower quality males. The trade-off between male attractiveness and other fitness components is mainly determined by the costs to the female in being choosy (Kokko et al. 2002; Kokko & Jennions 2003). Therefore, not all the sexually selected traits will correlate with body condition or other measurements of viability, but they should correlate with the fitness.

In the spotless starlings, the throat feathers (TF) of males are thought to be under sexual selection. The differences in the shape and the length of the throat feathers have been shown to be sufficient to correctly sex adult spotless starlings (Hiraldo & Herrera 1974; Lezana et al. 2000). Aparicio *et al.* (2001) tested the hypothesis that mate choice is indirectly based on heterozygosity (Brown 1997). To do this, Aparicio *et al.* assumed that the TF were sexually selected traits. They based this assumption on a correlation found between TF length and mating success, but the association of TF length with reproductive success was not assessed. To our knowledge, there are no other studies that provide evidence supporting the role of throat feathers as sexually selected ornaments.

This is a correlative study aimed at finding evidence for the role of TF as a sexually selected trait in the spotless starling. In addition to the TF length, we considered two other metrics: tarsus length and the condition of the males.

The tarsus length of males was used as a measurement of size. Spotless starlings are size dimorphic, males being around 6% heavier and 3% longer than females (Cordero et al. 2001). This means that the optimal size for males and females is different, which could be the result of sexual selection. The condition of the males was analysed since sexually selected traits are in many cases assumed to be condition dependent (Candolin 2005; Griffith et al. 1999; Kotiaho 2001). Therefore, controlling for condition would also indirectly control for other possible sexually selected traits that reflect the condition of the males (e.g. song repertoire size, song output and display rate and intensity.). The relationship between these three traits (TF length, size and condition) and the male reproductive success was assessed. Reproductive success was measured as the quantity and quality of offspring a male sired in a year, as determined by microsatellite genotyping.

Methods

Site

Field-work was conducted in Soto del Real, Spain. The study site was an open forest, mainly of ash (*Fraxinus angustifolius*) and oak (*Quercus spp*.), with some areas of pasture.

Between December 2003 and the beginning of March 2004, 78 nest-boxes were installed at the study site. All nest-boxes were made of wood and with the same dimensions (17 x 17 x 35 cm.) and arranged in groups of three (trios). Each box was located 7 to 11 metres from the other two boxes of the same group and groups were at least 65 metres apart. In total, there were 26 groups of three (trios).

Captures

During March, nest-boxes were regularly checked to see if there was fresh nesting material in them. We used this as a means to indirectly assess when males began to defend nest-boxes. Once a nest-box was seen to be in use,

we attempted to trap the occupying male. Birds were captured in the nest-boxes using a trap mechanism triggered by a tripwire that blocks the entrance when the bird enters.

Trapping was performed before the females started to lay eggs and again when the offspring were between 4 and 11 days old, to minimize abandonment resulting from the disturbance. Male starlings are readily caught by this method prior to egg-laying since they defend and prepare the nest. Once the eggs have hatched, brooding females will visit the nest-box more frequently than the males. Thus the two complementary periods of trapping allowed optimal sampling of the breeding population.

Additional nest-boxes were deployed to capture males that were difficult to capture in their own box. These nest-boxes were set close to established trios and left for one or two days to allow birds to become familiarized with them. Traps were then set, catching any exploring or defending adults that entered the boxes.

During captures we:

- Tagged the adult using a unique combination of plastic coloured leg-rings and a numbered aluminium leg-ring.
- Took a blood sample
- Removed four feathers from the central part of the chest (Throat feathers, TF)
- Recorded weight, beak length and width, wing length and tarsus length.

In total, 74 male and 126 female adult birds were captured during 2004 and, of these, blood samples were obtained from 67 males and 116 females.

Following the reproductive events

Nest-boxes were checked every second day in order to determine: the presence of green material, the time egg-laying initiated and the final clutch size. Eggs that were laid consecutively (less than 48 hours gaps between them) in a nest were assumed to belong to the same clutch. When no new eggs appeared for two days, the clutch was considered to be complete. The

nest was then checked on the eighth day of incubation (eight days after the last egg was laid), to ensure that the clutch was not lost. Then the nest was checked on the eleventh and twelfth day of incubation to determine hatching date. Hatching order was not recorded since most chicks hatch within 24 hours.

On days 6 and 14 after hatching, we weighed all chicks and measured their tarsus, wing and beak. On the 6th day they were ringed with numbered metal rings. Blood samples were taken on the 6th and 15th days, in order to prevent the loss of paternity data in cases where chicks did not survive until day 15. All un-hatched eggs observed in the nest were also collected. A tissue sample was taken from the embryos of un-hatched eggs and from any chick found dead prior to a blood sample being taken.

The strength of T-cell-mediated immune response in chicks was also quantified. On the 14th day, the thickness of one wing web of nestlings was measured with a thickness gauge (MITUTOYO CO., Japan). Then nestlings were injected subcutaneously with 0.05 ml of a 5 mg/ml solution of phytohaemagglutinin (PHA) (SIGMA ALDRICH) in the wing web. Around twenty-four hours later the thickness of the same area was measured to estimate the swelling response. We did not inject the other wing with phosphate buffered saline (PBS) as a control since this has been found to be unnecessary (Smits et al. 1999).

Nests were not disturbed after the chicks were fifteen days old, to prevent them from leaving the nest prematurely. Therefore, all chicks that reached fifteen days are considered in this study as fledglings and counted as successfully fledged.

When an event finished before the 15th June, the nest-box was checked again ten days later (when the chicks were 25 days old and it was certain that they had fledged) to determine if the nest was beginning to be used again (the old material was removed and/or new material had been added). Then it was checked every second day. When an event was not completed due to the loss of the eggs or chicks, nest-boxes were checked

every two days for these signs of re-occupation. The same protocol was followed with intermediate and second clutches as for the first clutches.

Observations

After hatching, observations were made to record which adults visited the nest-boxes. Any adult observed visiting the nest was assumed to be a social parent of the brood. These observations were also used to focus the trapping of adults in nest-boxes where un-ringed adults had been observed. Observations were made for 43 broods between the 5th and the 7th days after hatching. Nests were observed through a telescope for 30 minutes during the morning from temporary hides located about 20 metres away. The identity of all adults that were observed going inside or close to the focal nest was determined from their unique combination of colour rings. When an adult was observed while the nest-boxes were checked the colour ring combination was recorded (when visible) and used to complement the other observational data (for details see chapter 3).

Parentage analyses

The genotypes from 9 microsatellites were obtained for the captured adults and the offspring using the DNA extracted from the blood samples. Paternity then was assessed using observational data and molecular data. The paternity analyses were done using NEWPAT XL (Summers & Amos 1997), and CERVUS 3.0.3 (Kalinowski et al. 2007). From the 438 offspring sampled, 233 (54%) had both parents assigned and 135 (31%) had just one parent assigned (For details see chapter 3).

Statistical analyses

Analyses were done to reveal the importance of the male traits for their reproductive success. The analyses encompassed:

Categorical data

a) Reproductive state: reproducing and not reproducing.

b) Reproductive strategy: monogamous or polygynous.

Quantitative data

c) Paternity loss : proportion of EP offspring per sired offspring.

d) Reproductive success per event:

- Number of eggs per clutch.
- Number of hatchlings per brood.
- Number of fledglings per brood.

e) Offspring quality:

- Mean hatchling weight per brood.
- Mean Fledgling weight per brood.
- Mean T-cell immune response (swelling) per brood.

f) Total reproductive success per year:

- Number of eggs.
- Number of hatchlings.
- Number of fledglings.

Data used

The adult characteristics used in the analysis were the length of TF, the tarsus length and the condition. TF were measured from the tip of the vanes to the end of the calamus (naked, basal section of the quill). Measurements were done using digital callipers (MITUTOYO, Japan). This kind of feather has a narrow tip, especially those of adult males (Lezana et al. 2000), which can break when the feathers are being pulled out. Therefore, the mean of the three longest feathers, out of four, was used to avoid underestimation due to artificially shortened feathers.

The tarsus length was used as a general measurement of skeletal size. Tarsus length was measured by holding the bird's toes folded against the tarsus, and taking the full distance between the outermost bend of the toes and the tibia-tarsus joint, held at 90 degrees with respect to the tibia. The

measurements were done using digital callipers (MITUTOYO, Japan) to the nearest 0.01 mm.

The residuals of the regression of the cube root of the weight (g) against the tarsus length (mm), multiplied by a thousand were used as the condition metric (Appendix 6). The condition was obtained just for the males, since a female's weight changes dramatically depending on the stage of the reproductive event she is in (e.g. females about to start laying are much heavier than when incubating).

There were no correlations between the condition, tarsus and TF length of the males (Appendix 7), which means that they can be used simultaneously in a statistical analysis without risking collinearity. No signs of assortative mating were found. Specifically, no correlations were found between the tarsus and TF length of the females (tarsus and TF length) and their mates' characteristics (tarsus, TF length and condition; Appendix 8). Additionally, no differences in biometry were found between monogamous females (females of monogamous males, ambiguously referred as monogamous females in the literature), primary females and secondary females (females of polygamous males; Appendix 9).

For the analyses reported in the results section, the characteristics of the females (tarsus and TF length) are not used, since the focus of this study is on male traits and the lack of assortative mating guarantees that the results will not be skewed due to female differences. This compromise was made to avoid over-parameterized statistical models. Nonetheless, males tend to provide more parental care to monogamous and primary females than to secondary females. In order to avoid the skew that differential parental care could cause, the status level of the females was used in some analyses, by the use of a factor that separated females in two groups: a) monogamous and primary females and b) secondary females.

The body weight of the chicks at days 6 and 14 and the swelling response to the PHA were used as measurements of chick quality. Body weight was cube root transformed to allow the direct comparison of this three-dimensional measurement with linear measurements (Clark 1995; Gil et al. 2008). The

statistical analyses were done using the average cube rooted weight per brood and the average Immune response per brood.

The number of chicks at day 6 was used as a measurement of the number of hatchlings, while the number of chicks at day 15 was used as the number of fledglings. Although nest mortality may also happen after day 15, it was not possible to obtain reliable data on actual fledging number without disturbing the brood and inducing premature fledging. Thus number of nestlings at day 15 is the best surrogate that we can use of fledging success.

The laying period was divided in three categories according to the date in which the first egg was laid (Appendix 10). Clutches of first events were laid between the 13th of April and the 3rd of May; intermediate clutches were laid between the 4th and the 27th of May and second clutches were laid from the 28th of May to the 23rd of June.

The proportion of paternity loss suffered by a male was defined as:

$P_c = C_O / (T_O - P_O)$

Where P_c refers to Proportion of parasitic offspring; C_O is the number of offspring in the male's nest(s) that were not sired by him, but whose mother is his social mate; T_O is the total number of offspring in the nest(s); and P_O is the number of parasitic offspring in the nest(s) (offspring are parasitic if sired by different parents to those attending the nest).

Description of analyses

Different kinds of models were used according to the nature of the data. Cases where a non-normal distribution (Poisson or binomial) was assumed are reported in the results. The residuals were checked for normality in models that assumed this. All models that included multiple data per male were nested by male identity to avoid pseudo-replication. The Satterthwaite procedure was used to calculate the degrees of freedom of mixed models with normal distributions. When a model could be built assuming several different structures of variance, the one chosen was that which produced the lowest AIC (Akaike information criterion) value for the model. Models were initially tested using all possible double interactions and reduced according to the AIC

values of the models, the P values and the biological importance of the terms. The interactions that were not significant in the models are not reported. Analyses were done using SAS v. 9.1.

Results

From the males captured in 2004, those that reproduced had longer throat feathers (Wald $\chi^2_1 = 4.20$, P = 0.041, using a binomial logistic regression) and were in better condition (Wald $\chi^2_1 = 4.24$, P = 0.040) than males that did not reproduce (Figure 2). There were no differences in the size (tarsus length) of these males (Wald $\chi^2_1 = 0.66$, P = 0.415).

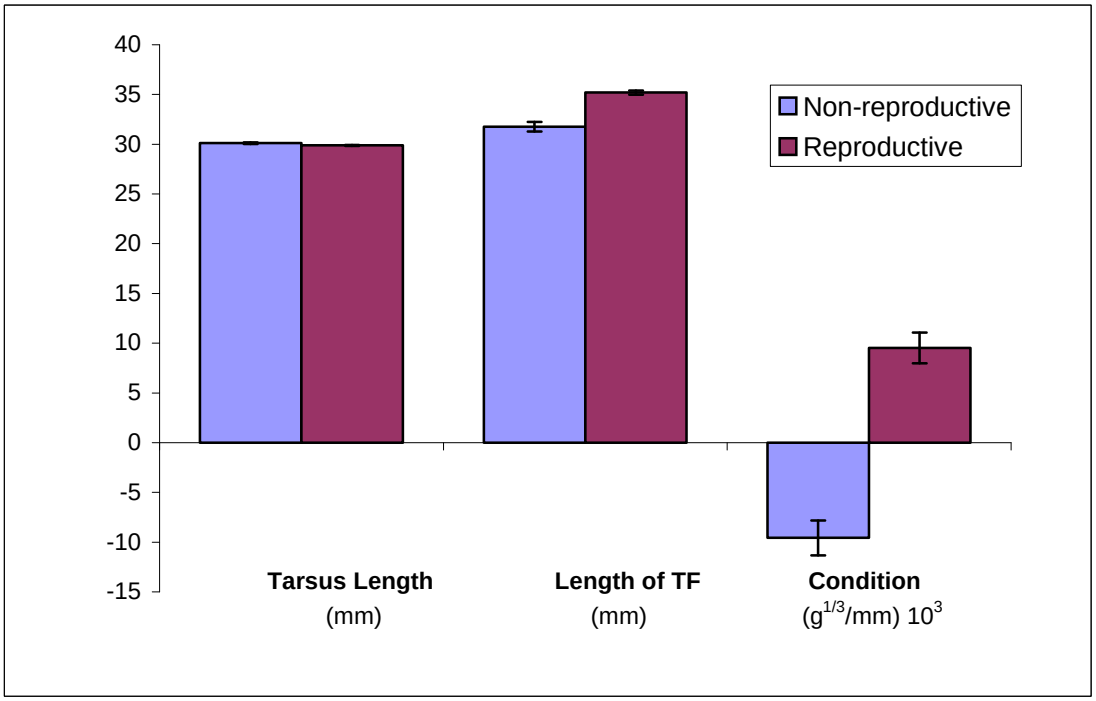


Figure 2. Tarsus length, length of the throat feathers and condition ± s.e. of males captured that did not reproduce (in blue) and that did reproduced (in purple).

Neither tarsus length (Wald $\chi^2_1 = 0.733$, P = 0.392) nor TF length (Wald $\chi^2_1 = 0.230$, P = 0.632) are good predictors of the reproductive tactic (monogamous or polygynous) that males adopted (Figure 3); but there was a

significant difference in condition between monogamous and polygynous males (Wald $\chi^2_1 = 4.52$, P = 0.034, using a binomial logistic regression).

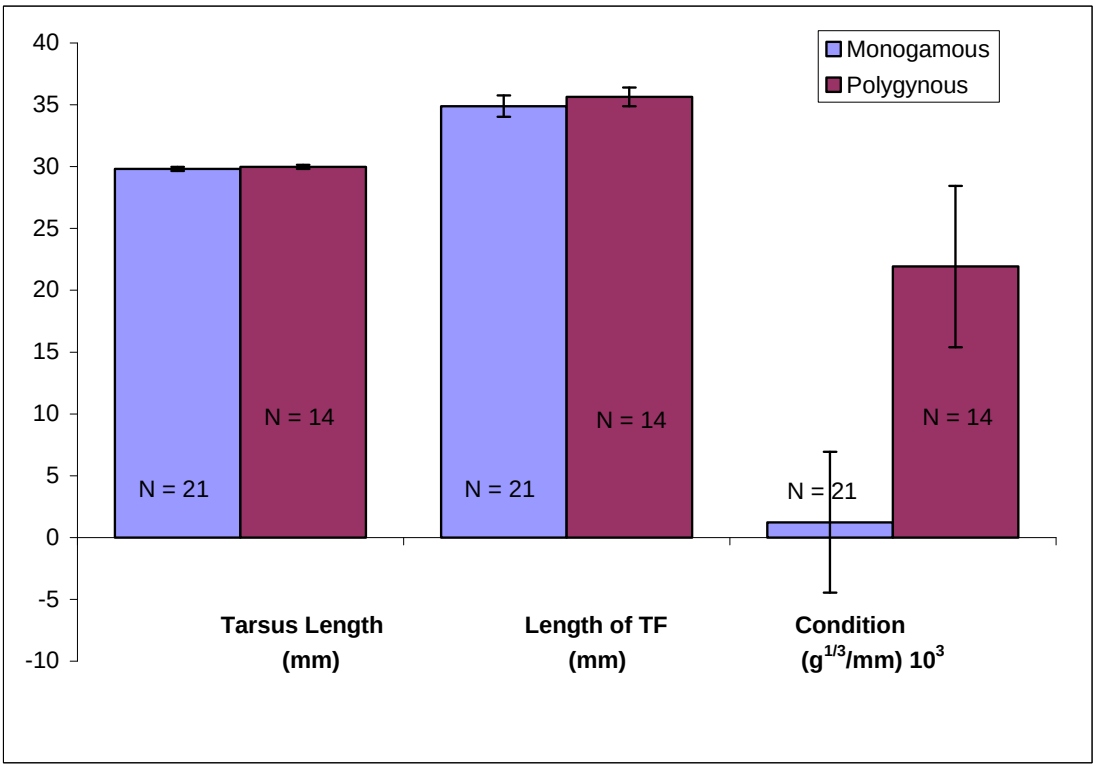


Figure 3. Tarsus length, TF length and condition ± s.e. of monogamous (in blue) and polygamous (in purple) males.

None of the male characteristics that we studied correlated with the proportion of paternity loss suffered by males in their nests (Wald $\chi^2_1 = 0.101$, P = 0.751 for tarsus length; Wald $\chi^2_1 = 0.260$, P = 0.610 for TF length; and Wald $\chi^2_1 = 1.637$, P = 0.201 for condition, using a binomial logistic regression). Similarly, no male characteristics correlated with the number of eggs, hatchlings or fledglings that males produced per brood (Appendix 11). The number of eggs was corrected using the genetic parentage of the offspring sampled. However, if eggs did not hatch and embryos were not sampled the parentage could not be assigned and these eggs are considered as having been sired by the attending male by default. Primary and monogamous females (females of monogamous males) tended to rear more fledglings ($F_{1, 63} = 3.78$, P = 0.056) than secondary females (Figure 4). Furthermore, the type

of reproductive event (first, intermediate or second) was a significant predictor of the number of fledglings reared per brood ($F_{2,63} = 3.42$, $P = 0.039$, GLM with Poisson distribution; Figure 5).

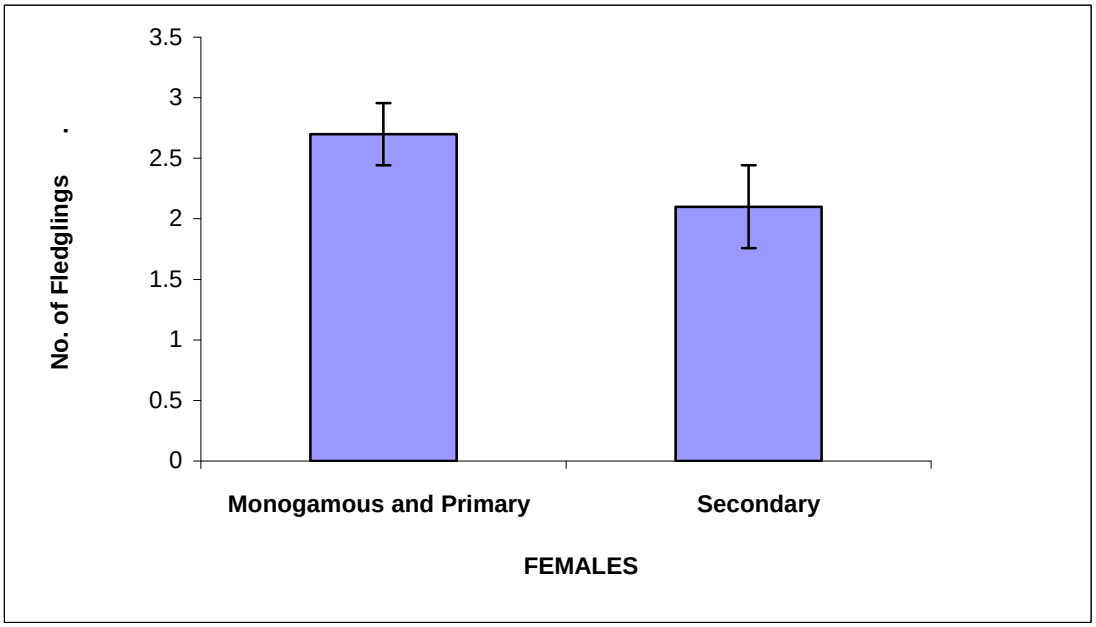


Figure 4. Mean number of fledglings per event ± s.e. reared by Monogamous (females of monogamous males) and Primary females and Secondary females.

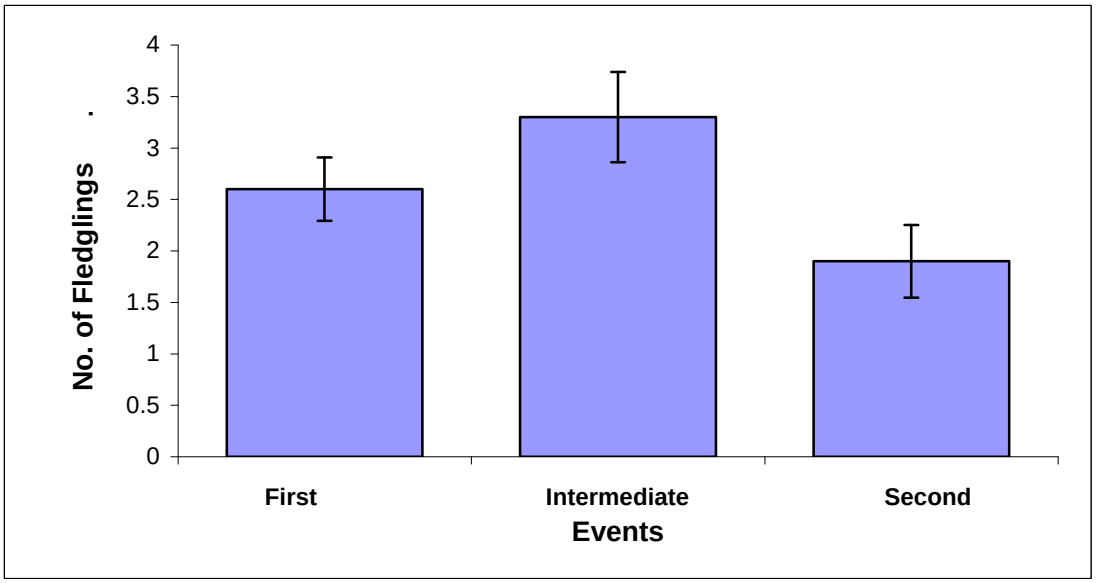


Figure 5. Mean number of fledglings per brood ± s.e. in first, intermediate and second breeding events. Only the significant difference on the number of fledglings is between the intermediate and the second events ($t_{64} = 2.61$, $P = 0.011$).

Neither male characteristics, nor female status (monogamous and primary vs. secondary) were related to the average weight of the chicks per event 6 days after hatching ($F_{1,44} = 0.59$, $P = 0.445$; $F_{1,44} < 0.01$, $P = 0.973$; $F_{1,44} < 0.01$, $P = 0.946$; and $F_{1,44} = 0.0$, $P = 0.903$ for tarsus length, TF length, condition and female status, respectively) or at 14 days old ($F_{1,15.8} = 0.76$, $P = 0.397$; $F_{1,7.3} < 0.01$, $P = 0.982$; $F_{1,18.1} = 1.09$, $P = 0.311$; and $F_{1,28.8} = 0.38$, $P = 0.544$ for tarsus length, TF length, condition and female status, respectively).

The mean weight of chicks per nest in first and intermediate events was higher than that of second events (Appendix 12) at age 6 days ($F_{2,61} = 5.12$, $P = 0.009$) and 14 days ($F_{2,46} = 25.35$, $P < 0.001$). The weight of the chicks at day 6 did not correlate with the brood size ($F_{1,44} = 1.75$, $P = 0.192$), but the weight at day 14 was negatively correlated with brood size ($F_{1,46} = 6.18$, $P = 0.017$; Figure 6).

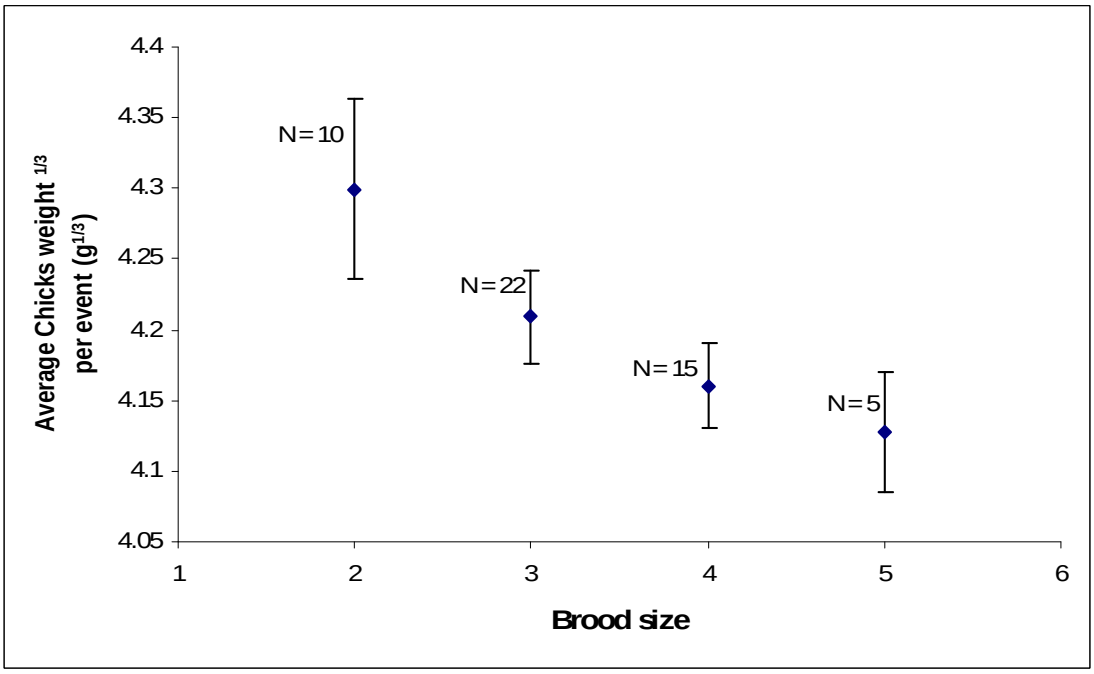


Figure 6. Mean ± s.e. of the average weight (cube root transformed) of fledglings (chicks on day 15) per brood according to brood size (number of chicks in the nest).

The strength of the T-cell-mediated immune response of the chicks did not correlate with any of the male characteristics ($F_{1,22.2} = 0.89$, $P = 0.356$; $F_{1,13.4} = 1.14$, $P = 0.305$; and $F_{1,20.1} = 1.10$, $P = 0.307$ for tarsus length, TF length and condition, respectively), nor did it correlate with the female status ($F_{1,28.8} = 0.38$, $P = 0.544$) or brood size ($F_{1,27.3} = 0.11$, $P = 0.743$). The immune response was higher in intermediate events, followed by second and first events ($F_{2,32.7} = 31.44$, $P < 0.001$; Appendix 13). Heavier chicks also exhibited greater immune responses than lighter chicks ($F_{1,36.9} = 21.79$, $P < 0.001$; Appendix 14).

The TF length and the condition of the male correlated positively with the number of eggs ($\chi^2_1 = 6.73$, $P = 0.009$ and $\chi^2_1 = 18.72$, $P < 0.001$, respectively), the number of hatchlings ($\chi^2_1 = 7.65$, $P = 0.006$ and $\chi^2_1 = 17.08$, $P <0.001$, respectively) and the number of fledglings a male sired during a year ($\chi^2_1 = 10.82$, $P = 0.001$ and $\chi^2_1 = 17.3$, $P = <0.001$, respectively; using only the data of reproductive males in a GLM with Poisson distribution). However, there were three outliers (marked in red in Figure 7). When these outliers were removed from the models, these trends were maintained, but the slopes were reduced. Condition was still significant for all models ($\chi^2_1 = 4.90$, $P = 0.027$; $\chi^2_1 = 4.56$, $P = 0.033$; and $\chi^2_1 = 5.31$, $P = 0.021$ for number of eggs, hatchlings and fledglings, respectively). However, TF length ceased being significant when the outliers were removed ($\chi^2_1 = 2.10$, $P = 0.147$; $\chi^2_1 = 2.19$, $P = 0.139$; $\chi^2_1 = 3.11$, $P = 0.078$ for number of eggs, hatchlings and fledglings, respectively).

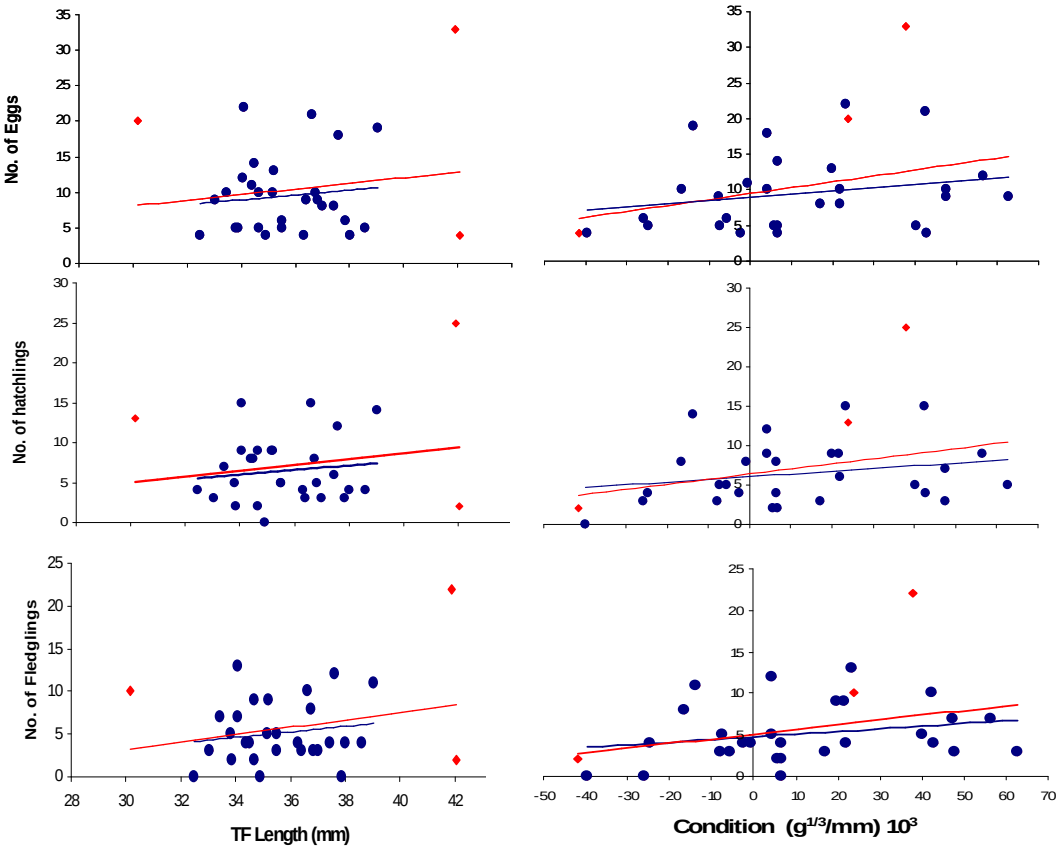


Figure 7. Graphs plotting the number of eggs (top row), hatchlings (middle row) and fledglings (bottom row) per male against the paternal characteristics: TF length (left column) and Condition (right column). The points marked in red are considered outliers. Regression lines in red include outliers, while the blue ones do not.

Discussion

The probability of a male reproducing was found to depend on their TF length and their condition. As can be seen in Figure 2, males that reproduced had longer throat feathers and were in better condition than those that did not reproduce. Body condition may be indirectly selected if other male sexually selected male traits are honest signals of condition. Condition could also be involved in intra-sexual selection, if males in better condition are able to defend a nest-site better than males with lower condition. Studies of sexual selection in the wild (in non-lekking species) look often only at the correlations using reproducing males but, in this case, the condition and the TF length of

the males also predict the failure of a male to reproduce. This shows that these characteristics are fundamentally important for the reproductive success of males and do not just influence the variability of success amongst reproducing males. Additionally, from those males that did reproduce, males with a higher condition were more likely to be simultaneously polygamous. Polygynous males will increase their reproductive success unless they experience a higher loss of paternity or if they have fewer offspring per nest.

The proportion of paternity loss did not correlate with any of the male characteristics that we studied. It has been proposed that polygamous males tend to lose more paternity, since the efficiency of mate-guarding and the number of copulations per female decreases with the number of females they have (Birkhead & Møller 1992; Cordero et al. 2003). In this study, males with a higher condition tended to be polygynous, but they did not tend to suffer more parental loss. This could reflect the higher capacity of these males to guard their females or the fact that these males benefit from a higher fidelity from their mates. This is compatible with the findings of studies of European starlings (*Sturnus vulgaris*), red winged blackbirds (*Agelaius phoeniceus*) and blue tits (*Parus caeruleus*) that showed no difference between the amount of paternity loss suffered by monogamous and polygynous males (Kempenaers et al. 1995; Smith & Vonschantz 1993; Westneat 1993). Cordero *et al.* (2003) showed that, in one out of the two years studied, spotless starling males with more simultaneous mates were cuckolded more frequently than those with only one mate at a time. However, in that study, males' testosterone levels had been experimentally manipulated so that males with increased testosterone levels were more polygynous even when the rest of the traits (e.g. TF length and condition) did not change. This artificial induction of polygyny could have produced polygynous males that did not have the condition necessary to guard their females or that were not "attractive" enough to guarantee their mates' fidelity. Therefore, experimentally elevated testosterone levels may increase polygyny but not necessarily reproductive success.

Our results show that, per brood, there was no difference in the number of offspring sired by males of differing quality. However, the number of fledglings

that a male sired with a monogamous or primary female was higher than the number sired with a secondary female. Males tend to provide less parental care to broods of secondary females (Moreno et al. 2002), which may decrease the survival rate of the offspring. This may explain why while the number of eggs and hatchlings were not significantly smaller for secondary females, the number of fledglings was.

The tendency for intermediate events (i.e. replacement clutches) to yield more fledglings was unexpected. Females and monogamous males may invest more in intermediate broods since these will be the only successful reproductive events they can have in that year (the intermediate event will finish too late to start a second one). An increase in investment during intermediate events may increase the parents' success, resulting in more fledglings per event. Alternatively, a greater number of fledglings from intermediate events could be an effect of the year. If the conditions in a given year are better for intermediate events than for first events (e.g. spring rainfall being delayed until late into the first event), it is possible that intermediate events would produce more fledglings than the earlier events. Intermediate events coinciding with good conditions could be misinterpreted as a fundamental increase in investment by parents in intermediate events. Additionally, females may lay eggs out of synchrony with the best breeding conditions if this increases their fitness by allowing them to have two reproductive events in a year. Moreover, if the high breeding synchrony of this species is due to the selective pressure of brood parasitism, even females with just one reproductive event per year may lay eggs out of synchrony with the best breeding conditions in order to breed in synchrony with other females.

Bigger males, males in better condition or with bigger ornaments (TF) did not seem to have offspring of higher quality, that is, offspring of greater weight or stronger immune response. Since this study was carried out in a wild population, there are numerous factors that could not be controlled, such as the amount of parasites in a nest, the quality of the mothers, the weather and the availability of food during different times of the breeding season. All these factors could obscure the relationship between the quality of the fathers and

their offspring.. A cross-fostering experiment would be necessary to see if the male quality traits (e.g. condition) are heritable. However, our data did not provide evidence for higher quality males producing higher quality offspring due to either good genes or direct benefits.

Additionally, the swelling response to PHA may not accurately reflect the T-cell-mediated immuno-competence of the offspring. Evidence has been found in a study of house sparrows (*Passer domesticus*), showing that the swelling response that followed an injection of PHA reflected innate and adaptive components of the immune system and that the swelling response correlated with the abundance of different immune cells and this depends critically on the length of time since the injection (Kennedy & Nager 2006; Martin et al. 2006). Therefore, the lack of correlation between the fathers' quality measurements and the offspring immune response may be caused by the inefficiency of the PHA test in measuring the innate immune response.

Chicks in second events were lighter than those in first and intermediate events (see also Gil et al. (2008)). This is almost certainly due to the environmental changes during the reproductive season (Salaberría *et al.* in prep.). By the time the chicks of the first events hatched the availability of food (especially insects) was high, but when the second clutches hatched it was poor (it was very hot and there was a severe drought). It is also understandable that the weight of fledglings decreased with brood size, since parents of bigger broods had to feed more chicks. However it might be surprising that even the difference in weight between broods of 2 and 3 chicks was very marked.

As predicted, heavier chicks also had a stronger immune response, since both are measures of the chicks' quality. Heavier chicks are expected to have more energy reserves or to have grown faster. Therefore, heavier chicks are expected to be of better quality and so better able to respond to immune challenges. Conversely, the variation in the strength of immune response relative to the event-type was not as expected. Chicks in intermediate events had the strongest immune responses, followed by chicks in second events and finally those of first events had the weakest responses. This could be an effect of parasite exposure, parental investment or a year effect, but there are

insufficient data to support any of these alternative non exclusive explanations.

Males in better condition had a greater reproductive success (a greater number of eggs, hatchlings and fledglings sired in a reproductive season). These males had also a greater probability of being polygynous. Even though the success of secondary broods was lower, these broods still made a substantial contribution to the number of chicks a male sired. Putting together all our results, males in better condition tended to sire offspring in more than one nest at a time and, therefore, had a greater reproductive success. It is unlikely that females can directly assess the body condition of the males. However, there are some other traits and behaviours that are thought to be sexually selected in the spotless starling, such as the display and addition of green materials to the nest (Polo & Veiga 2006; Veiga et al. 2006), the colour of the feathers and the beak, the song repertoire, the time spent singing, etc. Some of these traits may be signalling the current condition of the individuals. Consequently, condition may be selected for indirectly.

Since models without outliers are not significant for TF, the results do not show clearly if males with longer TF have a greater reproductive success. However, even when the outliers are disregarded, the tendency is maintained. These results, together with the finding that the probability of reproducing (vs. not reproducing at all) was related to the TF length, support the hypothesis that TF length is an important predictor of the reproductive success of male spotless starlings. Therefore, TF length appears to be a sexually selected trait or correlated with one (only the use of an experimental manipulation can distinguish between these).

In conclusion, the size (tarsus length) of males does not have an influence on male reproductive success. The condition and the TF length of males are important factors that determine (directly or indirectly) male reproductive success, males of higher condition and longer TF length siring more offspring per year. However, the quality of the offspring was not related to these male traits.

Chapter 5. Male Throat Feather Length and Reproductive Success: An Experimental Approach

Introduction

To explain the evolution of conspicuous male traits that do not confer a survival advantage, Darwin (1859) introduced the concept of sexual selection (SS). SS operates on traits that increase the reproductive success of an individual, even if they decrease its survival (Andersson 1994; Darwin 1859). There have been many studies examining whether particular traits are under sexual selection. Some have evaluated the correlation of the level of expression of a trait with: a) the level of preference of females for these males; b) the success of the males in intrasexual competition (e.g. acquisition and defence of territories); or c) the reproductive success or number of offspring sired by males. Selection can act in a direct or indirect way (see chapter 1). Therefore, a correlation between these measurements of fitness and the expression of a trait can be found even when the trait is not under direct selection, but correlates with one that is. To avoid confounding a correlated trait with a sexually selected trait, an experimental approach (and not just a correlative one) is needed.

Perhaps the most famous experiments aimed at finding evidence of sexual selection acting on trait, where done by artificially altering the feathers of male birds. Andersson (1982) modified the length of the tail feathers of the long-tailed widowbird, *Euplectes progne*. He found that males with elongated tails had a higher reproductive success than controls and males with shortened tails. Møller (1988) also modified the length of the tails, this time in swallows (*Hirundo rustica*). Males with elongated tail feathers obtained mates faster, experienced a higher reproductive success in their nests and were also preferred, by females, for extra-pair copulations. Another famous experiment

was performed by Petrie *et al.*, whom after finding a correlation between the number of eye-spots in the train of peacocks (*Pavo cristatus*) and their mating success (Petrie et al. 1991) proceeded to experimentally reduce the number of eye-spots (Petrie & Halliday 1994). They found that males of this negative treatment experienced a decrease in their mating success. Since then, additional studies on this species have found additional evidence supporting the role of male trains as sexually selected traits (Loyau et al. 2005b; Loyau et al. 2007), although some have failed to support this hypothesis (Hasegawa 1995; Takahashi et al. 2008).

Recent studies have shown that sexual selection often acts on coexisting multiple sexually selected traits in the same species. For instance, studies in the barn swallow (*H. rustica*) and the peafowl (*P. cristatus*) have found a role of multiple traits. Female swallows seem to choose males based on the length of the tail, but also on song rate (Møller et al. 1998). On the other hand, peahens appear to base their mate choice also on the vigour of male displays and not just the length and ornamentation of male train (Loyau et al. 2005b).

Several hypotheses have been proposed to explain the evolution and maintenance of multiple sexually selected traits (see Chapter 1 for details), and different studies have found supporting evidence for some of them. One of the principal hypotheses states that multiple traits evolve because some of them are selected by an intra- sexual process, while others are selected by inter-sexual selection. Evidence for this has been found in the water boatman, *Sigara falleni* (Candolin 2003) and the scarlet-tufted malachite sunbird, *Nectarinia johnstoni* (Evans & Hatchwell 1992a; Evans & Hatchwell 1992b). Another hypothesis states that different traits may reflect different aspects of male condition. Loyau *et al.* (2005b) and Møller and Petrie (2002) found evidence in this direction. A study by Marchetti (1998) found that yellow-browed leaf warbler females, *Phylloscopus inornatus,* were able to assess male quality through many traits, so when one of the traits was experimentally manipulated, they were still able to assess the quality of the males using the other signals, thus rendering the manipulation inefficient. A study in lark buntings, *Calamospiza melanocorys*, presents even more complex patterns (Chaine & Lyon 2008). Females in this species seem to select for multiple

traits, but the intensity and even the direction of selection vary from year to year, probably in relation to changes in the ecological and social environment.

In the spotless starling (*Sturnus unicolor*), there are several traits that seem to be under sexual selection. These traits include physical characteristics such as coloration (including UV reflectance) and feather length; and behaviours such as singing and the provision of green materials in the nest. This study focuses on the length of the throat feathers; a trait that has been assumed to be under sexual selection since there is a pronounced sexual dimorphism in this trait. A study by Aparicio *et al.* (2001) reported contradictory results. In the methods, as a justification of their hypothesis, they reported a correlation between mating success and average length of TF. However in the results they reported that the length of TF was positively correlated with heterozygosity and that there was a quadratic correlation between heterozygosity, mating success and number of offspring sired per year. So, their study results pointed to medium values of heterozygosity and, therefore medium length of TF, resulting in a higher mating and reproductive success, while the correlation that they present in the methods showed higher mating success associated with longer TF. These contradictory results have not been explored further, and the relationship between TF length and male reproductive success (observed or genetically verified) has not been assessed. Observational data (Chapter 4) suggest that TF length is under sexual selection.

In this study, we aimed to find experimental evidence that the length of the TF was under sexual selection, by altering the length of the throat feathers of male spotless starlings and measuring the effect on their reproductive success. In an attempt to control for other sexually selected traits (that may correlate with current condition of the males), a condition metric was included in the analyses. Reproductive success was measured as the quantity and quality of offspring a male sired in a year.

Methods

Site

Field-work was conducted at Soto del Real, Spain in an open forest, mainly of ash (*Fraxinus angustifolius*) and oak (*Quercus spp*.), with some areas of pasture.

At the end of 2003 and beginning of 2004, 78 nest-boxes were installed at the study site. These boxes were used by spotless starlings in 2004 for a previous study (Chapter 4). During 2005 and 2006, 69 of them were used for this experimental study. All nest-boxes were made of wood and with the same dimensions (17 x 17 x 35 cm.) and arranged in groups of three (trios). Each box was located 7 to 11 metres from the other two boxes of the same group and groups were at least 65 metres apart. In total, there were 23 groups of three (trios). This arrangement of boxes was used to try increasing the level of polygyny.

Captures

Form March the 22nd until the first female in the colony laid an egg (23rd of April in 2005 and 20th of April in 2006), we attempted to capture adults (especially males) in the nest-boxes. We used traps with a mechanism triggered by a tripwire that blocks the entrance when the bird enters. Captures were done mostly during the mornings when the birds are more active. The traps were set and then checked after 15 to 20 minutes. If they had not been triggered they were left for another similar period of time. Males captured before egg-laying were assigned to an experimental treatment (see below).

Trapping was performed again when the offspring were between 4 and 11 days old, to minimize abandonment resulting from the disturbance. Male starlings are readily caught by this method prior to egg-laying since they defend and prepare the nest. Once the eggs have hatched, brooding females will visit the nest-box more frequently than the males. Thus the two

complementary periods of trapping allowed optimal sampling both sexes in the breeding population.

Additional nest-boxes were deployed to capture males that were difficult to capture in their own box. These nest-boxes were set close to established trios and left for one or two days to allow birds to become familiarized with them. Traps were then set, catching any exploring or defending adults that entered the boxes. It was important to capture as many adults as possible to ensure having a DNA sample for them for the paternity analysis.

During captures we:

- Tagged the adult using a unique combination of plastic coloured leg-rings and a numbered aluminium leg-ring.
- Took a blood sample
- Removed four feathers from the central part of the chest (Throat feathers, TF)
- Recorded weight, beak length and width, wing length and tarsus length.

Treatments

Males that were captured before egg-laying started were assigned to one of the following treatments (Figure 8):

- Control: No modification was done to these males.
- Negative: Their throat feathers (TF) were cut to lengths that resemble that of females and young males (20.09 ± 0.18mm not including the rachis).
- Positive: Nine elongated feathers in groups of 3 were attached to the skin using super-glue, in the middle, right and left parts of the throat. Elongated feathers were made using 2 TF (clipped from other males) attached to each other to increase the length (44.51 ± 0.59 mm). This treatment was only performed in 2005, since we observed that feathers did not hold on for long (a male recaptured 10 days later had just 3 elongated feathers left and another recapture 1 month later did not

have any left). Therefore, I have dropped data regarding this experimental group in the remainder of the chapter.

Positive Treatment

Control

Negative Treatment

Figure 8. Males were assigned to one of three different treatments. Males on the positive treatment had nine longer throat feathers attached. Throat feathers of control males were not altered. Throat feathers of negative treatment males were shortened.

Only males captured 10 days before the first egg was laid are included in this study, to allow sufficient time for the treatments to have an effect. Ten days before egg-laying, couples may already have formed and there may be not enough time to allow females to change mates in response to the treatment. Males captured both years were assigned to the same treatment in both years, except for males belonging to the positive treatment in 2005, which were assigned to the control group in 2006. Of 72 males captured in 2005, 36 are included (19 controls and 17 negatives), while in 2006, 53 of the 67 males captured are included (28 controls and 25 negatives), 11 of the males are included in both years.

Following the reproductive events

Nest-boxes were checked every second day in order to determine the presence of green material, the initiation of egg-laying and the final clutch size. Eggs that were laid consecutively in a nest (with less than 48 hours between them) were assumed to belong to the same clutch. The clutch was considered to be complete when no new eggs appeared for two days. The nest was then checked on the eighth day of incubation (eight days after the last egg was laid) to ensure that the clutch had not been lost. Then the nest was checked on the eleventh and twelfth day of incubation to determine the hatching date (date that first egg hatched = day 1).

On days 6 and 14 after hatching, all chicks were weighed and their tarsus, wing and beak were measured. On the 6th day they were ringed with numbered metal rings. Blood samples were taken on the 6th day post-hatching. All un-hatched eggs observed in the nest were also collected. A tissue sample was taken from embryos of un-hatched eggs and from any chick found dead before a blood sample was taken.

The strength of T-cell-mediated immune response in chicks was also quantified. On the 14th day, the thickness of one wing web of nestlings was measured with a thickness gauge (MITUTOYO CO., Japan). Nestlings were then injected subcutaneously with 0.05 ml of a 5 mg/ml solution of phytohaemagglutinin (PHA) (SIGMA ALDRICH) in phosphate buffered

saline (PBS) into the wing web of one of the bird's wings. Around twenty-four hours later the thickness of the same area was measured to estimate the swelling response. This technique does not require a control injection in the other wing, and has been shown to provide reliable results (Smits et al. 1999).

Nests were not further disturbed after the chicks were fifteen days old, to prevent them from fledging prematurely. Therefore, all chicks that reached fifteen days are considered in this study as fledglings and scored as successfully fledged.

When a reproductive-event was completed before the 15th June, the nest-box was checked again ten days later (when the chicks were 25 days old and it was certain that they had fledged) to determine if the nest was going to be used again (the old material had been removed and/or new material was added). Then it was checked every second day. When an event was not completed due to the loss of the eggs or chicks, nest-boxes were checked every two days to determine re-occupation. The same protocol was followed with intermediate and second clutches as for the first clutches.

Observations

After hatching, observations were made to record which adults were the probable social parents of the broods. During 2005 all nest-boxes containing chicks were observed at least once but usually twice, firstly between the 4th and the 8th day, and secondly between the 10th and the 14th day after hatching. Most observations were done with video cameras; but some were done from a hide using a telescope. Observations were done during the morning for an hour. In 2006, observations were performed exclusively using video cameras. In this year, only nest-boxes that contained chicks in first reproductive events were recorded. Therefore, these recordings were shorter (around 30 minutes) and were not repeated once the adults had been identified. No observations were made of subsequent reproductive events at nest-boxes after they had been observed once.

The identity of all adults that were observed going inside or close to the focal nest was determined from their unique combination of colour rings. When an

adult was observed while the nest-boxes were checked, the colour ring combination was recorded (when visible) and used to complement the other observational data (for details see chapter 3). If one of the attending parents was not ringed, we attempted to capture it inside the nest-box when the chicks were between 4 and 11 days old or in an extra nest-box nearby, when the chicks were older.

Parentage analyses

Genotypes from 9 microsatellites were scored for adults captured and all offspring using the DNA extracted from the blood samples. Paternity then was assessed using observational and molecular data (For details see chapter 3). The paternity analyses were done using NEWPAT XL (Summers & Amos 1997), and CERVUS 3.0.3 (Kalinowski et al. 2007). From the 793 offspring sampled during these two years, 457 (57%) had both parents assigned and 217(27%) had just one parent assigned.

Analyses

Analyses were conducted to see whether the treatments (negative vs. control) had an effect on the reproductive success of males. The analyses focused on the effect of the treatments on:

a) Reproductive state: probability of reproducing against not reproducing.
b) Reproductive strategy: probability of being monogamous or polygynous.
c) Paternity loss: proportion of EP offspring relative to sired offspring in their nest or nests.
d) Reproductive success per event:
    - Number of eggs per clutch.
    - Number of hatchlings per brood.
    - Number of fledglings per brood.
e) Offspring quality:
    - Mean hatchling weight per brood.

- Mean fledgling weight per brood.
- Mean T-cell immune response (swelling diameter) per brood.

f) Total reproductive success per year form their nests:

- Number of eggs.
- Number of hatchlings.
- Number of fledglings.

Data used

The central part of the analysis focuses in the differences between treatments (control and negative). Two variables that were found to explain reproductive success in the correlative study (Chapter 4) , body condition and the original size of male TF, were controlled for in the analysis. Male size (tarsus length) was not included, since there was no evidence that it affected the reproductive success of males in the correlative study (Chapter 4).

TFs were measured from the tip of the vanes to the end of the calamus (the naked portion of the quill or shaft). Measurements were done using digital callipers (MITUTOYO, Japan) to the nearest 0.01 mm. TFs have a narrow tip, especially in adult males (Hiraldo & Herrera 1974; Lezana et al. 2000), which can break when the feathers are being pulled out. Therefore, the mean of the three longest feathers, out of four, was used to prevent artificially shortened feathers being considered.

The residuals of the regression of the cube-root transformed weight (g) against tarsus length (mm), multiplied by a thousand were used as the condition (See Appendix 6). The condition was obtained just for the males, since a female's weight changes depending on the stage of the reproductive event she is at (e.g. females about to start laying are much heavier than when incubating) and we were not able to obtain female mass data at comparable stages. Male TF length was not correlated with condition ($F_{1,57.4} = 2.36$, $P = 0.13$, using a mixed model nested by individual and controlling for year). This allows the simultaneous use of these terms in a statistical analysis

without problems of collinearity. Measurements of condition and TF length were used only for the year the male was captured, since they are expected to change through time.

No signs of assortative mating were found. Specifically, no correlations were found between the characteristics of the females (tarsus and TF length) and the treatments or with other characteristics of their mates (TF length, condition and reproductive tactic; Appendix 15). Additionally, no differences were found between TF length of monogamous females (mates of monogamous males), primary females and secondary females ($F_{3, 31.5} = 0.54$, $P = 0.662$) or between the lengths of their tarsi ($F_{3, 29.9} = 0.65$, $P = 0.591$).

For the analyses reported in the results section, the characteristics of the females (tarsus and TF length) are not used, since they are not the focus of this study and the lack of assortative mating guarantees that the results will not be skewed due to female differences. This compromise was made to avoid over-parameterized statistical models. Nonetheless, males tend to provide more parental care to monogamous and primary females than to secondary females. In order to avoid the skew that differential parental care could cause, the status level of the females was used in some analyses (a factor that separated monogamous and primary females from secondary females).

Body weight of the chicks at days 6 and 14 was used as well as the swelling response to the PHA as measurements of chick quality. Body weight was cube-root transformed to allow the direct comparison of this three-dimensional measurement with linear measurements (Clark 1995; Gil et al. 2008). Statistical analyses were conducted using the average cube-rooted weight per brood and the average immune response per brood.

The events were divided into three categories according to the date at which the first egg was laid (Appendix 16). In 2005 clutches of first events were laid between the 23rd of April and the 3rd of May; intermediate (or replacement) clutches between the 4th and the 31st of May and second clutches from the 5th to the 16th of June. In 2006 clutches of first events were laid between the 20th

and the 30th of April; intermediate clutches between the 1st and the 28th of May and second clutches from the 31st of May to the 13th of June.

The proportion of paternity loss suffered by a male was defined as:

$P_c = C_O / (T_O - P_O)$

Where P_c refers to proportion of parasitic offspring; C_O is the number of offspring in the male's nest(s) that were not sired by him, but whose mother is his social mate; T_O is the total number of offspring in the nest(s); and P_O is the number of parasitic offspring in the nest(s) (offspring parented by different individuals to those attending the nest).

Statistical analyses

Different types of model were used according to the nature of the data. In the cases where a non-normal distribution (Poisson or binomial) was assumed it is reported in the results. Where models assumed a normal distribution, the residuals were checked for normality. Most models included multiple data per male (in different events or years) and were, therefore, nested to avoid pseudo-replication. The Satterthwaite procedure was used to calculate the degrees of freedom of mixed models with normal distributions. When a model could be built assuming several different structures of variance, the one chosen was that which produced the lowest AIC (Akaike information criterion) value for the model. Most models were initially tested using all possible double interactions and reduced according to the AIC values of the models, the P values and the biological importance of the terms. The models for offspring quality (weight and immune response) were started with only a selection of the two-way interactions (11 and 15 interactions, respectively). In these two models, the interactions were selected according to their biological importance. The reduction of these models was done as stated before. The interactions that were not significant in the models are not reported. Analyses were done using SAS v. 9.1.

Results

The probability of reproducing was independent of the treatment (Wald $\chi^2_1 = 1.172$, P = 0.279), the original TF length (Wald $\chi^2_1 = 0.339$, P = 0.561) and the year (Wald $\chi^2_1 = 0.092$, P = 0.762). However, males in better condition had a better chance of reproducing (Wald $\chi^2_1 = 6.324$, P = 0.012; using a binomial logistic regression N = 74; Figure 9).

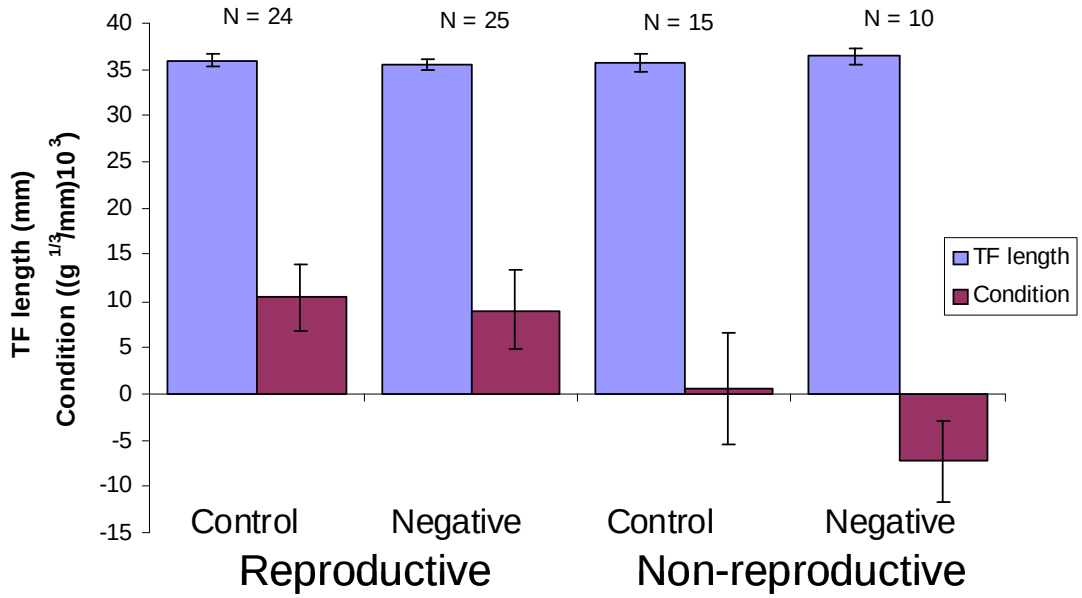


Figure 9. Mean ± s.e. throat feather (TF) length (blue) and condition (purple) for males that reproduced (first 2 pairs of bars) and that did not reproduce (last 2 pairs of bars) and that belonged to the negative treatment (2nd and 4th pairs of bars) or to the control group (1st and 3rd pairs of bars).

Control males were not more likely to be polygynous than males in the negative treatment (Wald $\chi^2_1 = 0.942$, P = 0.3319; using a binomial logistic regression N = 43). The original TF length and body condition did not explain the probability of being monogamous or polygynous (Wald $\chi^2_1 = 0.428$, P = 0.513; Wald $\chi^2_1 = 0.021$, P = 0.885, respectively).

Males of the negative treatment did not experience a higher loss of paternity in their nest than control males (Wald $\chi^2_1 = 0.174$, P = 0.6762; model with

binomial error, N = 49). The original TF length and body condition did not explain the proportion of paternity loss suffered by males (Wald $\chi^2_1 = 1.095$, P = 0.295; and Wald $\chi^2_1 = 1.3839$, P = 0.239, respectively).

The number of eggs, hatchlings and fledglings that a male sired per reproductive event, did not differ significantly between negative and control males ($F_{1,80} = 0.03$, P = 0.871; $F_{1,78} = 0.71$, P = 0.403; and $F_{1,76} = 0.15$, P = 0.701, respectively). The original TF length and the body condition of males were not associated with the number of offspring per event (Appendix 17). However, the number of fledglings is marginally non-significantly related with the body condition of the father ($F_{1,76} = 3.29$, P = 0.074; Figure 10). Monogamous and primary females had more eggs and more hatchlings per event than secondary females ($F_{1,80} = 4.53$, P = 0.036; $F_{1,80} = 4.00$, P = 0.049, respectively). This tendency is maintained but is not significant for the number of fledglings per event ($F_{1,76} = 2.22$, P = 0.170; Appendix 18). For the model concerning the number of hatchlings, the year and the interaction between year and event-type are both significant (Appendix 17 and Appendix 19). The year, the event-type and their interaction are significant for the model of the number of fledglings (Appendix 17 and Appendix 19).

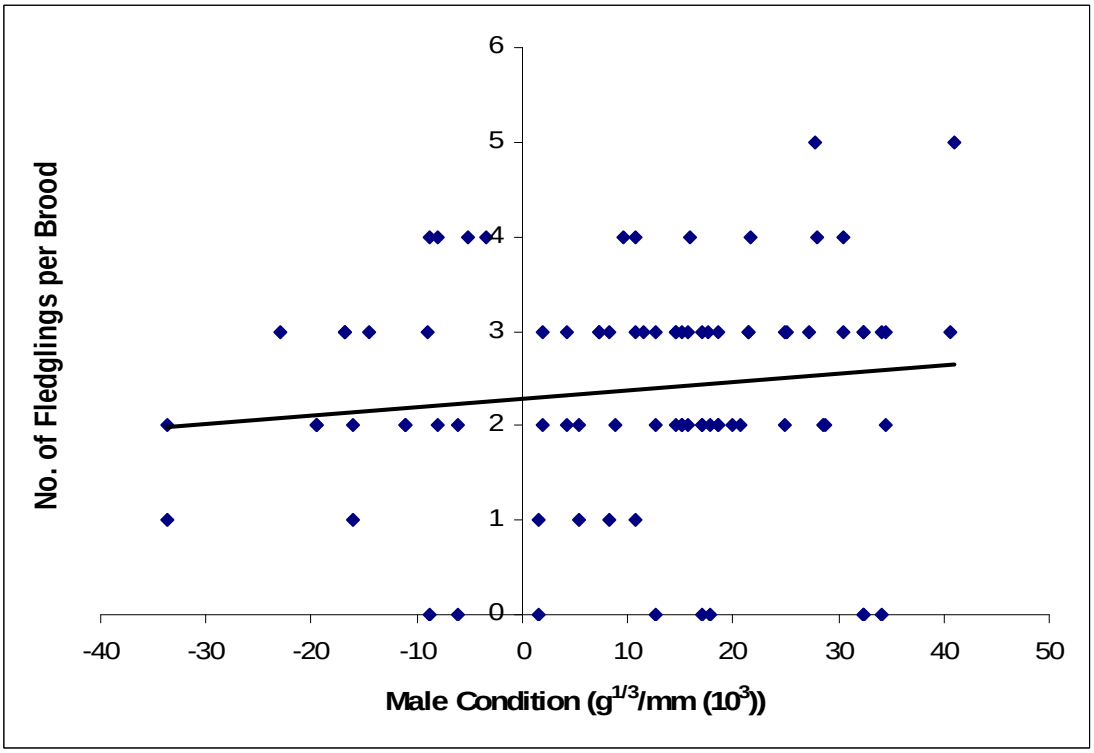


Figure 10. Number of fledglings sired per event, plotted against the condition of the fathers.

The weight of the hatchlings was not explained by the treatment ($F_{1,46.7} < 0.01$, $P = 0.980$) or by any of the male characteristics, female status or year (Appendix 20). The weight of the hatchlings differed according to the event-type ($F_{2,65.9} = 14.49$, $P < 0.001$; Appendix 21), chicks were heavier in first than in intermediate events and chicks of second events were the lightest.

The weight of fledglings sired by males of the negative treatment was lower than that of fledglings sired by control males ($F_{1,30.3} = 10.55$, $P = 0.003$; Figure 11) when controlling for other factors (see Appendix 20). The mean weight of fledglings decreased with brood size ($F_{1, 63.8} = 7.23$, $P = 0.009$; Appendix 22). The interaction between year and event-type was also significant ($F_{2,60} = 9.84$, $P < 0.001$; Appendix 23). There was also a tendency for chicks to be heavier in broods of primary and monogamous females than in those of secondary females ($F_{1,65} < 3.24$, $P = 0.077$).

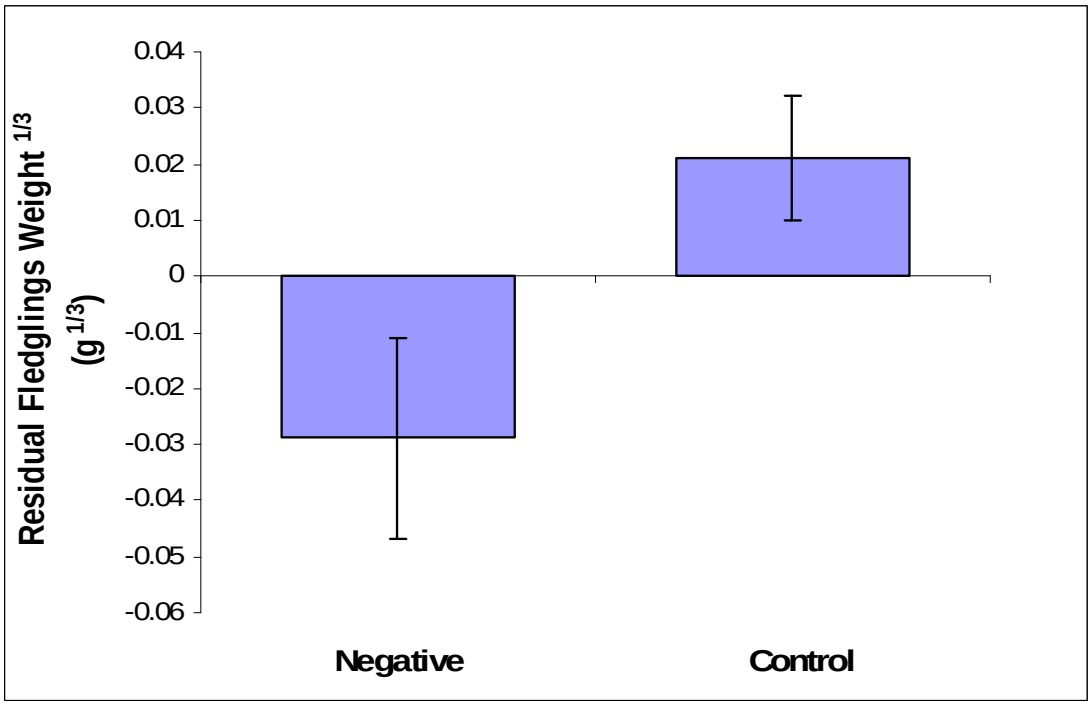


Figure 11. Residuals of fledgling cube-rooted weight obtained from a simplified model taking out the condition of the fathers, but retaining the other variables and significant interactions.

The T-cell immune response of the fledglings was not explained by treatment ($F_{1,63} = 0.10$, $P = 0.747$), or any of the paternal characteristics, brood size, female status or fledgling weight (Appendix 20). The T-cell immune response

differed between the different event-types and the different years of the study: the interaction of event-type and year was significant ($F_{2,63.8} = 5.05$, $P = 0.009$; Appendix 24).

Males of the negative treatment sired in total (from all the events) fewer eggs in their own nest or nests ($F_{1,43.5} = 5.14$, $P = 0.028$; using a Poisson mixed model) than control males. The average numbers of hatchlings and fledglings were slightly higher for control than for negative males (Figure 12), but these differences were not significant ($F_{1,36.1} = 0.35$, $P = 0.560$; and $F_{1,29.5} = 0.55$, $P = 0.465$, for hatchlings and fledglings respectively). The original male TF length and body condition, did not explain number of offspring that they sired (Appendix 25). There was also a significant year effect; males sired fewer hatchlings and fledglings in 2005 than in 2006 ($F_{1,23.6} = 4.74$, $P = 0.04$; and $F_{1,20.6} = 6.34$, $P = 0.02$, respectively; Appendix 26).

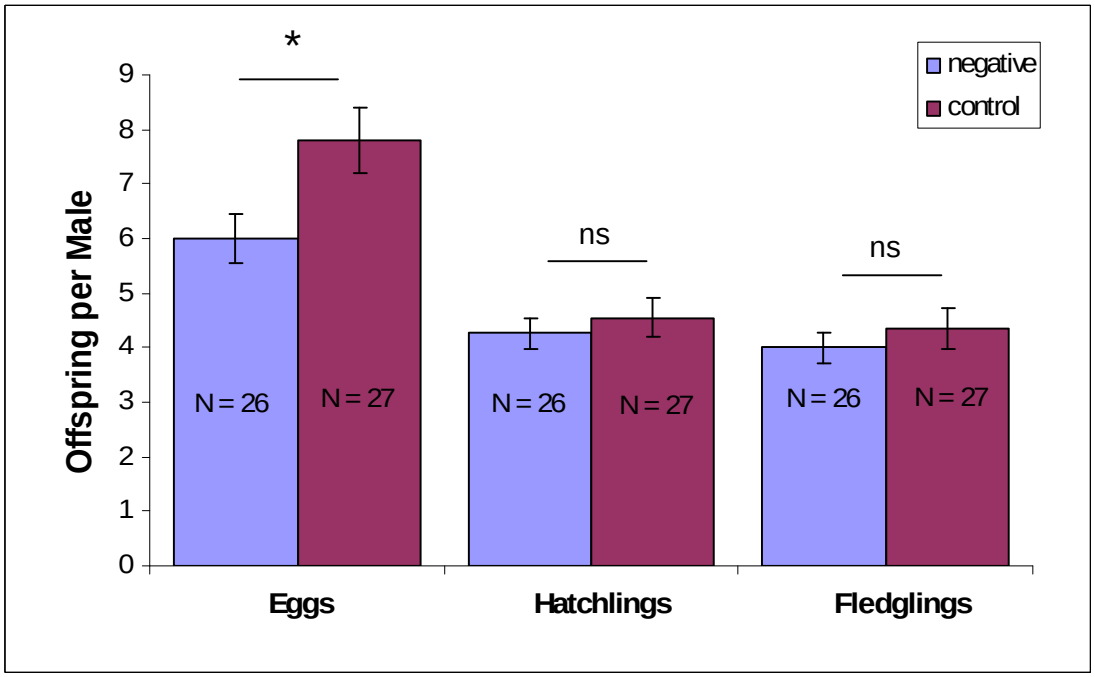


Figure 12. Mean (± s.e.). number of eggs, hatchlings and fledglings per male by treatment: negative treatment (blue), control treatment (purple). The probability values of the differences were calculated using the least squares means of the final model shown in Appendix 25. * $P < 0.05$; and ns $P > 0.05$.

Discussion

Contrary to expectations, our treatment did not have any effect on the probability of males reproducing. According to the correlative study (Chapter 4) males in better condition and with longer TF had a higher probability of reproducing. In the present experiment, male condition was found to be a good predictor of a male's reproducing. However, the length of the TF, either the original length or the experimentally manipulated length, did not predict the probability of reproducing. Additionally, neither the treatment nor the condition or the original TF length had a significant effect on the status of the male (monogamous or polygynous). This analysis included 43 males of which only 8 were polygynous (5 controls and 3 negatives), which limited the power of the test and could have resulted in a lower probability of detecting a significant difference. However, it is also possible that females are evaluating multiple male traits and when we experimentally modified one of these traits, females chose males based in other traits (e.g. song), as they have been reported to do in other species (Marchetti 1998).

Males of the negative treatment did not lose more paternity than controls. This could mean females were not more likely to seek or accept extra pair copulations if paired with a male with reduced feathers. On the other hand, even if females increased their extra-pair behaviour, males could have compensated by increasing mate-guarding or the frequency of copulations.

Similarly, males of the different treatments did not differ in the number of eggs, hatchlings and fledglings that they sired per event. However, there was a tendency for males in better condition to have more fledglings per event. This greater number of fledglings could reflect higher parental care from males in better condition. Events of primary and monogamous females had more eggs and more hatchlings than those of secondary females. The number of fledglings of secondary nests was also lower, but not significantly so. Therefore, primary and monogamous females, invested more in their clutches, laying more eggs, but did not rear significantly more fledglings than secondary females.

Males of the negative treatment did not differ from control males with respect to the weight of the hatchlings that they sired. However, males of the negative treatment sired lighter fledglings than those of the control treatment. A possible explanation is that, if feather length signals resource holding potential, males of the negative treatment would need to display higher levels of nest-box defence activities, thus leading to a lower rate of chick feeding. Additionally, a lower investment from females could also result in lighter chicks. That is, if females that paired with males with reduced TF length, invested less by feeding the chicks less, one would expect them to be lighter. This possibility would be predicted by a differential allocation mechanism (Burley 1988).

The swelling response to PHA of chicks did not differ according to the treatment or the characteristics of their fathers. Immune response was just explained by the event type and the year. The way that the immune response changed throughout the year differs between 2005 and 2006 (Appendix 24) and is completely different to the pattern observed in the correlative study (2004, Chapter 4 Appendix 13). It was expected that the extent to which later events show relatively lower immune response would vary between years as the condition of nestlings will vary with variation in seasonality. However, it was not expected that the gross ranking of the different periods in the breeding season (reflected by event type) would vary between years.

Males of the negative treatment sired significantly fewer eggs than control males. The numbers of hatchlings and fledglings were also slightly lower, but not significantly so. This trend is very similar to the one observed for number of offspring per event. Females of control males seem to have been laying more eggs than those mated with negative males. However, they did not succeed in rearing significantly more hatchlings or fledglings

The treatment does not seem to have a strong effect on the reproductive success of the males. Our failure to detect the effect of the treatment could be partly do to the decrease in polygyny as the colony “matures”. The age of the colony (time since the introduction of nest-boxes in the area) may influence the levels of polygyny, since prior to the installation of the nest-boxes in 2004 the availability of nest sites for this species (natural holes in trees and walls)

was considerably lower. The number of available nest sites in a given year may influence the number of individuals visiting the area the following year. Consequently, during 2004 fewer individuals may have arrived in the area and competed for nest sites than in subsequent years. This may explain why more males managed to defend more than one nest in that year. Forty percent of the males were polygynous in the correlative study in 2004 while, for this experimental study (2005 and 2006), just 18.6% were polygynous. As the level of polygyny decreased, the differences in the reproductive success also decreased, making it more difficult to detect them. Additionally, females could be using other cues to assess the quality of males, like their plumage colour and song complexity; diminishing the effect of the treatment. Furthermore, the message conveyed by multiple traits may be affected if one of the traits is experimentally modified, making the process of mate-selection random. For example, if a female was to see a male singing at a high rate (signalling good condition) but displaying very short feathers (signalling low quality), her evaluation process may be confounded and she may end choosing a mate randomly. This scenario could be further complicated if females show flexibility in their preferences according to the environmental changes or their individual characteristics (age, current condition, etc.). If the intensity and even the direction of preference changes through a female's life, or from year to year as shown in lark buntings (Chaine & Lyon 2008), a much longer study that meticulously controls for other traits will be needed to detect the strength of the selection on a particular trait.

In conclusion, the observational study presented in Chapter 4 shows stronger effects of condition and of TF length on the reproductive success of the males than the effects found in this experimental study. There are several possible explanations for this. The detection of differences in reproductive success may be more difficult due to a decrease in polygyny as the colony in this study area gets older. Females' selection of mates may be based on multiple traits and when the TF length is modified they may rely on other signals. Alternatively, females may exhibit some flexibility in preference depending on their individual condition or the environmental conditions, which may confuse and weaken the effects of selection on this trait. However, even when we did

not find a broad effect of the manipulation of feather length on the reproductive success of males, some effects were detected; fledglings sired by males with shortened feathers were lighter and these males also sired significantly less eggs and less (but not significantly so) hatchlings and fledglings. Therefore, it seems that the length of the TF is under sexual selection and has an effect on the maternal investment in the offspring sired by those males, even though this effect seems to be diluted by the presence of other sexually selected traits.

Chapter 6. Reproductive Success and Male Traits in the Spotless Starling (*S. unicolor*): Concluding Remarks

The general aim of this study was to assess some traits of male spotless starlings for their importance in attaining reproductive success. Specifically, we wanted to test the hypothesis that the length of the throat feathers (TF) is under sexual selection. Since both extra-pair paternity and intra-specific parasitism have been reported to be common in this species (Calvo et al. 2000; Cordero et al. 2003), it is highly likely that calculating the reproductive success of males using just observational data will be inaccurate. Determining the real parentage of the chicks is necessary to obtain a more accurate measurement of reproductive success. Microsatellite markers, the most popular genetic tool used for parentage analysis, were selected to meet this challenge (Jarne & Lagoda 1996; Sunnucks 2000). Therefore, the first step of this thesis was to develop these genetic tools for their use in spotless starling parentage analysis.

We obtained nine highly polymorphic microsatellite markers (Chapter 1). Two of these were isolated in other species, but showed cross-species amplification, while the other seven were isolated directly from the spotless starling. These seven microsatellites also amplify and are polymorphic on European starling. The European starling is one of the most popular models in biological studies, from environmental and physiological studies (Akins et al. 1993; Arena et al. 1999; Arenal & Halbrook 1997; Bentley et al. 2000; Cheng et al. 1999; Congiu et al. 2000; Connors & Nickol 1991; Gautsch et al. 2000; Kumar et al. 2000; Parker & Goldstein 2000; Riters et al. 2001; Vyboh et al. 2001; Ward et al. 2001; Young et al. 2001) to behavioural and sexual selection studies (Adrethausberger 1983; Adrethausberger 1984; Adrethausberger 1988; Adrethausberger 1989; Adrethausberger & Guttinger 1984; Adrethausberger et al. 1989; Adrethausberger et al. 1990; Adrethausberger & Jenkins 1988; Braaten 2000; Buchanan et al. 2003;

Christians 2000; Christians et al. 2001; Christians & Williams 2001; Duffy & Ball 2002; Eens & Pinxten 1990; Eens & Pinxten 1995a; Eens & Pinxten 1995b; Eens & Pinxten 1996; Eens et al. 1989; Eens et al. 1990; Eens et al. 1991a; Eens et al. 1991b; Eens et al. 1992a; Eens et al. 1992b; Eens et al. 1993; Eens et al. 1994; Eens & Verheyen 1988; Gwinner et al. 2000; Gwinner & Schwabl 2005; Reid et al. 2000; Reid et al. 2002; Remage-Healey & Romero 2000; Robinson et al. 2005; Romero & Remage-Healey 2000; Smith & Bruun 2002; Tobler & Smith 2004; Vasquez & Kacelnik 2000). Future studies in the European starling could benefit from the use of these microsatellites.

The genotypes of all the adults captured for this study and their sampled offspring were scored using these 9 microsatellites and subsequently used to obtain the parentage of the chicks with two different analytical programs, NEWPAT and CERVUS. In addition to the genetic data, observational data were used to confirm the results obtained with these programs. The assignment of the parentage in this study was conservative; the failures to assign a parent were sometimes due to adults that were not genotyped, but in other cases a parent may have not been assigned because of the lack of supporting observational evidence.

Some of the markers used were not ideal for their use in parentage analysis. One of these was sex linked and three had a high proportion of null alleles. Recently, a study of spotless starlings (García-Vigón et al. 2008), used six microsatellites, four of which where isolated by Rubenstein (2005) from the superb starling, *Lamprotornis superbus* and are not used in our study (the other 2 were tried and one of them used in our study too). García-Vigón *et al* (2008) did not report the characteristics of these four markers in the spotless starling. If these markers are highly polymorphic and do not have high proportions of null alleles, using them in addition to the markers obtained in this study would further improve the resolution of parentage analysis in this species. Furthermore, should sufficient resolution be acquired with additional markers, the use of observational data could become unnecessary and a greater proportion of the parentage assignments could be made with a high level of confidence in future studies.

Although the results presented here are conservative, the proportion of parentage assigned was high, with 85% of the offspring having at least one parent assigned. The results show that extra-pair paternity and intra-specific brood parasitism were common in *S. unicolor*, with 7.0% of EPP and 7.4% IBP offspring, affecting 19% and 21% of broods, respectively. Quasi-parasitism was also detected, but at a much lower percentage (less than 1% of the offspring). The percentage of EPP offspring reported here is lower than that reported by Cordero et al. (2003). Several explanations for this are discussed in Chapter 3, ranging from the conservative nature of the analysis in this study, to changes between populations and between years on the amount of EPP occurring. Conversely, the level of parasitism found by Cordero et al. (2003) was extremely low compared with the levels found in this study and those reported by Calvo et. al (2000): the reasons I have suggested that may explain this disparity include the lower accuracy of DNA fingerprinting as used in Cordero's study, and of observational data used in Calvo's study, compared with the microsatellite analysis used in this study. However, changes within and between populations may be the main reason for the disparity between the studies.

This parentage analysis also made possible the detection of polygyny. The proportion of polygynous males and the degree of polygyny decreased with year. 48% of the nests were defended by polygynous males in 2004, 39% in 2005 and only 20 % in 2006. Different hypotheses explaining this phenomenon are discussed in chapter 3. We favour the age of the colony hypothesis, which states that the number of individuals visiting an area the following year depends on the number of nests available that year. Therefore, in 2004, the first year in which the nest-boxes were installed, fewer birds visited the area and competed for nest sites than in subsequent years. A similar pattern was observed in another colony near Madrid, where many nest-boxes remained unoccupied during the first year after they were installed: the proportion of occupied nest-boxes increased the next year and almost all of them were occupied in subsequent years (Veiga et al. 2006). It is particularly interesting to see that the decrease in polygyny coincides with an

increase of IBP and QP. Birds may be responding to the increase in competition by adopting these alternative reproductive strategies.

The paternity assignment (Chapter 3) provided the foundation from which the correlative and the experimental studies were conducted (chapter 4 and 5). The aim of the correlative study was to find evidence that some male characteristics are important predictors of male reproductive success. Specifically, this was to establish if the throat-feather (TF) length is a sexually selected trait in the spotless starling. In addition to the TF length, two other metrics were considered. The tarsus length was used as a measurement of size and the body condition of the males was assessed in an attempt to control for other sexually selected traits that may be condition dependent (e.g. song repertoire size, song output and the rate and intensity of display).

The size of the males did not correlate with any of the measurements used to assess male reproductive success. Additionally, none of the male characteristics correlated with offspring quality, measured as the weight of hatchlings or fledglings or T-cell immune response. That is, higher quality males do not seem to produce higher quality offspring (due to either good genes or direct benefits). These results could be reflecting a null or low heritability of quality and a preferred investment in more offspring rather than in better quality offspring. However, the failure to detect a correlation between paternal and offspring quality may be the result of working with a wild population, where a numerous environmental factors are uncontrolled.

Even when no correlations between the quality of the males and their offspring were detected, strong evidence was found for male TF length and body condition being a predictor of the quantity of offspring sired by a male in a given year. Males with higher condition (defined in Appendix 6) and with longer TF were much more likely to reproduce and males with higher condition were also more likely to be polygynous. Additionally, males with higher condition sired more offspring and therefore had a higher reproductive success. The results for the analysis of TF length were similar, with males with longer TF siring more offspring. There were some outliers that could have had disproportionate influence on this analysis. However, the trend was retained when these were removed from the analysis. Since there was no

biological reason to remove these data and the probability of reproducing (versus not reproducing) was predicted by TF length, it seems that the TF length was correlated with reproductive success and, hence, was a trait either under sexual selection itself or correlated with one that was. To demonstrate a trait is sexually selected and not just a correlate of another trait, requires an experimental manipulation of the trait.

Chapter 5 presented an experimental study, manipulating the length of the throat feathers of male spotless starlings was altered and measuring the effect on their reproductive success. To begin with, positive, negative and control treatments were used. The positive treatment was intended to increase the perceived length of the TF by affixing some elongated feathers. However, we quickly found that the attached feathers did not last enough for the treatment to have an effect. Therefore, the positive treatment was suspended in the second year of the experiment and the data from these males were not used. The negative treatment consisted of cutting the feathers of the males to resemble the length of those of females and young males. The TF of males in the control group were not modified.

The results of this experimental study were not as decisive as those of the correlative study. Even though condition remained a good predictor of a male's likelihood of reproducing, the TF manipulation had no effect; males in the negative treatment did not have a lower probability to reproduce. Additionally, neither the treatment nor the condition of the males affected the probability of the males being monogamous or polygynous. Similarly, the extent of paternity loss through extra-pair mating and the number of offspring per brood were not related to the treatment or the body condition of the males. However, males of the negative treatment sired significantly fewer eggs in total from all their nests than control males did, but the numbers of hatchlings and fledglings were not significantly lower. Additionally, males of the negative treatment reared lighter fledglings.

One of the reasons why the effects of the treatments were not as strong as expected from the correlative study, may be that the power of the test was decreased due to the decrease in polygyny as the colony 'matures'. As mentioned previously, the levels of polygyny were higher the first year the

nest-boxes were set out than in consecutive years. Since the correlative study was done in this first year, when the level of polygyny was higher and therefore, the differences in the reproductive success of males may have been greater and easier to detect. For the correlative study, in 2004, 40% of the males were polygynous while, in 2005 and 2006, for the experimental study only 18.6% of the included males were polygynous. The level of polygyny and any differences in the reproductive success decreased and, thus, the power of the experiment to detect these differences was reduced. However, this may not be the only reason why the treatment did not provide as powerful results as expected. Multiple sexually selected characters often appear in a given species, and the spotless starling was no exception. There are several male traits that are thought to be under sexual selection in this species. Females could be using several cues to assess the quality of males, like their plumage colour and song complexity; this diversity of cues diminishing the effect of the treatment which altered just one. Furthermore, the overall message conveyed by multiple traits may be affected if one of the traits is experimentally modified. The female evaluation process may be confounded and females may resort to base their decision in a different trait or even to randomly choosing a mate. Some other confounding factors may arise from environmental changes. Since this study used a wild population, many factors could not be controlled, from weather to the parasite loads, or levels of predation in the different nests. This scenario could be further complicated if females show flexibility in their mate-preference according to environmental changes or the individual characteristics of the female (age, current condition, etc.). This has been shown to occur in lark buntings (Chaine & Lyon 2008), where a change on the intensity and the direction of female preference occurred between consecutive years.

In conclusion, I have presented evidence that the length of the TF is a sexually selected trait, found from the correlative study and, more weakly, from the experimental study. Body condition was also found to be related to the reproductive success of males. Since it is unlikely that the birds could directly assess the body condition of a male, it is probable that other sexually selected traits that correlate with condition may have been used by females

(or competing males) to assess male quality. The use of cues other than the TF length for intra and inter-sexual selection and the decrease in the variability of male reproductive success may have weakened the power of the experimental study. Nonetheless, I propose that the evidence presented in this thesis supports the hypothesis that TF length is under sexual selection, even when its importance relative to other secondary sexual traits still needs to be assessed.

Further work

When planning this study we were rather ambitious and our expectations for the use of the nest-boxes by spotless starlings were exceeded, with all but one of the nest-boxes being occupied. This allowed us to establish a model system in the wild, and to be able to collect many samples and a large body of data, some of which has already been used as part of other studies including studies of: colour of eggs in relation to female characteristics (Lopez-Rull et al. 2007); developmental plasticity in chicks experiencing different levels of competition (Gil et al. 2008); and parent-absence begging in sibling cooperation (Bulmer et al. 2008). Additionally we hope to use other data collected in parallel, to test further hypotheses.

Sex allocation theory (Trivers & Willard 1973) predicts that parents should produce more offspring of the sex with the greater fitness benefits. Differential sex allocation has been reported in *S. unicolor* (Cordero et al. 2001; Polo et al. 2004; Veiga et al. 2008). If the length of the TF is under intra-sexual selection, the sex ratio of broods produced by females mated to males with long TF are expected to be skewed towards males. Using the data collected for this thesis we would be able to test this hypothesis. The primary sex ratio of around 40% of the broods cannot be obtained because some embryos and chicks were not sampled since they disappeared from the nest (due to intra-specific sabotage, predation or parental removal of un-hatched eggs and dead chicks). Nevertheless, the sample may be sufficiently large to test this hypothesis using the correlative and the experimental approach.

Androgen hormone assays will be performed on blood samples, collected from adult males during 2005 and 2006, that are large enough to permit these essays in addition to their use for genotyping. Testosterone levels have been shown to influence male behaviour and reproductive success in this species (Moreno et al. 1999). We intend to test if the length of TF is correlated with the level of androgens and if the androgen levels in our study population correlate with reproductive success.

The TF collected for measurement have also been kept with the intention of measuring wave length reflectance at a later date. The colour of these feathers has proved difficult to measure due to their small size and their iridescent properties. Controlling for the colour of the TF in our analysis may be important. The colouration of general plumage, not just the throat feathers, and the beak colouration are likely to be under sexual selection. From studies of zebra finches (McGraw & Ardia 2003; McGraw & Parker 2006) it has been shown that beak colouration reflects current condition while feather coloration can reflect long-term or over-wintering condition. The need for modern and expensive equipment to study this colouration may have hampered its study in the spotless starling until now. However, the topic appears to have great potential in lending a temporal aspect to the assessment of condition.

A captive study where environmental factors can be controlled and where other male traits such as colouration, song rate and repertoire can be measured could give us a better idea of female preference. However, studies in captivity do not always reflect patterns in the wild. Females may base their choice on different male traits or sexual selection for a trait may change direction according to environmental and social conditions. However, captive studies may be of great importance for studying environmental effects on female preferences. For example, we could hypothesise that females will change their preference for males according to food availability, with monogamous males or males with lower testosterone levels being preferred under low food conditions, since they provide better parental care (Moreno et al. 1999). Conversely, when food is abundant females may prefer highly ornamented, polygynous males. Trying to test this hypothesis in the wild will be hard since food availability will be difficult to manipulate and correlative

studies between rich and poor years will have an open-ended timescale and involve many other confounding variables, from temperature changes, parasite loads, winter survival, competition between males, etc. It is possible to have a much greater control of all such factors in an aviary. So, captive studies are of great value for testing some hypotheses.

Even though our study was of a wild population, we were able to obtain data from most of the adults and offspring in the surroundings and we were able to control many factors (e.g. nest size, laying-date and female status). As a result the correlative and experimental studies provided reliable evidence supporting the hypotheses that male condition correlates with male reproductive success and that the length of the TF is a sexually selected trait. Therefore, this species and this study population are likely to prove a valuable model for similar studies, which would be more difficult in other species where such factors cannot be so practically controlled. Furthermore, studying this population in the wild during three consecutive years allowed us to better understand the population dynamics and the differential use of reproductive strategies, and has also generated many additional questions for future study.

Appendices

Appendix 1. Captured Adults

	2004		2005		2006	
	Males	Females	Males	Females	Males	Females
Captures [a]	74	126	72	51	67	79
Recaptures[b]	-	-	22	15	27	37
No. blood samples[c]	67	116	69	48	66	78

[a] The number of different adults captured within a year. [b] The number of adults that had been captured in previous years and were recaptured. [c] The Number of blood samples taken each year, for some of the captured adults no sample was taken or it was lost or mislabelled.

Appendix 2. Offspring Samples Taken.

	2004	2005	2006	Total
No. of occupied Nests[a]	77	68	69	214
No. of Events[b]	178	149	148	475
No. of chicks[c]	389	286	343	1018
No. of dead chicks[d]	32	8	1	41
No. of embryos[e]	17	110	58	185
No. of samples[f]	438	404	402	1244

[a] The number of nests where at least one egg was laid. [b] The number of reproductive events (reproductive attempts). An event starts with the egg laying and, if completed, ends at fledgling. Both complete and incomplete events were recorded. [c] The number of chicks that reached at least 6 days of age. [d] The number of chicks that died before the 6th day post-hatching and that were found in the nest, making it possible to take a tissue sample. [e] The number of embryos (from un-hatched or biopsied eggs) from which a tissue sample was taken. [f] The total number of offspring (chicks and embryos) from which a tissue sample was taken.

Appendix 3. Offspring Genotyped

Year	Total offspring genotyped [a]	Offspring genotyped for ≥ 5 loci	Offspring genotyped for < 5 loci	Offspring that did not amplify	Loci per individual genotyped [b]
2004	432	384	48	6	7.64 ± 2.02
2005	401	384	17	3	8.2 ±1.45
2006	392	337	55	10	7.49 ± 2.15
Total	1225	1104	120	19	7.88 ± 1.82

[a] The number of chicks and embryos for which at least one locus was successfully amplified. [b] The average and standard deviation of the number of loci amplified per individual.

Appendix 4. Changes of Parents Between Reproductive Events During the Same Year.

Year	Nests with two informative events	Mother changed	Father changed	Both parents changed	Total No. nests with changes
2004	48	13 (27.08%)	1 (2.08%)	13 (27.08%)	27 56.25%
2005	40	6 (15%)	7 (17.5%)	9 (22.5%)	22 55%
2006	28	4 (14.29%)	2 (7.14%)	2 (7.14%)	8 28.57%
Total	116	23 (19.83%)	10 (8.62%)	24 (20.69%)	57 (49.13%)

The number and percentage of nests where the parents (one or both) changed between events during a given year.

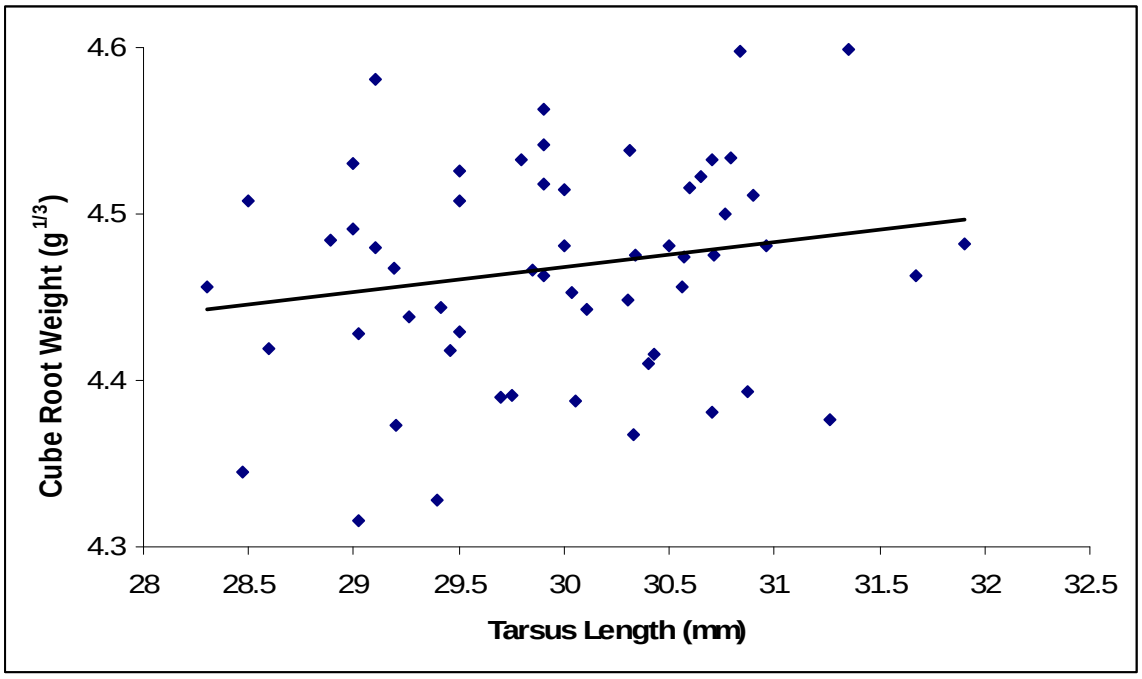
Appendix 5. Parents and Pairs Siring Chicks in One or More Years.

	Fathers	Mothers	Same pair
Parents in only one year	62	63	
Parents in all of the 3 years	18	12	0
Parents in 2004-05	34[a]	38[a]	12
Parents in 2005-06	33[a]	33[a]	7[b]
Parents in 2004,06	19[a]	16[a]	0
Total no. of individual parents	112	125	

The number of males and females with assigned parentage; in just one year; in all of the three years of the study; in consecutive and not consecutive years; and in total. [a] parents identified in all three years are also included in these figures. [b] Two of these pairs shared one male.

Appendix 6. Male Body Condition

Male condition was assessed for all the males captured during the three years of this study using a generalised linear mixed model. The model was nested by subject (since many males were captured in more than one year); the dependent variable was the cube root of the weight of the males while the tarsus length and the year were independent variables. Both variables were significant (tarsus length $F_{1,194} = 5.23$, $P = 0.023$ and year $F_{2,78.1} = 4.58$, $P = 0.013$). The residuals of this regression were then multiplied by a thousand. The resulting figure is the condition value used. This last multiplication was done to make the condition values (residuals) easier to handle during the analyses and easier to graph. This does not alter the value *per se*, just the units. Furthermore, this transformation allows SAS to provide useful odds ratio estimates. The odds ratio estimate is the magnitude by which the probability of the binomial response variable changes when the predictor is increased by one, i.e. the factor by which the probability of reproducing increases when the condition increases by one unit. As the predictor variable here (condition) has (raw) values that range from around –0.06 to 0.07, an increase of 1 is not biologically realistic and yields massive change in the probability of reproducing. SAS does not give the exact estimates of the odds ratios in binomial analyses when they are greater than 1000, as is the case here. Amplifying the residuals by three orders of magnitude solves this problem. The condition calculated for males in one year was used only for the analysis of that year, since male condition is expected to change through time.



This graph shows the increase in weight with body size for males captured in 2004. The metrics used for condition are the distance from the points to the regression line (i.e. the residuals of the regression between the cube root weight and the tarsus length) multiplied by a thousand.

Appendix 7. Pearson Correlation of Male Characteristics

	Pearson Correlation Coefficient	Number of observations	P value
Condition and Tarsus length	-0.05	58	0.707
Condition and TF length	0.243	57	0.073
TF length and Tarsus length	-0.102	74	0.385

There was no correlation between any of the male characteristics (Condition, Tarsus and Throat Feather (TF) length) even before Bonferroni correction. α value after Bonferroni correction was equal to 0.017.

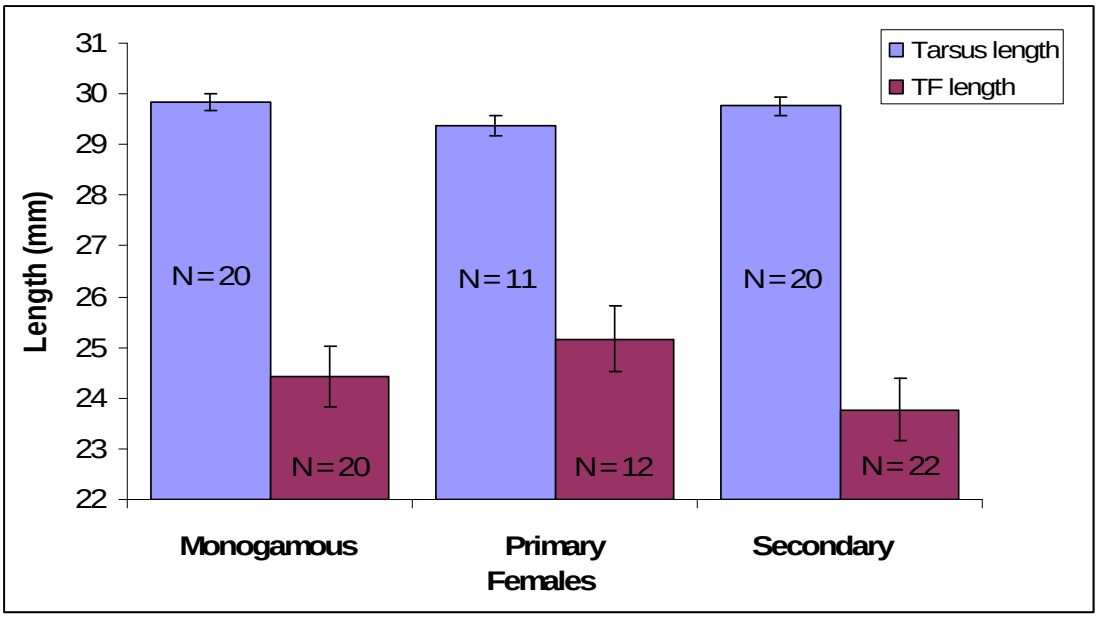
Appendix 8. Detecting Assortative Mating

		Male Condition	Male Tarsus length	Male TF length
Female Tarsus length	Pearson Corr. Coef.	0.319	0.118	-0.269
	No. observations	24	26	27
	P value	0.129	0.559	0.185
Female TF length	Pearson Corr. Coef.	0.127	0.167	0.202
	No. observations	25	28	27
	P value	0.546	0.395	0.313

No correlation was found between any of the females' characteristics and the characteristics of their mates. Only primary and *monogamous females were used for this analysis. (* Females that mated with monogamous males are referred to as monogamous females). α value after Bonferroni correction was equal to 0.008.

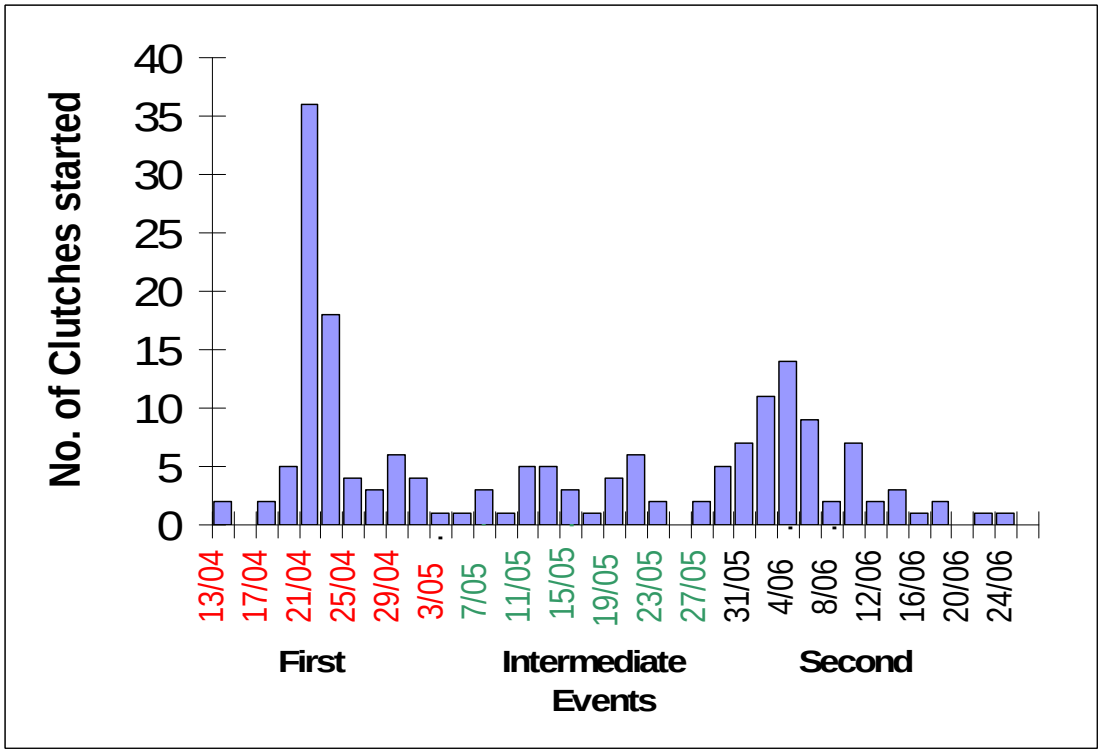
Appendix 9. Characteristics of Female with Different Reproductive Status

There were no differences in size (tarsus length; $F_{2,48} = 1.32$, $P = 0.276$) or in throat feather (TF) length ($F_{2,51} = 1.09$, $P = 0.343$) of *monogamous, primary and secondary females. (* Females that mated with monogamous males are referred to as monogamous females even when these females could have consecutive broods with different males).



The graph shows the means ± s.e. of the tarsus (blue) and TF (purple) lengths of monogamous, primary and secondary females.

Appendix 10. Event Type



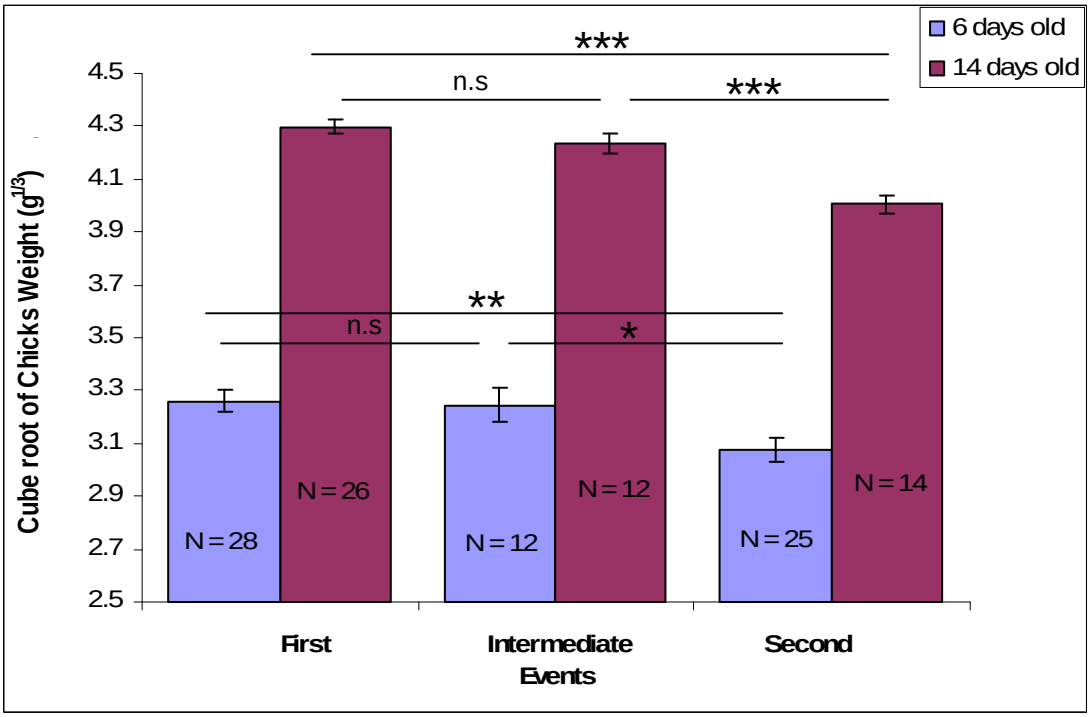
This histogram shows the distribution in time of the individual events (clutches) during the breeding season of 2004. Events began during the period of 13th April to 24th June. Each histogram bin contains two days. The first peak (dates in red) corresponds to the First events and the last one (dates in black) to the Second events. All the events that are in the middle (dates in green) are treated as Intermediate (or replacement) events.

Appendix 11. Models Testing for Predictors of the Number of Eggs, Hatchlings and Fledglings per Event

Dependent Variable = number of eggs per event				
Effect	**Num. df**	**Den df**	**F value**	**P value**
Tarsus length	1	63	0.34	0.561
TF length	1	63	0.46	0.501
Condition	1	63	2.47	0.1214
Event kind	2	63	1.16	0.321
Female kind	1	63	0.30	0.586
Dependent Variable = number of hatchlings per event				
Effect	**Num. df**	**Den df**	**F value**	**P value**
Tarsus length	1	63	<0.01	0.976
TF length	1	63	0.08	0.778
Condition	1	63	0.01	0.929
Event kind	2	63	1.77	0.178
Female kind	1	63	1.32	0.254
Dependent Variable = number of fledglings per event				
Effect	**Num. df**	**Den df**	**F value**	**P value**
Tarsus length	1	63	0.02	0.900
TF length	1	63	0.78	0.380
Condition	1	63	0.16	0.691
Event kind	2	63	3.42	**0.039**
Female kind	1	63	3.78	**0.056**

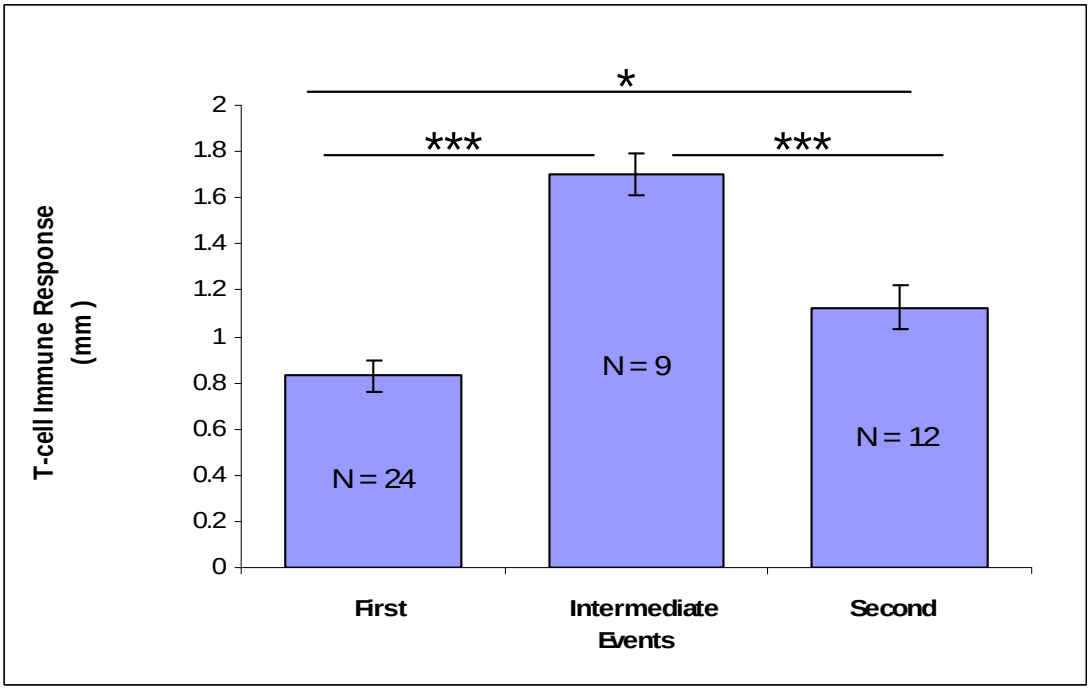
The table shows the results obtained for the fixed effects of three different mixed models (GLMM). Models assumed a Poisson distribution and were nested by male. The total number of observations used was 70. Throat feathers are abbreviated to TF, the numerator and denominator degrees of freedom are abbreviated to Num df and Den df respectively. Significant effects are indicated with their P values in bold.

Appendix 12. Chicks' Mean Weight on First, Intermediate and Second Events



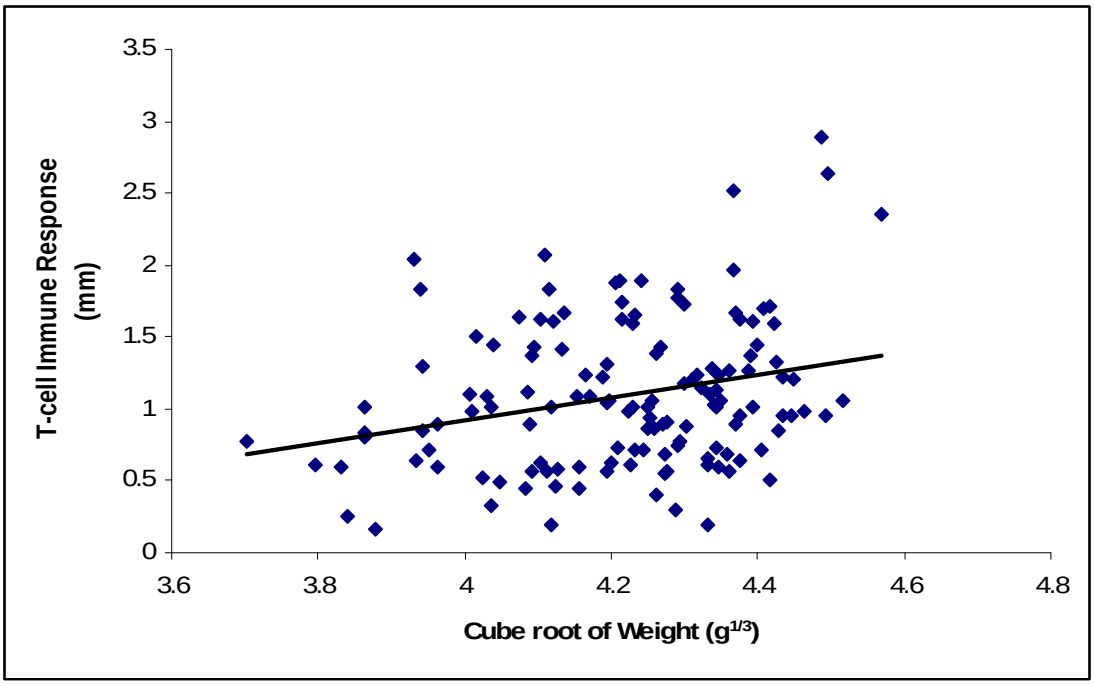
The graph shows the mean ± s.e. of the average weight (cube root transformed) per brood of chicks at day 6 (blue) and 14 (purple) of age, for first, intermediate and second events. The probability values of the differences were calculated using the least squares means of the final model. Levels of significance indicated are: n.s. = not significant; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$.

Appendix 13. Mean T-cell Response on First, Intermediate and Second Events



The graph shows the mean ± s.e. of the average per brood of T-cell response for first, intermediate and second events. The probability values of the differences were calculated using the least squares means of the final model. Levels of significance indicated are: * P < 0.05; *** P < 0.0001.

Appendix 14. Mean T-cell Response and Chick Weight



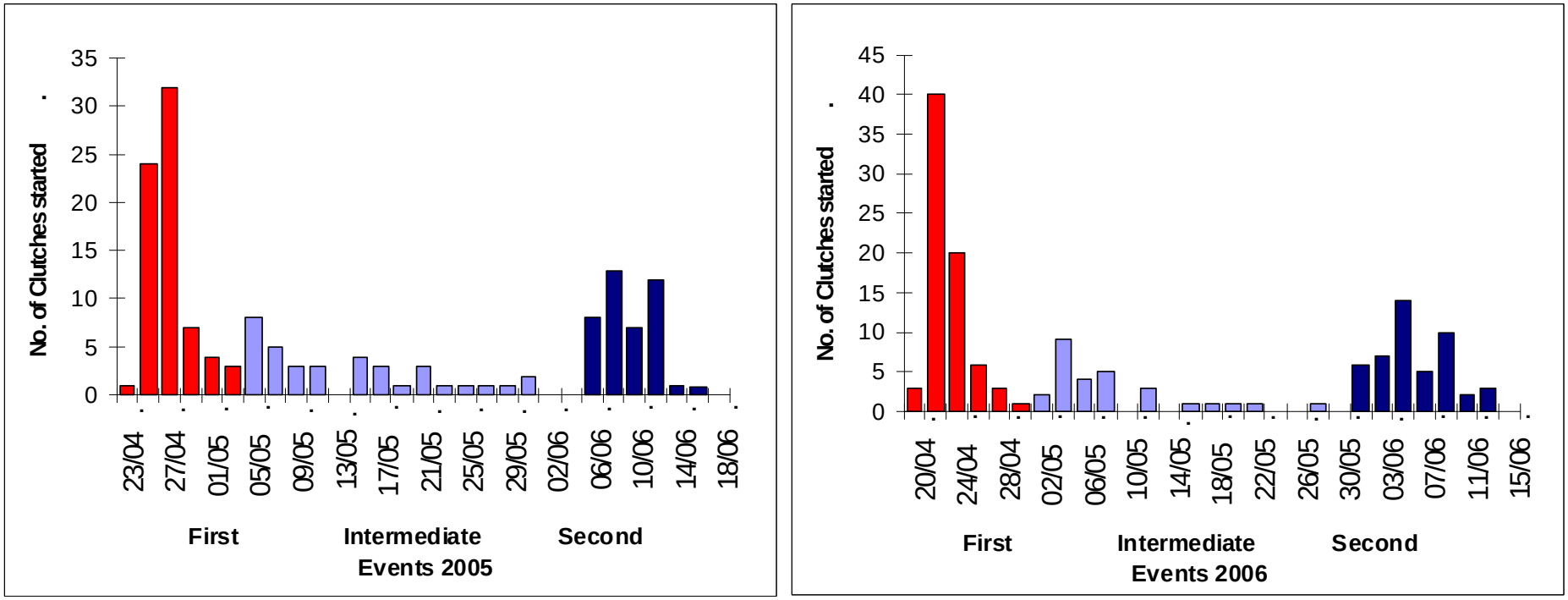
In this graph the mean (cube-root transformed) weight of fledglings per brood is plotted against the mean T-cell immune response per brood; showing an increase in the immune response with increasing chick weight.

Appendix 15. Detecting Assortative Mating

Dependent variable		Treatment	Male Condition	Male TF length	Reproductive Tactic	year
Female TF length	F value	0.12	0.81	0.15	<0.01	0.76
	Num df.	1	1	1	1	1
	Den df.	26.6	25.1	9.92	6.07	6.02
	P value	0.728	0.378	0.707	0.958	0.417
Female Tarsus length	F value	1.20	0.13	3.08	0.01	0.16
	Num df.	1	1	1	1	1
	Den df.	32	32	32	32	32
	P value	0.282	0.579	0.089	0.936	0.690

No relationship was found between the female tarsus and TF lengths and the treatment group, condition, TF length, the reproductive tactic (monogamy vs. polygyny) of their mates or the year. Only primary and *monogamous females were used for this analysis. (* Females that mated with monogamous males are referred to as monogamous females).

Appendix 16. Event Type



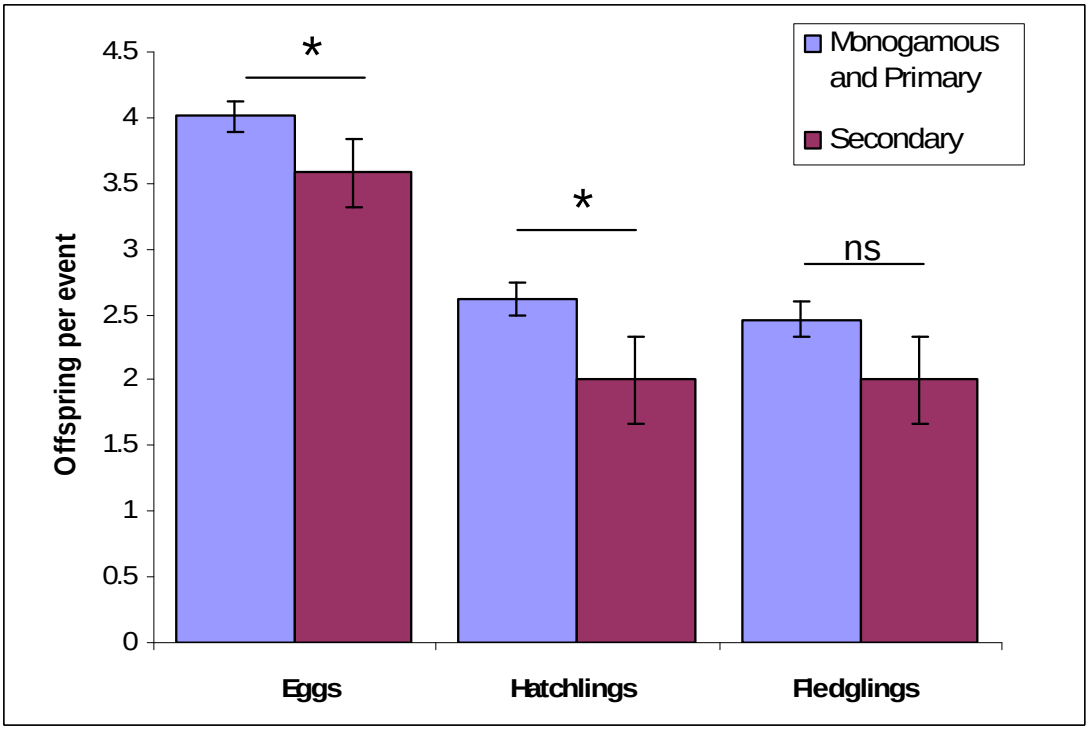
The histograms show the distribution of events (clutches) by their start date in 2005 (left) and 2006 (right), with start-date intervals of 2 days (width of histogram bins). The first peak (red) corresponds to the First events and the last one (dark blue) to the Second events. All the events in the middle (light blue) are considered to be intermediate (or replacement) events.

Appendix 17. Offspring per Event

Dependent variable		Treatment	Male Condition	Male TF length	Female Status	Event type	Year	Interaction event*year
Eggs per Event	**F value**	0.03	1.34	0.04	4.53	2.30	1.47	n.a.
	Num df.	1	1	1	1	2	1	n.a.
	Den df.	80	80	80	80	80	80	n.a.
	P value	0.871	0.251	0.837	**0.036**	0.107	0.228	n.a.
Hatchlings per Event	**F value**	0.71	2.99	0.56	4.00	1.76	9.45	4.80
	Num df.	1	1	1	1	2	1	2
	Den df.	78	80	78	80	80	80	80
	P value	0.403	0.088	0.459	**0.049**	0.178	**0.003**	**0.011**
Fledglings per Event	**F value**	0.15	3.29	0.30	2.22	5.75	11.61	3.88
	Num df.	1	1	1	1	2	1	2
	Den df.	76	76	76	76	76	76	76
	P value	0.701	0.074	0.589	0.140	**0.005**	**0.001**	**0.025**

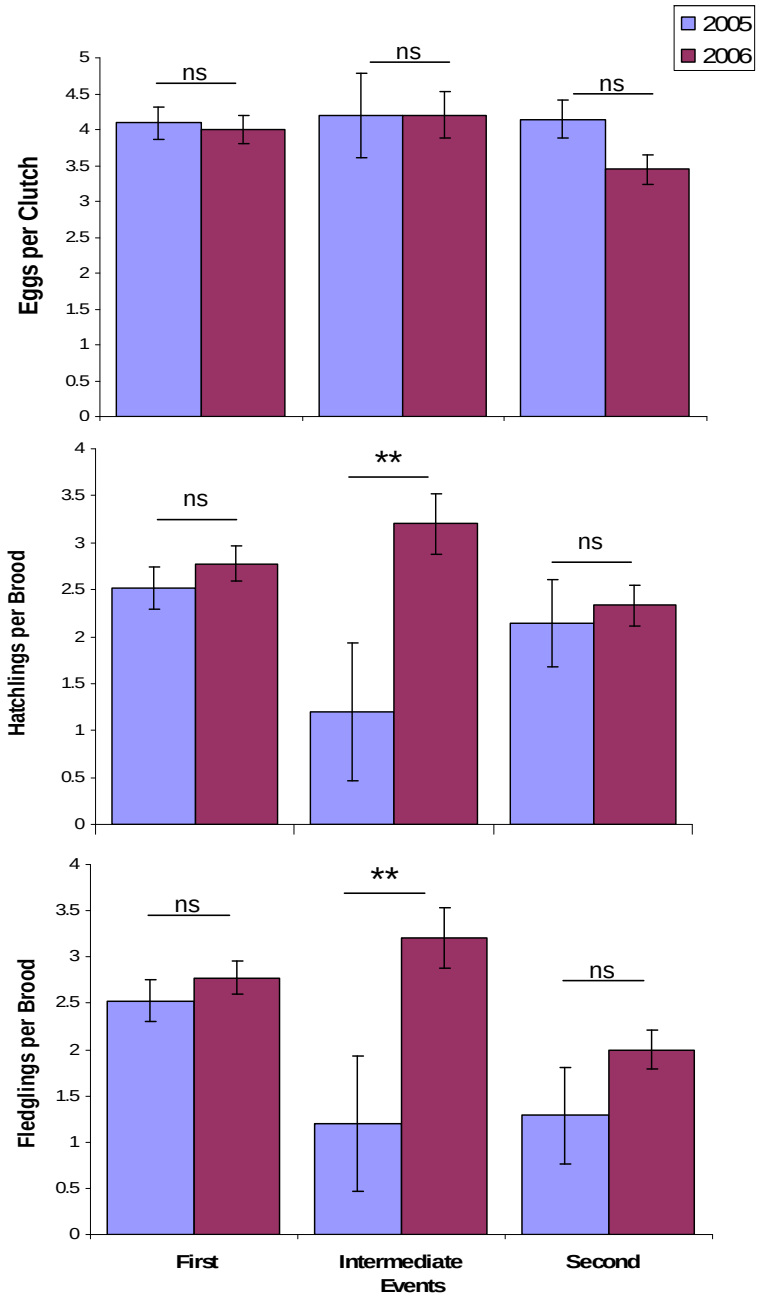
This table shows the results for the fixed effects of three different mixed models (GLMM). Models assumed a Poisson distribution and were nested by male. Abbreviations in the table are as follows: TF = Throat feathers; Num df. and Den df. = the numerator and denominator degrees of freedom, respectively; Non applicable = n.a. (used if final model did not include the interaction). Significant effects are indicated with their P values in bold.

Appendix 18. Female Status and Number of Offspring



This graph shows the mean (± s.e.) number of eggs, hatchlings and fledglings per event, for monogamous and primary females (blue) and for secondary females (purple). The probability values of the differences were calculated using the least squares means of the final model shown in Appendix 17. Levels of significance indicated are: * $P < 0.05$; and ns = $P > 0.05$

Appendix 19. Offspring per Event and Year



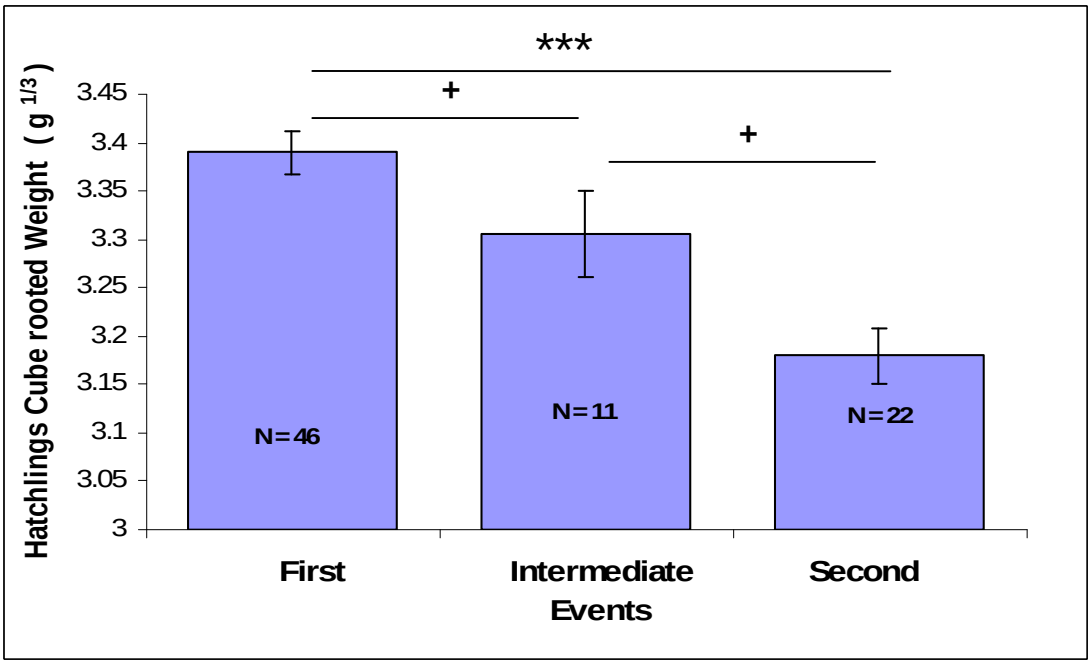
The graphs show the mean (± s.e.) number of eggs (top), hatchlings (middle) and fledglings (bottom) per event, in the first, intermediate and second events in 2005 (blue) and 2006 (purple). The probability values of the differences were calculated using the least squares means of the final model shown in Appendix 17. Levels of significance indicated are: ** $P < 0.005$; * $P < 0.05$; and ns = $P > 0.05$.

Appendix 20. Offspring Quality

Dependent variable		Treatment	Male Condition	Male TF length	Female Status	Brood size	Event type	Year	Interaction event*year	Fledglings Weight $^{1/3}$
Hatchlings Weight $^{1/3}$	**F value**	<0.01	0.76	2.26	4.53	0.22	14.49	1.76	n.a.	n.a.
	Num df.	1	1	1	1	1	2	1	n.a.	n.a.
	Den df.	46.7	61.2	44.4	69	69	65.9	64.1	n.a.	n.a.
	P value	0.980	0.388	0.140	0.934	0.64	**<0.001**	0.386	n.a.	n.a.
Fledglings Weight $^{1/3}$	**F value**	10.55	0.45	0.61	3.24	7.23	63.02	18.26	9.84	n.a.
	Num df.	1	1	1	1	1	2	1	2	n.a.
	Den df.	30.3	46.2	29.4	65	63.8	58	64.7	60	n.a.
	P value	**0.003**	0.507	0.440	**0.077**	**0.009**	**<0.001**	**<0.001**	**<0.001**	n.a.
T-cell Immune Response	**F value**	0.10	0.64	0.65	2.04	<0.01	4.93	6.86	5.05	0.87
	Num df.	1	1	1	1	1	2	1	2	1
	Den df.	63	63	63	63	63	63	63	63	63
	P value	0.747	0.425	0.423	0.158	**0.993**	**0.010**	**0.011**	**0.009**	0.355

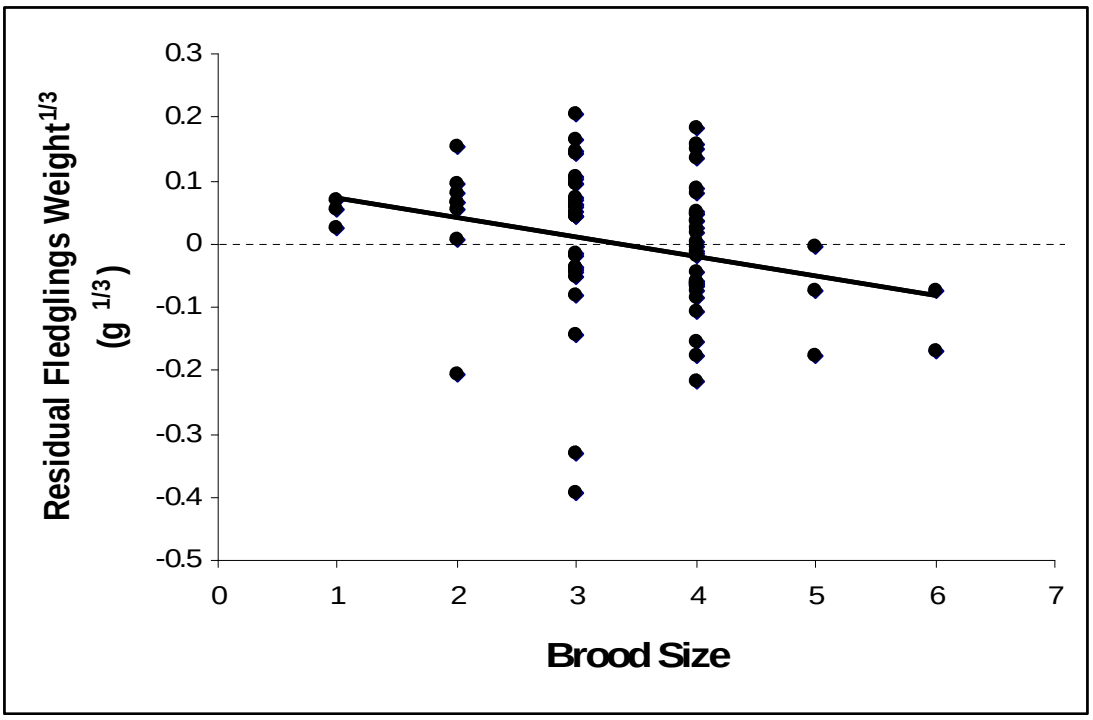
This table shows the results for the fixed effects of three different mixed models (GLMM) nested by male. Throat feathers are abbreviated to TF, the numerator and denominator degrees of freedom to Num df. and Den df. respectively and non applicable to n.a. (used if final model did not include the interaction). Significant effects are indicated with their P values in bold.

Appendix 21. Hatchling Weight and Event Type



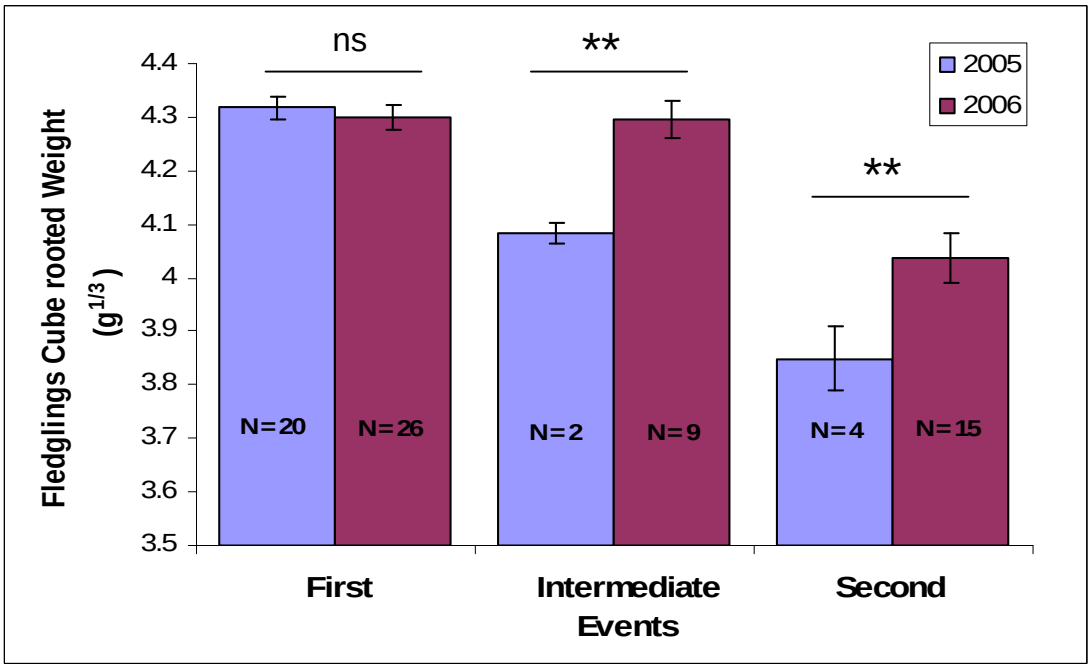
This graph shows the mean (± s.e.) cube-root transformed weight of hatchlings in the first, intermediate and second events. The probability values of the differences are calculated using the least squares means of the final model shown in Appendix 20. The levels of significance are indicated as follows: *** P < 0.0001; and + P = 0.072.

Appendix 22. Fledgling Weight and Brood Size



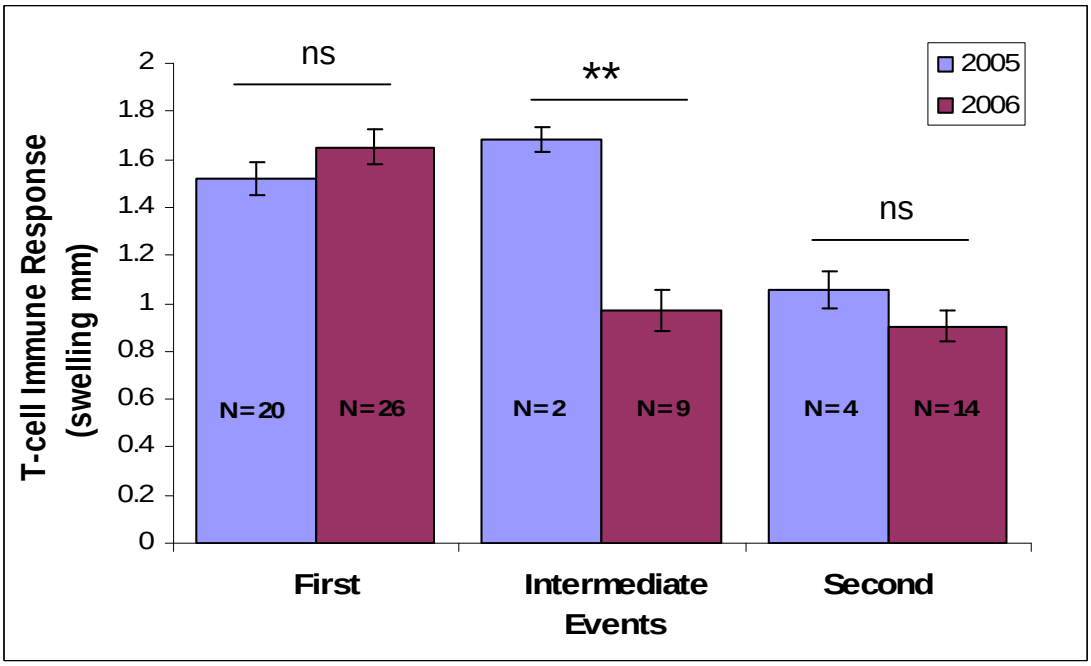
The residuals of fledgling cube-root transformed weight obtained from a simplified model without brood size included as a factor, but retaining the rest of the variables. The original model and all the variables are shown in Appendix 20.

Appendix 23. Fledgling Weight and Event Type per Year



This graph shows the mean (± s.e.) cube-root transformed weight of fledglings in the first, intermediate and second events of 2005 (blue) and 2006 (purple). Sample size is shown inside the bars. The probability values of the differences were calculated using the least squares means of the final model shown in Appendix 20. Levels of significance are indicated as follows: ns = P > 0.1 and * P < 0.05.

Appendix 24. T-cell Immune Response and Event Type per Year



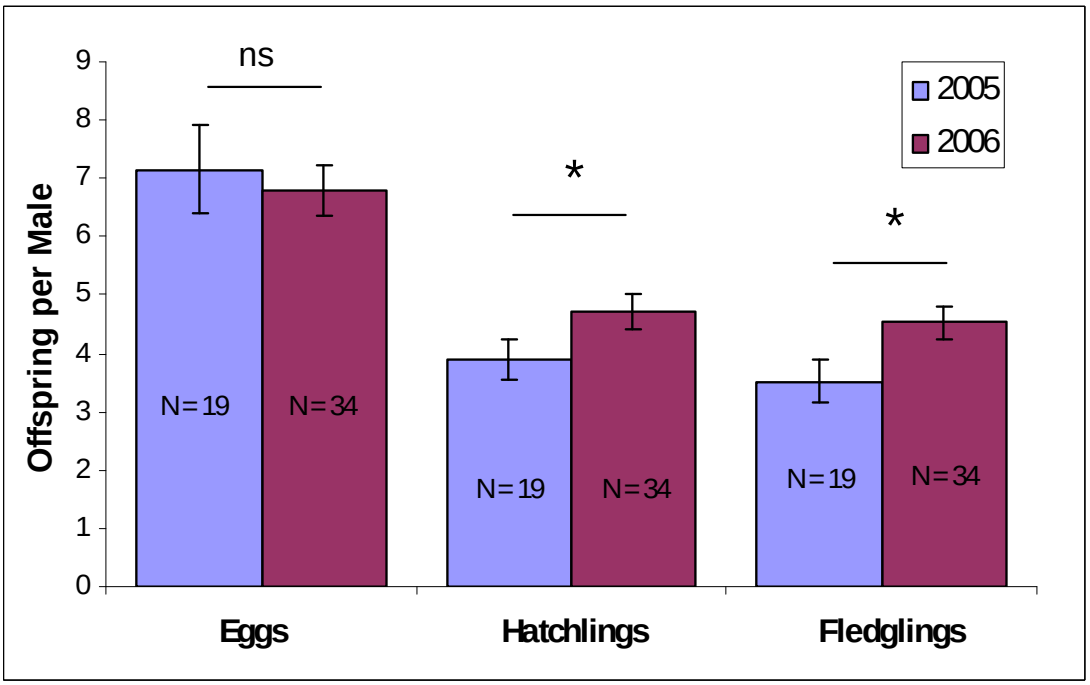
This graph shows the mean (± s.e.) swelling response to phytohaemagglutinin (T-cell immune response) of fledglings in the first, intermediate and second events of 2005 (blue) and 2006 (purple). Sample size is shown inside the bars. The probability values of the differences were calculated using the least squares means of the final model shown in Appendix 20. Levels of significance are indicated as follows: ns = P > 0.1 and ** P < 0.005.

Appendix 25. Total Offspring per Male

Dependent variable		Treatment	Male Condition	Male TF length	year
Total No. of Eggs per Male	**F value**	5.14	0.26	1.37	0.01
	Num df.	1	1	1	1
	Den df.	43.5	41.8	46.6	26.2
	P value	**0.028**	0.616	0.247	0.913
Total No. of Hatchlings per Male	**F value**	0.35	1.17	0.50	4.74
	Num df.	1	1	1	1
	Den df.	36.1	25.9	42.3	23.6
	P value	0.560	0.289	0.485	**0.040**
Total No. of Fledglings per Male	**F value**	0.55	2.22	0.39	6.34
	Num df.	1	1	1	1
	Den df.	29.5	23	44.2	20.6
	P value	0.465	0.150	0.534	**0.020**

The table shows the results obtained for the fixed effects of three mixed models (GLMM) for total number of eggs, hatchlings and fledglings sired by a male per year. Models assumed a Poisson distribution and were nested by male. Treatment was only significant for the total number of eggs. In 2005 the average number of hatchlings and fledglings per male was lower than in 2006. Throat feathers are abbreviated to TF, the numerator and denominator degrees of freedom are abbreviated to Num df. and Den df, respectively, and significant effects are indicated with their P values in bold.

Appendix 26. Total Offspring per Year



This graph shows the mean (± s.e.) number of eggs, hatchlings and fledglings per male in 2005 (blue) and 2006 (purple). The probability values of the differences were calculated using the least squares means of the final model shown in Appendix 25. Levels of significance are indicated as follows: ns = P > 0.05 and * P < 0.05.

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