FACTORS AFFECTING NESTLING GROWTH IN THE COLLARED FLYCATCHER (*FICEDULA ALBICOLLIS*)

PhD Thesis

by

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1. INTRODUCTION

One of the most important questions for iteroparous species is that how much to invest into current reproduction so that they have enough energy for self-maintenance, survival and future reproduction. Environmental and social conditions, furthermore the actual health status of the individuals can all have strong effect on the availability of resources which may affect the investment between and within reproductive events. Birds offer an exciting model system to study reproductive allocation, since prenatal development of embryos is separated both from their mother and their siblings. Differential allocation before birth is thus possible only during egg formation. After hatching, females are further able to alter their parental investments, however, in many species males also feed their offspring thus have possibilities to interact.

1.1. Environmental constraints in reproductive investment

It has been demonstrated that reproductive investment has serious energetic costs, which in turn affect also the survival chances of the parents. During egg laying 35-60% of the daily energy intake of the females is allocated for egg formation, while their protein ingestion is increased by 86-230% (Ojanen 1983, Robbins 1983). In addition, the energy requirements of incubation and feeding of the young are also large. It has been shown that incubating females spend 20-30% more energy than non incubating individuals (Williams 1996), while the basic metabolic rate of the parents during chick rearing is three-four times larger than those individuals which do not care for their young (Clutton-Brock and Godfray 1995, Nilsson 2002).

Environmental conditions have been shown to affect energy availability to the parents. In warmer weather the activity of flying insects is increased (Taylor 1963, Bryant 1975), while the availability of insect prey decreases during cold days. In addition, under unfavourable weather conditions females need to allocate more energy also to their own thermoregulation, which further reduces the amount of energy available for reproduction. Both direct (Otto 1979) and indirect measures (daily average temperature (Haftorn 1986) or rainfall (Ludvig 1993)) of food availability during egg formation were shown to affect females' reproductive investment into their clutch. These findings were supported by a study on the Collared Flycatcher (*Ficedula albicollis*), where females laid smaller eggs in years with cold temperature during egg formation (Hargitai et al. 2005). This suggests that nutrition

investment into the eggs was constrained by food availability also in this species. This may have strong fitness consequences on nestling growth and survival.

1.2. Adaptive decisions in reproductive investments

Adaptive decisions may also have an important role in shaping the reproductive investment of the animals. For example females mated to a high quality male or a mate carrying "good genes" may invest more into the current reproduction to increase the probability of male retention (*differential allocation hypothesis*, Burley 1988) or because the estimated reproductive success of young inheriting "good genes" will be higher (Sheldon 2000). However, reproductive value of the young may differ not only between but also within reproductive events. For instance females often start to incubate their eggs before clutch completion. Thus nestlings from last laid eggs hatch later and often remain smaller throughout the nestling period. This is because last laid eggs often act as insurance in the case of hatching failure occurring in the clutch (Clifford and Anderson 2001). Nestlings from last laid eggs may also act as surplus, which can be reared under favourable circumstances but can be sacrificed if environmental conditions deteriorate (Lack 1954). In these cases, less investment into last laid offspring is expected. However, if the adaptive value of certain young is expected to be higher, e.g. because females cuckold their social mate with a higher quality male or the survival probability of one of the sexes is expected to be better, preferential investment in the more valuable young can be advantageous.

1.2.1. Hatching asynchrony

Females in many bird species start to incubate their eggs before clutch completion. This results in hatching asynchrony and in turn a pronounced size hierarchy among nestlings. As a result later hatching offspring experience competitive disadvantages compared to their nestmates. Many hypotheses have been proposed to explain the adaptive function of this phenomenon (reviewed in Nilsson 1993, Stenning 1996).

Some of the hypotheses suggest that hatching asynchrony acts as a tool by which females are able to influence the survival probabilities of their young. The *brood reduction hypothesis* (Lack 1954), for example, predicts that hatching asynchrony is advantageous in unpredictable environments. When food is abundant, all nestlings can fledge independent of hatching order. However, in cases of food-shortage, older nestlings outcompete their younger siblings and

consequently, younger ones might quickly starve to death. By sacrificing the smallest nestlings, the rest of the brood can survive and fledge in better condition. This confers benefits both to the surviving young and the parents, since fledglings in better condition might survive better (Pettifor et al. 2001) and therefore increase the fitness of their parents more than fledglings in poorer condition. The *sibling rivalry reduction hypothesis* (Hahn 1981) predicts that in broods with an already established size hierarchy among nestlings (due to hatching asynchrony), sibling competition and thus energy expenditure of the nestlings is reduced. This results in faster growth or better body condition than in synchronous broods. Furthermore, the pronounced age hierarchy among siblings may reduce the peak energetic costs of the parents when feeding their young, since nestlings reach their maximum growth rate and thus the highest food demand at different times (*peak load reduction hypothesis*: Hussell 1972).

Another group of hypotheses argues that hatching asynchrony is only a by-product of starting the incubation before clutch completion, which is adaptive for reasons other than establishing sibling size asymmetry. If there is heavy nest predation or food resources are strongly declining during the chick-rearing period, by starting the incubation before clutch completion females can shorten the average time that offspring spend in the nest (i.e. the combined length of the egg and nestling phase). Thus females can reduce the risk of predation on their broods and prevent starvation at least of those nestlings which hatch and thus fledge earlier (*nest failure hypothesis*: Clark and Wilson 1981; *hurry-up hypothesis*: Hussell 1972). According to the *egg viability hypothesis* (Arnold et al. 1987, Veiga 1992) females start to incubate before completing their clutches in order to protect the hatchability of their eggs, since the viability of unincubated eggs may decline with time.

When looking specifically at the effects of hatching asynchrony on the last hatched nestlings, it is often found that the size handicap with which these nestlings start their life results in disadvantages when competing for food (Ostreiher 1997, Pettifor et al. 2001). Thus they might fledge with a smaller weight (Cotton et al. 1999, Clotfelter et al. 2000) and experience a lower survival probability later in life (Oddie 2000). Therefore in species where size asymmetry among siblings is only a by-product of earlier onset of incubation, compensation for the detrimental effects of hatching asynchrony by laying larger eggs at the end of the laying sequence is beneficial (Howe 1976, Hillström 1999, Rutkowska and Cichoń 2005). However, if females follow a brood reduction strategy or sibling size hierarchy is adaptive among nestlings, allocating less nutrients into the last laid eggs can be advantageous (Slagsvold et al. 1984, Williams et al. 1993, Schwabl et al. 1997).

1.2.2. Egg components and offspring fitness

Several studies have demonstrated that egg size reflects the lipid and/or protein content of the eggs (e.g. Meathrel and Ryder 1987, Williams 1994, Hill et al. 1995, Royle et al. 1999, Jager et al. 2000, Badzinski et al. 2002, Reynolds et al. 2003). Thus, egg size has serious consequences on the growth and survival of developing embryos. Nestlings from larger eggs are structurally larger, have more resource reserves, grow more quickly and have higher survival probabilities (Parsons 1970, Williams 1994, Blomqvist et al. 1997) therefore nestlings would clearly benefit from hatching from large eggs. However, both environmental conditions and adaptive decisions (Burley 1988) shape egg size. For example, in the Mallard (*Anas plathyrynchos*) and the Zebra Finch (*Taeniopygia guttata*), females laid larger eggs when mated to high quality males (Cunningham and Russell 2000, Rutstein et al. 2004) and as a result, produced offspring of higher survival prospects when paired with more attractive males. By producing eggs of different size females may adaptively manipulate the level of sibling competition in the brood (Howe 1976, Clark and Wilson 1981, Slagsvold et al. 1984). This was discussed above.

Though the main sources generating phenotypic differences within broods are hatching asynchrony and egg size, there are other egg components which may have serious fitness consequences. Carotenoids can act as antioxidants (Edge et al. 1997), protecting tissues of bird embryos from the attack of free radicals, which are the by-products of rapid oxidative metabolism (von Schantz et al. 1999) and they may also stimulate and regulate immune response through various mechanisms (Bendich 1989). It was found in the Barn Swallow (*Hirundo rustica*) that nestlings hatching from eggs with higher carotenoid concentration had better cellular immune response (Saino et al. 2003), while results on Blue Tits (*Parus caeruleus*) showed that higher carotenoid level in the eggs helped the maturation of the immune system (Biard et al. 2005). Hatching and fledging success of Zebra Finch nestlings were also positively related to the amount of carotenoids in the egg (McGraw et al. 2005). However, carotenoids are also a limiting resource in natural environment (Olson and Owens 1998) thus females may incur significant costs by allocating carotenoids into their eggs. The amount of carotenoids allocated into the egg yolk depends on the availability of carotenoid rich food on the breeding territory (Blount et al. 2002a, b) and also on the own need of the females (Thompson et al. 1997).

Mothers can influence the survival chances of their offspring also by allocating various immune components into their eggs providing passive defence against diseases in the first

days of nestlings' life, since the immune system of the chicks is underdeveloped upon hatching (Gasparini et al. 2001) but it may also have long-lasting effects on the offspring's own immune system (Grindstaff et al. 2006, Reid et al. 2006). Egg white contains an antibiotic component, the lysosime, and also immunoglobulin A and M, while the yolk contains immunoglobulin G (Apanius 1998, Grindstaff et al. 2003). Since producing these components is probably costly to the females it is not surprising that immunoglobulins in the eggs were found to be correlated with body condition or health status of the mothers (Blount et al. 2002a, Buechler et al. 2002) and were also affected by mate quality (Saino et al. 2002).

Beneficial effects of yolk testosterone on nestling development have also been shown for many species. Testosterone increases the competitive ability (Schwabl 1993, Lipar and Ketterson 2000) and the development of nestlings (Schwabl 1996, Eising et al. 2001). Thus elevated testosterone might increase also the survival prospects of the young. In asynchronously hatching species it is often found that females invest testosterone differentially into later laid eggs thus enhancing or reducing the survival probabilities of later hatching offspring (Schwabl et al. 1997, Eising et al 2001). Female Zebra Finches laid eggs containing more testosterone when mated to attractive males (Gil et al. 1999) which can be interpreted as preferential allocation into more valuable broods. While a study on the Collared Flycatcher found that females allocated more testosterone into clutches of subadult males, which may be interpreted as a "help" to nestlings of inexperienced, young males (Michl et al. 2005).

1.2.3. Adaptive decisions after hatching

Food allocation patterns may be the outcome of nestling competition (Ostreiher 1997, Viñuela 1999) and/or the result of active parental decisions (Lyon et al. 1994, Kölliker et al. 1998). The importance of these two factors may vary considerably among species, but if parents have any control over food allocation, they are expected to base their decision on the need of their young (Godfray 1991), while also taking into account the possible costs and benefits of rearing an individual offspring (Kilner and Johnstone 1997). Therefore after hatching of the chicks, females may either continue to support the nestlings already preferred by early maternal effects, e.g. by unevenly distributing the food, or they may alter their preference according to environmental changes during the incubation period. Feeding the chicks, however, is not exclusively the task of the females in most bird species, therefore male parents also have the opportunity to influence the survival and reproductive prospects of their

young. Paternal preferences may be in agreement with maternal preferences, however conflict could arise over food allocation if certain young increases the fitness of one parent more than the other (e.g. extra-pair nestlings increase maternal, but not paternal fitness).

1.2.4. The role of mate choice in offspring quality

Mothers can increase their reproductive success also by choosing high quality males as partners based on the expression of their secondary sexual characters (e.g. by inheriting good genes). However, the question arises how females can assess the quality of their prospective mates through their secondary sexual characters. With other words, how the physiological status/genetic quality of the males are reflected through their secondary sexual characters. According to the Hamilton and Zuk (1982) hypothesis the expression of male secondary sexual characters may be limited by blood parasites. Thus males with elaborate secondary sexual characters are presumably less parasitized. This may enable females to assess the quality of their prospective mates based on signal quality and to choose mates resistant to blood parasite infections, thereby increasing their own reproductive success. Several studies have supported the predictions of this hypothesis and showed that parasites had indeed negative effects on the expression of secondary sexual characters (Milinski and Bakker 1990, Thompson et al. 1997, Figuerola et al. 1999, Spencer et al. 2005), and in some studies females were found to choose more ornamented mates that are less parasitized (Kennedy et al. 1987; Wiehn et al. 1997). This is advantageous for several reasons. Offspring of less parasitized males might inherit resistance genes to parasitic infections (Barber et al. 2001, Langefors et al. 2001, Lohm et al. 2002) or these nestlings just simply grow better because their fathers can provide better parental care (Buchanan et al. 1999). Some other studies, which did not investigate parasite infection in the birds, also found that males with elaborate ornamentation provided more food during courtship (Hill 1991), had better territories (Keyser and Hill 2000) and proved to be better parents when rearing their young (Linville et al. 1998, Buchanan and Catchpole 2000). Therefore the nestlings of attractive males grow (Petrie 1994) and survive better (Norris 1993, Hasselquist et al. 1996) and also have better reproductive success than those of less ornamented individuals (Hill 1991).

If during social mate choice females fail to find a good quality male then they can still increase the quality of their young by extra-pair copulations. Extra-pair copulations can result in direct (e.g. parental investment in the offspring by the extra-pair mate) or indirect benefits for the females (for a review see Griffith et al. 2002). Indirect benefits may involve fertility

insurance (Sheldon 1994, Gray 1997) and various types of genetic benefits. Females may for example participate in extra-pair copulations to increase the genetic variability of their offspring as a risk spreading strategy (Williams 1975, Westneat et al. 1990), or they may seek for extra-pair fathers with compatible genes (Johnsen et al. 2000, Foerster et al. 2003). However, the most thoroughly studied hypothesis is that females try to gain "good genes" to their offspring, which could then improve the survival (Hasselquist et al. 1996, Kempenaers et al. 1997) and/or future reproductive performance of the young (Schmoll et al. 2005).

One example of sexual character dependent extra-pair fertilizations was found in the Swedish population of the Collared Flycatcher. It was shown that the forehead patch size of the males (a heritable and condition dependent secondary sexual character) predicted the paternity in the broods (Sheldon et al. 1997). Extra-pair young sired by large patched males fledged in better condition than their half-sibs and the difference between the chicks increased with the difference in the forehead patch size between the social and extra-pair father.

1.3. The role of parasites in reproductive success of the birds

Parasites can have severe negative impacts on their bird hosts. It was demonstrated that ectoparasite load in the nests affects hatching success (Oppliger et al. 1994, Tomás et al. 2007) and parental body mass at the end of the nestling period negatively (Christe et al. 2002). While infestation by Blowfly larvae (*Protocalliphora* spp.) had negative effects on body size and mass of fledglings (Hurtrez-Boussés et al. 1998). Furthermore, blood sucking Dipterans can transmit microparasites (e.g. avian Haemosporidian parasites) between birds which also affect the health and reproductive success of their hosts. Biting Midges (*Ceratopogonidae*) and Hippoboscid Flies (*Hippoboscidae*) transmit *Haemoproteus* parasites, while Mosquitoes (*Culicidae*) and Simuliid Flies (*Simuliidae*) are the vectors of *Plasmodium* and *Leucocytozoon* species, respectively.

Haemosporidian parasites have complex life-cycles. They develop in two groups of host: in the birds and the vectors. Vectors inoculate sporozoites into the birds' blood stream, which after multiple asexual divisions in various tissues produce merozoites. Merozoites invade the blood cells of the birds where gametocytes are formed, which are sucked up by Dipterans when feeding on infected birds. The fertilization takes place in the midgut of the vector where the zygotes develop further. Here sporozoites are formed which penetrate the salivary glands of the vectors and later infect the birds (Valkiūnas 2005). The major difference in the life-cycle of these parasites is that asexual division of merozoites does occur in hosts' blood cells

if *Plasmodium* species infect the birds but not in the case of *Haemoproteus* and *Leucocytozoon* infection when only gametocytes are formed.

Once infected, parasites usually persist in the birds for many years or even the whole life (the dynamics of parasitemia of *Plasmodium* parasites can be seen on Figure 1). Generally, birds which survived the acute infection (thus maintaining chronic or latent infections) are detected in the wild. In these stages parasites can be detected from the blood when short-term recrudescence or relapse takes place (usually at the beginning of bird's breeding season). These parasites during various stages of their life-cycle can cause anaemia, block up capillaries in different organs and decrease the oxygen-binding capacity of haemoglobin. The symptoms are more severe during acute infections and as a consequence several individuals perish (Valkiūnas 2005).

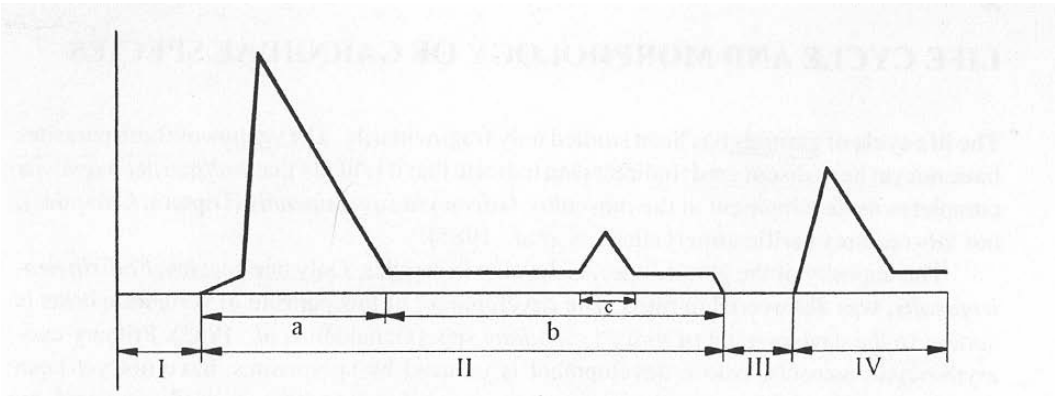

Figure 1. Schematic representation of the dynamics of parasitemia of *Plasmodium* parasites in birds: I - prepatent period (parasites develop in internal organs); II - primary parasitemia; III - latent stage of infection (parasites are absent in the blood but persist in internal organs); IV - secondary parasitemia due to relapse (synchronized with the breeding period of birds); *a* - *c* – stages of parasitemia: *a* – acute including the crisis (lasts from one week up to several weeks), *b* – chronic (the length of this stage is variable), *c* – recrudescence. The abscissa is a calendar and the ordinate is a relative intensity of parasitemia (*from Valkiūnas 2005*).

Behavioural ecological studies also reported negative effects of these parasites on bird reproduction. Females, infected with *Haemoproteus* species lay significantly smaller clutches than non-infected individuals (Marzal et al. 2005), and as a result of changes in their thermoregulation and incubation ability, significantly fewer nestlings hatch from their fertile eggs (Sanz et al. 2001, Marzal et al. 2005). Moreover, it has been demonstrated that blood parasite infection has negative effect also on parental care (Buchanan et al. 1999), and as a result fewer nestlings fledge from broods reared by infected parents (Merino et al. 2000).

2. AIMS OF THE STUDIES

2.1. The effect of egg size and hatching asynchrony on nestling performance

In ***study 1*** we aimed to investigate whether Collared Flycatcher females enhance or reduce the disadvantages of their last hatched chicks. If the pronounced size hierarchy is adaptive from the viewpoint of parental fitness (e.g. in the case of *brood reduction* and the *sibling rivalry hypotheses*) parents have no interest in compensating for this disadvantage. They may even reduce the investment into the last laid eggs (for testosterone see: Schwabl et al. 1997, for nutrients see: Arnold 1989, Heeb 1994, Viñuela 1997), thereby exaggerating the competitive disadvantage of the surplus offspring. However, if the size hierarchy among nestlings is only the by-product of earlier start of incubation before the whole clutch has been laid down parents would benefit from such a compensation. The most obvious way is the preferential feeding of the later hatched offspring by one or both of the parents (Gottlander 1987). However, compensation may also act through preferential maternal investment into the later laid eggs. This differential investment may manifest in an elevated level of testosterone to increase the competitive ability and the development of nestlings (Schwabl 1993, Schwabl 1996, Lipar and Ketterson 2000, Eising et al. 2001) and in preferential nutrient investment into the eggs (Howe 1976, Cichoń 1997, Royle et al. 1999, Reynolds et al. 2003).

In the Collared Flycatcher parents do not discriminate between nestlings of different size when allocating food in the brood (Rosivall et al. 2005), thus parental compensation for hatching asynchrony, if it happens, is possible only during the egg-laying period. Since females do not allocate testosterone differentially in relation to laying order (Michl et al. 2005) we assessed the nutrient contents of the eggs (by measuring egg size) and investigated its effect on nestling growth. Though some earlier studies have already attempted to investigate the effect of laying order on the growth and fledgling condition of nestling passerines, only a few of them linked the growth of individual nestlings to the exact laying and hatching order (Badyaev et al. 2002).

In ***study 1*** we found successful compensation in terms of egg size for the detrimental effects of hatching asynchrony. Furthermore, Hargitai and her colleagues (2005) also showed that egg size increased with laying order in years with a warm pre-laying period, however in colder years there was no such relationship. Since ambient temperature affects the availability of insect prey in general (Taylor 1963, Bryant 1975), and pre-laying temperature was positively correlated with the food abundance during the nestling period in the study

population (Spearman rank correlation: N = 6, R = 0.89, p = 0.019) we refer to warm and cold years as good and bad quality years, respectively.

Two explanations arise for the difference in the egg size pattern between good and bad years. First, females follow different strategies when they allocate nutrients into the eggs. This is because the adaptive value of size hierarchy differs between good and bad years with sibling size asymmetry being disadvantageous in good years but advantageous or neutral in bad years. Second, although compensation for hatching asynchrony would be beneficial independent of year type, because of energetic constraints during egg laying (i.e. less food is available) females are simply not able to lay larger eggs at the end of the laying sequence in poor years. In ***study 2*** we aimed to test the first hypothesis so that we altered the rearing conditions of the chicks by conducting a brood-size manipulation experiment and measured the effects of size hierarchy on nestling growth, fledging size and parental survival.

If the strategy of the females differs between good and bad quality years (first hypothesis), we would expect that hatching asynchrony and the resulting size hierarchy is either advantageous or has no effect on the fitness of the parents rearing enlarged broods (i.e. females did not compensate for hatching asynchrony in poor years (Hargitai et al. 2005); see Table 1). The benefits could arise through the reduced peak load of the parents or decreased nestling competition (see above), which might have an important role under severe conditions. On the other hand, we expect that hatching asynchrony has negative effects on parental fitness in reduced broods, as in previous studies hatching asynchrony was compensated for in good years (Hargitai et al. 2005, ***study 1***). In cases of good food supply maximum parental workload may not be an issue and females may prefer to decrease the detrimental effects of hatching asynchrony on the growth of the last hatched young (for details see ***study 1***). However, hatching asynchrony is expected to have negative effects on both enlarged and reduced broods if the lack of maternal compensation in poor years is only due to energetic constraints imposed on females during egg laying (second hypothesis; Table 1).

Table 1. Predictions on the effects of hatching asynchrony (HA) in experimentally simulated good and bad years in the view of former studies on this species (+ means positive relationship, 0 means no relationship and – means negative relationship in the table).

	Relationship of egg size and laying order	Effect of egg size pattern on the disadvantage of the last chick	Predicted effect of HA on parental fitness / brood performance	
			Year dependent female strategy	Energetic constraint
Good year	+	-	-	-
Poor year	0	not applicable	+ / 0	-

2.2. The effect of parental quality and extra-pair copulations on nestling performance

We also aimed to investigate whether the size of secondary sexual characters of the social mates predicts females' participation in extra-pair copulations and whether extra-pair young perform better than their nestmates. Therefore in ***study 3*** we examined the growth and fledging size of extra-pair and within-pair offspring. We also controlled for the possible confounding effect of offspring sex, because females may adjust their brood sex ratio in relation to the same attractivity signals as they use in extra-pair mate choice (Ellegren et al. 1996, but see Rosivall et al. 2004), and sexes may differ in their growth rate even in monomorphic species (Martins 2004). In addition, the effect of sex on offspring performance is interesting *per se*, because it may explain previously found seasonal shift in brood sex ratios in this population (Rosivall et al. 2004).

We were also interested in how parental quality affects nestling growth and performance and whether secondary sexual characters signal the ability of males to avoid parasitic infections. Therefore in ***study 4*** we investigated the relationship between male attractiveness (measured as the size of the forehead and the wing patch) and avian malaria (*Haemoproteus* and *Plasmodium* parasites sensu Pérez-Tris et al. 2005) infection, and the association between male secondary sexual characters, parental malaria infection and nestling performance. To control for environmental conditions nestlings were reared in simulated good and bad environments so that chicks were cross-fostered in reduced or enlarged broods. The cross–fostering design helped us to separate early maternal and genetic effects from rearing effects.

Collared Flycatcher males have two heritable, sexually selected, white plumage characters (forehead patch and wing patch; Sheldon et al. 1997, Sheldon and Ellegren 1999, Qvarnström et al. 2000, Michl et al. 2002, Garamszegi et al. 2006), however, their function differ significantly between populations. In a Swedish population of this species, the white wing

patch is known to be important in extra-pair mate choice (Sheldon and Ellegren 1999) but it was unrelated to the body condition of the males (Garant et al. 2004). In our Hungarian study population, however, wing patch size is a condition dependent signal (Török et al. 2003) and has an important role in male territorial behaviour (Garamszegi et al. 2006) but its role in extra-pair mate choice is not known. On the other hand forehead patch is a condition dependent signal in the Swedish population (Gustafsson et al. 1995, Sheldon et al. 1997, Qvarnström 1999) but not so in our population (Hegyi et al. 2002, Hegyi et al. 2006a). Still, there is some indication also in our population that males with large forehead patch are of better quality because the song rate of these males decreased less after immune challenge (Garamszegi et al. 2004a) and large patched males start to breed earlier in the season (Hegyi et al. 2007). In the Swedish Collared Flycatcher population, the forehead patch play an important role in mate choice (Sheldon et al. 1997) but in our Hungarian population results are mixed (Michl et al. 2002 vs. Garamszegi et al. 2004a). One study showed that mates of small patched males were more likely to participate in extra-pair copulations (Michl et al. 2002) just like in the Swedish study, but another study found no such relationship (Garamszegi et al. 2004a). Therefore it is not clear how females benefit from extra-pair fertilizations.

2.3. Between-year dynamics of Haemosporidian parasites

Though previous studies showed that avian malaria parasites might have important consequences on the host none of these investigations aimed to assess the long-term effects of these parasites. Thus information on the real fitness effects of these parasites is rather scarce. To get more complete picture on the effects of these parasites I aimed to study how acute infections detected from nestlings affect their own growth and performance. I screened also adult samples collected from different years for the presence of avian malaria. I aimed to assess parasite composition in different years and the long-term effects of different parasites on the host. However, because of the low prevalence and high diversity of these parasites in Collared Flycatchers, in ***study 5***, I present only some preliminary results on the distribution of these parasites between years. The discovery of their long-term effects on the hosts remains the task of future studies.

2.4. A methodological note

Recently several studies have demonstrated that polymerase chain reaction (PCR)-based methods have higher sensitivity at low levels of parasitemia (Richard et al. 2002, Waldenström et al. 2004), though they are not flawless (Cosgrove et al. 2006, Valkiūnas et al. 2006). By the use of nested PCR (i.e. when the screening is conducted using two PCRs that are performed sequentially) the sensitivity is even more increased (Waldenström et al. 2004). However, this approach is more costly and takes additional time. In addition, along with the increase in sensitivity comes the risk of contaminations and amplification of non-target DNA, i.e., genes for which the primers were not designed (reviewed in Burkardt 2000, Freed and Cann 2006). To ensure that the correct target gene has been amplified, most studies also sequence the PCR product. However, as the sample sizes in datasets used for molecular, biological, and ecological studies steadily increase, combined with a decrease in the cost of running PCRs, large scale ecological and biological studies may use nested PCR protocols just to screen samples for positive or negative amplifications for a group of parasites or microorganisms. To ensure the validity of such studies, it is therefore, of importance to investigate and note any shortcomings or pitfalls when using nested PCR methods to screen for microorganisms. Therefore I report a cautionary note in ***study 6*** regarding misleading amplifications when using a highly sensitive nested PCR protocol (described in Hellgren et al. 2004) for the detection of Haemosporidian parasites.

3. METHODS

3.1. Description of the study area and the species

The studies in this thesis were conducted in an artificial nest box plot in the Pilis Mountains, Hungary (47˚43′ N, 19˚01′ E) in 2002, 2003 and 2004. The study plot is a part of a continuous, unmanaged, oak-dominated woodland, a protected area of the Duna-Ipoly National Park. The dominant trees are the Sessile Oak (*Quercus petraea*) and the Turkey Oak (*Quercus cerris*) (Figure 2). 80-90% of the almost 800 nest boxes is occupied each year mainly by Collared Flycatchers and in smaller numbers by Great Tits (*Parus major*) and Blue Tits. My study species, the Collared Flycatcher, is a small hole nesting, long-distance migratory passerine bird, ideal study object of behavioural studies, since it prefers to breed in

artificial nestboxes (Gustafsson 1988). Furthermore, the high site fidelity of the species (Könczey et al. 1992) allows us also to follow the individuals' reproductive success over years and thus to estimate their lifetime reproductive success.

Males are black on their back, are white on their ventral part and have a conspicuous white collar on their neck. They also have two sexually selected white plumage characters, the wing patch and the forehead patch. The forehead patch appears when males emerge from their female-like cryptic plumage in late winter. Wing patches, on the other hand, are renewed during the complete post-breeding moult in summer (Cramp and Perrins 1993). The adult (more than one-year-old) males can easily be distinguished morphologically from subadult (one-year-old) males because adult males have blacker wing feathers and have bigger wing patches. Females are brownish coloured on their back and white on their ventral part. They do not have a forehead patch but have a wing patch, which is as small as that of the subadult males (Svensson 1992) (Figure 2).

In mid April adult males arrive from their wintering sites (Middle and South Africa) to their breeding territories and occupy a territory (which is a nest box and its close surroundings). Females arrive a few days later and choose males (nest boxes). Subadult males arrive even later and generally breed in the second half of the breeding season (Hegyi et al. 2006a). Males breed socially monogamously but a few of them (6-10%) are polygynous (Török et al. 1998, Garamszegi et al. 2004b) and the rate of extra-pair fertilizations can be as high as 40% in this population (Michl et al. 2002). Collared Flycatchers breed once in a year, females usually lay 5-7 eggs and they incubate alone. The eggs hatch approximately 12 days after the last egg was laid. Both parents feed their nestlings that usually fledge 14-15 days after hatching.

Figure 2. Typical picture of the habitat (upper part, left). Female Collared Flycatcher incubating her clutch (upper part, right). Subadult male (bottom, left), adult male Collared Flycatcher (bottom, right) (*photos by Miklós Laczi*).

3.2. Field methods

3.2.1. Morphological measurements of the parents

When nestlings were 10-12 days old, adults were captured at their nests by spring trap and standard morphological measurements were taken. Their tarsus length was measured to the nearest 0.1 mm and their body mass to the nearest 0.1 gram. The size of the forehead patch of the males, which is the product of its maximum height and width, was measured with a digital calliper to the nearest 0.1 mm. We also estimated the wing patch size of the males as the sum of the lengths of white bars on the outer vanes of the 4-8th primaries measured from the tip of the coverts. A small blood sample (10-50 µl) was also taken from each parent and stored in SET-buffer (0.15 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0) or in absolute ethanol and kept in either in a small transportable refrigerator (between 0°C and 10°C) or on room temperature in the field and later on -20°C until analyses.

Blood samples were also collected from various African resident and European migratory bird species in Jos, Nigeria (9˚56′ N, 8˚52′ E) during autumn 2003 by researchers at Lund

University in order to screen these samples for the presence of Haemosporidian parasites (for the complete list of bird species and their parasites see Appendix).

3.2.2. General nestling handling procedures

Body mass was measured (to the nearest 0.1 g) every second day from hatching or from 2-day-age of the nestlings (specified later) until 14 days of age or until fledging. The length of the 3rd outer primary was measured from day 8 until day 14 or until fledging (to the nearest 0.5 mm). On day 14 tarsus length was also measured (to the nearest 0.1 mm). A small blood sample (10-50 µl) was taken from nestlings at 10-12 days of their age and stored in SET-buffer or in absolute ethanol and kept in a refrigerator in the field and later on -20°C until analyses. From embryos and nestlings that died during our studies (specified later in the data analyses sections) tissue samples were collected and preserved as the blood samples.

3.2.3. Field methods in the Maternal compensation study (study 1) and the Paternity study (study 3)

Eggs were numbered with a permanent marker from the laying of the first egg until clutch completion. The length and width of the eggs were measured to the nearest 0.1 mm using a calliper. All clutches were placed into an incubator (PL Machine SK75) one day before the expected hatching date and replaced with plastic eggs of approximately equal size and weight. All females accepted these dummy eggs as their own and continued the incubation. The original eggs were hatched in separated compartments at 37.2ºC and 70-80% humidity. All embryos, which were still alive when placed into the incubator, hatched successfully. We checked hatching every hour from 4:15 am to 9:00 pm. For eggs hatching during the night we assumed that they hatched halfway between the last and the first checking.

Each hatchling was weighed to the nearest 0.01 g with an electronic balance (Mettler PM4800), marked individually on their breast with a permanent non-toxic pen and returned to their nest immediately or early the following morning if they hatched during the night. Colour marking was randomized in relation to hatching order. We followed body mass increase from the day when the first chick(s) in a brood hatched (day 0) and the growth of the wing feathers from day 8 every second day until fledging. On day 14 tarsus length was also measured.

3.2.4. Field methods in the Hatching asynchrony study (study 2) and the Parental quality study (study 4)

To analyse the effects of hatching asynchrony (***study 2***) or parental quality (***study 4***) under simulated good and bad food supply pairs of enlarged and reduced broods with the same hatching date were created. The original brood size of the brood pairs was the same in all but one case (in this case the difference was one chick between the two broods). We partially cross-fostered broods two days after hatching so that 4 chicks were moved from nest A to nest B and 2 chicks were moved from nest B to nest A. As a result we had enlarged (+2 chicks) and reduced (-2 chicks) broods consisting of approximately equal numbers of their own and foster chicks which were selected randomly with respect to their size. Earlier studies have shown that though parents are to some extent able to adjust their provisioning rate according to the altered demand of their brood, which results in a change in work load, brood size manipulations can successfully alter the feeding rate to individual nestlings (for Collared Flycatchers see: Török and Tóth 1990; for other bird species see e.g.: Cronmiller and Thompson 1980, Nur 1984, Martins and Wright 1993) and change the level of nestling competition (Neuenschwander et al. 2003). Thus enlarged and reduced broods have already been used to simulate years with bad and good food supply, respectively, e.g. by Merilä (1996) and Råberg et al. (2005).

Each nestling was weighed on the day of swapping and marked individually by clipping tufts of down on its head and back. Body mass of the nestlings was measured from day 2 and the length of the third outer primary from day 8 every second day. On day 14 tarsus length was also measured.

3.3. Laboratory methods

3.3.1. Sex determination

Since sex identification of the nestlings is not possible via visual characters we used molecular markers for this purpose. These primers simultaneously amplify homologous parts of CHD-W and CHD-Z genes.

DNA from blood samples was extracted by the standard phenol-chlorophorm or by ammonium-acetate methods (Nicholls et al. 2000) and concentration of genomic DNA was adjusted to 25 ng/µl. We applied one of these extraction protocols and DNA concentration

was adjusted in the same way in all the studies in which DNA work was performed (see later paternity analysis and parasite detection).

Some of the adult samples were used as controls during molecular sexing. The sex of these birds was always correctly determined. Our thermal profile differed from the original protocol (Fridolfsson and Ellegren 1999) in that we used 10ºC and 5ºC lower annealing temperatures for the "touch down" and the following cycles respectively (for more details see Rosivall et al. 2004). PCR products were run in 2% agarose gels, pre-stained with ethidium-bromide, and detected in a FluorImager (Vistra). In some of the samples (that were collected in 2002), DNA was partially degraded due to storing problems, preventing us from sexing these offspring with the above protocol. In these cases, we used a special asymmetric nested PCR protocol (for details see Rosivall et al. 2004).

3.3.2. Paternity analysis

We assessed paternity by using four highly variable microsatellite loci (FhU2–4 [Ellegren 1992, Primmer et al. 1996] and PdOμ5 [Griffith et al. 1999]). We modified the original thermal profiles slightly to improve PCR amplification. PCRs were performed in 10 μl volumes on a 9700 Thermal Cycler (Applied Biosystems). In case of using the PdOμ5 and Fhu4 primer pairs the reaction volumes contained 25 ng DNA, 0.5 units of Taq DNA polymerase, 0.4μM of each primer, 1x PCR buffer, 0.125 mM of each nucleotide and 1.5 mM $MgCl_2$. When using the Fhu3 primer pairs the reaction volume contained 25ng DNA, 0.5 units of Taq DNA polymerase, 0.4μM of each primer, 1x PCR buffer, 0.125 mM of each nucleotide and 1.0 mM $MgCl_2$. And finally, when Fhu2 primer pairs were applied the reaction volume contained 25ng DNA, 0.5 units of Taq DNA polymerase, 0.3 μM of each primer, 1x PCR buffer, 0.125 mM of each nucleotide and 1.25 mM $MgCl_2$.

The thermal profile for PdOμ5, FhU2 and FhU4 primer pairs started with 2 min of denaturation at 94ºC, followed by 30 cycles at 94ºC for 30s, 60ºC for 30s and 72ºC for 30s, and ended with an elongation step at 72ºC for 10min. The thermal profile for FhU3 primer pairs was different: the denaturation lasted for 3 min at 94 ºC and was followed by 10 "touch down" cycles in which the denaturation step at 94 ºC lasted for 15s, the annealing temperature decreased by 0.8 ºC in each cycle starting from 63 ºC. This was followed by an elongation step at 72ºC for 30s in each cycle. After the "touch down" cycles 22 cycles were applied with the following profile: 94ºC for 15s, 55ºC for 30s and 72ºC for 30s. The protocol ended with an elongation step at 72ºC for 10min.

The PCR products were run on 6% polyacrylamid gels and visualized using a FluorImager (Vistra). The samples of all members of a family were run on the same gel so any mismatch in the genotypes between offspring and putative parents could be detected. Assuming Mendelian inheritance, we classified offspring as extra-pair young if they showed genotype mismatch with their putative father. Mutations are very unlikely to confound our results, because no single-locus mismatch has been found between mothers and offspring, which could have been the indicative of high mutation rate.

3.3.3. Molecular detection of Haemosporidian parasites

Nested PCRs were performed using the protocol described by Waldenström et al. (2004) and Hellgren et al (2004). The two protocols differ in the fact that the Waldenström et al. protocol detects only avian malaria (*Haemoproteus* and *Plasmodium*) parasites while the Hellgren et al. protocol detects both avian malaria parasites and also *Leucocytozoon* parasites. In the first step of these nested PCRs the primer pair targets at the *mt*DNA of the parasites and amplifies a longer fragment of the cytochrome *b* gene which is present in avian malaria parasites (Waldenström et al. 2004) or in avian malaria parasites and *Leucocytozoon* species (Hellgren et al. 2004). The length of the fragment after the first reaction is either 580 basepair long or 617 basepair long (including primers) when using the Waldenström et al. or the Hellgren et al. protocol, respectively. The second primer pair which is internally nested, increases the specificity of the first reaction but still amplifies fragments from both parasite genera of *Haemoproteus* and *Plasmodium* when using either protocol, however, the amplification of the *Leucocytozoon* genus is separated from the detection of avian malaria parasites species in the Hellgren et al. protocol. In this reaction a shorter, 524-basepair-long fragment (including primers) for avian malaria parasites is amplified by the Waldenström et al. protocol while 527-basepair-long and 526-basepair-long fragments (including primers) are amplified for avian malaria parasites and for *Leucocytozoon* species respectively, in the Hellgren et al. protocol.

In all PCRs both negative (ddH_2O) and positive controls (samples from birds which were previously confirmed to be infected) were included among the samples to control for possible contaminations and failures during PCRs, respectively. To reduce the risk of loosing infections either because of low quality DNA (e.g. due to degradation) or because of sampling error we sexed all individuals and screened all samples twice for blood parasites. All samples with positive amplification were sequenced directly using BigDye® Terminator v3.1 cycle

sequencing kit and products from the sequencing reactions were run on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems). Sequences were edited and aligned using the program BioEdit (Hall 1999) and identified to genus level by comparing sequence data with that of previously identified parasites. Samples included in ***study 4*** showing at least one double peak in the raw sequence data (Figure 3) were subjected to a second, independent amplification, using the same procedure as described above, and the resulting PCR products were cloned using a TOPO TA-Cloning kit® (Invitrogen) as described by Pérez-Tris and Bensch (2005).

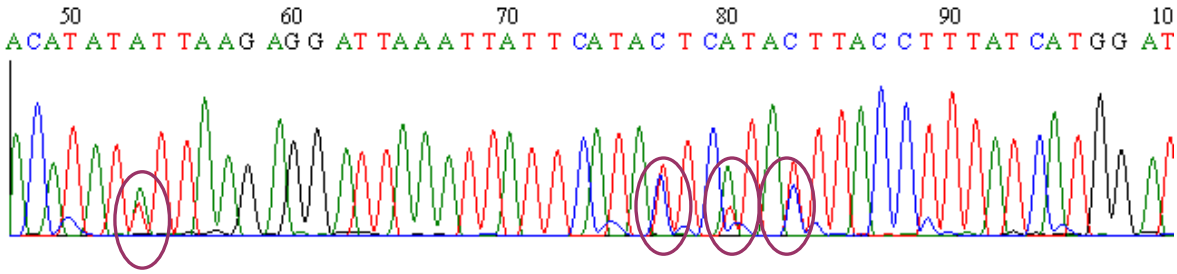

Figure 3. Sequence chromatogram indicating the presence of at least two different parasite lineages in the sample. Different malaria lineages were distinguished by one or more nucleotide differences based on agreement between scientists working in the area (Bensch et al. 2004).

3.4. Data analyses

3.4.1. General methods

It has been shown previously that the age of the males might indicate their quality, since older males have already lived longer, can occupy better territories or prove to be better parents when rearing their young (Forslund and Pärt 1995, Sætre et al. 1995). Furthermore, one study on our Hungarian Collared Flycatcher population showed that nestlings originated from subadult males grow slower and are in worse body condition at fledging (Hegyi et al. 2006b). Thus based on plumage characteristics we identified the binary age of the males during courtship (i.e. subadult or adult) and included only broods of adult males in our experiments. Identification of female age is not possible through plumage characters (only by using capture-recapture data), thus in our studies we did not control for female age.

Since food conditions change as the season progresses we always tried to limit the number of days elapsed between the laying date of the first and the last broods included in our studies.

The first egg was laid within a 6-, 4- and 8-day interval in studies conducted in 2002, 2003 and 2004, respectively. Furthermore, those broods, which were provisioned by only one parent or were secondary broods of polygynous males were not included in our studies.

Feather growth was found to increase linearly so we calculated the slope of a linear regression for each nestling to describe feather growth rate (Nilsson and Svensson 1996). However, we analysed individual body mass growth in two different ways depending on the models fit best to our dataset. We either entered the body mass data between day 2 and day 12 into the General Linear Mixed Model of SAS as a dependent variable while using age as a repeated measure variable or used the logistic growth model of Starck and Ricklefs (1998): $W=A/(1+\exp(-K*(t-t_i)))$, where *K* is the rate of mass increase, *A* is the asymptotic mass and *t* is the age of the individual. All statistical analyses were performed in Statistica for Windows, in SPSS and in SAS. Details of different statistical analyses are given at the data analysis section of the different studies.

3.4.2. Maternal compensation for hatching asynchrony (study 1)

In this study we used 45 clutches consisting of 6 or 7 eggs (22 in 2002 and 23 in 2003). Because it may be different being laid sixth in a clutch of 6 eggs or 7 eggs, we ranked each egg into one of the following five categories: first, second, middles, penultimate and last laid egg. See e.g. Magrath et al. (2003) for a similar grouping.

Egg volume (V) was calculated according to the formula $V= -0,042+0,4976*L*W^2$, where L= egg length and W= egg width, described by Ojanen et al. (1978) for a sibling species, the Pied Flycatcher (*Ficedula hypoleuca*). Place of a chick in the hatching order was described by the hatching time, which was calculated as the time elapsed between the first hatching in a brood and the hatching of the chick in question. Hatching time was therefore 0.0h for the chicks hatched first in a brood. Mass was found to increase logistically with time, so we calculated the rate of mass increase (from day 0 until fledging) using a logistic growth model (see above) where the accurate age of the individuals was calculated from their hatching time. The growth of the primaries was described with data collected from day 8 until fledging. Here we did not control for the accurate hatching time.

Unless stated otherwise, analyses were performed using general linear mixed models with laying order as repeated measure factor and year and brood size as factors. The covariance structure of the model was selected on the basis of Schwarz's Bayesian Information Criterion (BIC). For all but one of the dependent variables the best fit was achieved by using first order

autoregressive covariance, therefore we used this covariance structure throughout the analyses. This covariance structure assumes that measurements closer in the repeated measure sequence are more similar to each other, which is our expectation if eggs are laid sequentially. Denominator degrees of freedoms are obtained by Satterthwaite approximation and are therefore not integers. Post hoc comparisons were performed contrasting the groups in question in the Test Subcommand of the SPSS. Statistical analyses were performed using SPSS 11.0 and Statistica for Windows 4.5.

Sample sizes varied among dependent variables. Five broods (2 and 3 in 2002 and 2003, respectively) were not included in the nestling growth analyses, because females reared their young alone. Inclusion of these broods in the investigation of egg size patterns did not change the result, therefore they were kept in this analysis. Feather growth was measured in 34 broods only, therefore this analysis is based on a smaller dataset. Because of unhatched, died or not measured nestlings some data were occasionally missing. However, hatching success and subsequent nestling survival (95.82% and 98.80%, respectively) were not related to laying order (χ^2-test were used to compare the distribution of hatched/survived chick to the number of observations throughout the laying order; hatching success: χ^2=0.615, df= 4, p=0.961; survival: χ^2= 0.080, df= 4, p= 0.999) and therefore they are not expected to affect the outcome of our analyses.

3.4.3. The effects of hatching asynchrony and rearing environment (study 2)

Altogether, we studied 48 broods with the most common brood size being of 6 or 7 nestlings. One brood which was depredated on day 4, was excluded from the analyses as were those broods, which were secondary broods of polygynous males or reared by only one parent. The remaining 43 nests were retained for the analyses. We analysed nestling growth and fledging size data both at the individual and brood levels in order to estimate the effect of hatching asynchrony and brood size manipulation on both the individual nestlings and the parental fitness.

In ***study 1*** we found that the extent of hatching asynchrony in a brood was correlated with the coefficient of variation (CV) of 2-day body mass when controlled for year (using GLM: $F = 18.84$, $df = 1, 38$, $p < 0.001$). Similarly, the hatching time of an individual nestling (i.e. the time elapsed between the first hatching in the given brood and the hatching of the chick in question) was correlated with the corrected deviation (CD) of nestling body mass from the

brood mean (CD = (a - ā) / ā, where ā = mean body mass of the brood on day 2, a = the 2 day body mass of the chick in question; using GLM: $F = 468.36$, $df = 1, 212$, $p < 0.001$). Therefore, in the present study we did not directly measure hatching asynchrony but used CV ("size variation" later on) and CD ("relative size" later on) to estimate the effect of hatching asynchrony on nestling performance at the brood and the individual level, respectively.

In the individual level analyses we used General Linear Mixed Models including manipulation category (i.e. enlarged or reduced) as a factor, relative size as a covariate and the interaction of these terms. Original and rearing broods were also included as random factors. Dependent variables were wing feather length (the length of the third outer primary), body mass and tarsus length on day 14, and wing feather growth rate. The growth of the primaries was described with the slope of a linear regression for each nestling using data collected between day 8 and day 12. Body mass growth was analysed by entering the body mass data between day 2 and day 12 into the model as a dependent variable while using age as a repeated measure variable. The interaction of explanatory variables with age indicates an effect on nestling growth. The covariance structure of the model was selected on the basis of the Akaike Information Criterion values (Burnham and Anderson 1998).

In the brood level analyses we used General Linear Models including the brood means of nestling size and feather growth rate as dependent variables, manipulation category as a factor, size variation as a covariate and the interaction of these terms. When analysing body mass growth we entered the brood means of body mass between day 2 and day 12 into the model as dependent variable and used age as a repeated measure variable. Similar analyses were performed for the mean value of the two largest chicks in the broods in order to estimate the effect of the explanatory variables specifically on the nestlings with a competitive advantage. The two largest chicks were the chicks, which weighed the most on day 2.

After the analyses of our initial models, we performed a step-wise backward deletion of non-significant terms. All analyses were performed using the Mixed Procedure of SAS 8.02 (SAS Institute, Cary, North Carolina).

Two broods were depredated after day 12, 6 nestlings had already fledged before the final measurements were taken, and some measurements were occasionally missing, therefore sample sizes varied among analyses. Nestlings which died (6 out of 162 and 2 out of 110 in enlarged and reduced broods, respectively) were excluded from the analyses. Brood means were calculated for the rest of the chicks in the brood.

The two manipulation categories did not differ in terms of the size difference between the largest and smallest chicks in the broods on day 2 ($t_{41} = -0.69$ $p = 0.496$). The average

difference was 1.44 ± 0.13 and 1.30 ± 0.02 g (mean ± SE in enlarged and reduced broods, respectively), which corresponds to a hatching span of 30.65 and 29.22 hours respectively (the hatching asynchrony (HA) estimate is based on data from ***study 1***; equation of the linear regression HA = 16.04 + 10.16 x $mass2d_{max\text{-}min}$, where $mass2d_{max\text{-}min}$ is the mass difference between the heaviest and the lightest chick at 2 days of age).

We also analysed the effect of manipulation and the estimated hatching asynchrony on the survival of the parents. Because of the high site fidelity of breeding individuals (Könczey et al. 1992) we considered those individuals which were recaptured in two years following the experiment as survivors while non-recaptured birds as non-survivors. Survival was analysed using Generalized Linear Models with binomial error and logit link including manipulation category as a factor, size variation as a covariate and the interaction of these terms. Since the dispersion parameter was larger than 1.0 we tested the significance of the parameters with an F test (Crawley 1993); d scale option was used in SAS 8.2.

3.4.4. The effects of extra-pair paternity and sex on nestling performance (study 3)

We obtained performance, sex and paternity data for 32 broods (13 and 19 broods in 2002 and 2003, respectively). Four of the parents were captured in both years, and to avoid pseudoreplication we included only one of their broods. In one brood, all the chicks were sired by foreign males (at least two different individuals). Since in this case we cannot exclude the possibility that another male than the social mate was captured and sampled in the nest box, this brood was omitted from all analyses.

Throughout the study, we used individual based analyses (General Linear Mixed Models) with brood identity as a random factor. When we analysed the effect of extra-pair paternity on offspring growth and fledging size, only nestlings from mixed paternity broods were included (n=87 chicks). The effect of sex, however, was analysed also on a larger data set including 170 chicks. Because the effect of possible confounding factors on nestling performance, such as laying order, brood size and year, were analysed in ***study 1*** using a larger dataset, we included only those factors in the present analyses that were significant in that study. Laying order had a significant effect on most of the growth and size parameters and it was therefore included in all analyses. We also included the laying order x paternity and laying order x sex interactions in our initial models. Because it may be different being laid sixth in different sized clutches, we ranked each nestling into one of the following five categories: hatched from the first, second, middle, penultimate or last laid egg.

Post-hatching performance of the young was investigated by analysing the effect of the above variables on measures of body size (tarsus length, body mass, feather length), and body condition on day 14 (just before fledging), and also on feather growth rate and body mass change during growth. As year had a significant effect on feather length at fledging in ***study 1***, we controlled for year when analysing feather length patterns (but not in other analyses). Body condition of the chicks was estimated as the residual body mass on tarsus length. Feather growth rate was analysed from 8 until fledging of the nestlings. In the case of body mass growth, we used body mass as dependent variable and nestling age (2-12 day) as a repeated measure variable. The interactions of age with other variables were also included in the model. Significant effects for age interactions indicated that the given variables affected nestling growth.

The speed of embryonic growth was investigated by analysing the hatching time of the young (i.e. the time elapsed between the first hatching in a brood and the hatching of the chick in question). When controlled for laying order, this value should clearly show whether there is any difference between the development rate of extra-pair and within-pair embryos. On a larger dataset, the brood size x year x laying order interaction affected hatching time of the nestlings, and therefore this interaction was also included in the hatching time analysis (but not in other analyses).

In all above analyses, non-significant variables (except those background variables which were significant on a larger dataset (see above)) were deleted from the models one by one starting with the highest order interactions. These analyses were performed using the SAS 8.2 program. Nestling mortality was not analysed, because in broods where we had data on paternity, only four nestlings/embryos died.

When testing the effect of laying order on sex and paternity of the offspring, we compared the observed versus expected number of male/extra-pair young in different positions using χ^2-test. The effect of paternity on offspring sex was tested using Generalized Linear Mixed Models (glimmix macro), with brood identity as a random factor.

When we investigated the effects of parental traits on paternity of the broods, we used some additional data collected in 2004. If a parent was breeding in multiple years, it was entered to our analysis only once. So the sample size in this analysis was 61 broods. To analyse our data, we used generalized linear models with backward-stepwise deletion of the non-significant terms.

3.4.5. The effects of parental quality and malaria infection on nestling performance (study 4)

When we analysed the effects of parental traits and malaria infection on nestling growth and fledging size we performed three sets of analyses. First, we investigated the effects of putative parental traits on nestling performance. In these analyses all nestlings that originated from a given brood (before cross-fostering) were included and malaria infection and morphological traits of the parents belonging to these broods were used as independent variables. The second analysis was restricted to those chicks, which were genetically related to their putative parents (all genetic offspring independent of cross-fostering). Finally, we also analysed the effects of traits of the rearing parents (i.e. the parents that reared the nestlings after cross-fostering) on nestling performance (all nestlings that were fed by a social pair). Altogether we identified 10 lineages of avian malaria in this study (different malaria lineages were distinguished by one or more nucleotide differences based on agreement between scientists working in the area (Bensch et al. 2004)) and the overall prevalence of these parasites was 30.2%. In the above analyses therefore birds were treated either infected or uninfected with avian malaria.

We used General Linear Mixed Models including manipulation category (i.e. enlarged or reduced) and malaria infection in female and male parents as factors, forehead and wing patch size of the male, tarsus length of the male and the female as covariates, and second order interactions of manipulation category with all the other independent variables. Original and rearing broods were also included as random factors. Dependent variables were wing feather length (the length of the third outer primary) and body mass on day 14, wing feather growth rate and mass growth rate of individual nestlings. The growth of the primaries was analysed between day 8 and 12. Body mass growth was analysed between day 2 until day 14, calculating the rate of body mass increase for each individual from the logistic growth model. Two broods were depredated after day 12, 6 nestlings had already fledged before the final measurements were taken, therefore for 15 individuals we did not have body mass data on day 14. For these nestlings we calculated *K* from day 2 to day 12. Because the inclusion of these individuals in the present analysis did not change the results they were not omitted. Nestlings that died during growth (6 out of 162 and 2 out of 110 in enlarged and reduced broods, respectively) were excluded from all analyses.

After the analyses of our initial models, we performed a step-wise backward deletion of non-significant terms. All analyses were performed using the Mixed Procedure of SAS 8.2 (SAS Institute, Cary, North Carolina).

When we analysed the relationship between malaria infection and the secondary sexual characters of the males we used Generalized Linear Models with binomial error and logit link. The size of male secondary sexual characters and tarsus length were continuous predictor variables. Since the dispersion parameter was larger than 1.0 we tested the significance of the parameters with an F test (Crawley 1993); the d scale option was used in SAS 8.2.

4. RESULTS

4.1. Maternal compensation for hatching asynchrony (study 1)

We found that in Collared Flycatcher clutches, egg volume increased with laying order (Table 2, Figure 4). Hatching mass showed a similar increase (Table 2), and it was the consequence of increasing egg volume, because when the residuals of hatching mass on egg volume were entered to the mixed model, laying order was not significant ($F = 0.40$, $df = 4$, 115.85, $p = 0.81$).

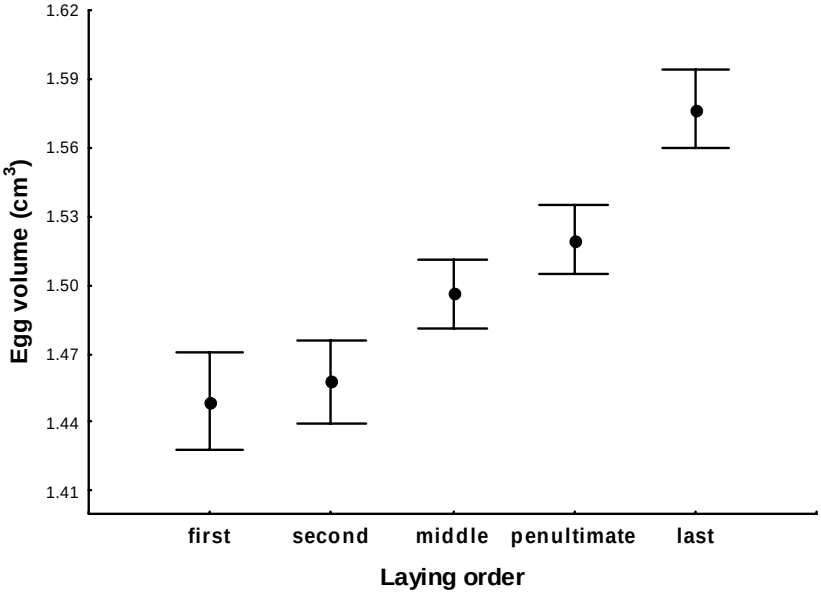

Figure 4. Egg volume (Mean ± SE) in relation to laying order.

The significant overall effect of laying order on hatching time indicates that broods hatched asynchronously, but there was a difference between the two study years (Table 2, Figure 5). In 2002 the last egg hatched later in clutches with 6 eggs ($p < 0.001$), while the last

two in clutches with 7 eggs (both for last-earlier laid and penultimate-earlier laid comparison $p < 0.001$). In 2003 the last two eggs hatched later independent of the brood size (all $p < 0.007$). When we entered the hatching asynchrony (the time difference between the first and last hatching) to an ANOVA with brood size and year as factors, the overall year effect was significant ($F = 5.62$, $df = 1, 33$, $p = 0.024$). In 2003 hatching asynchrony was higher (Mean ± SE was 25.20 ± 1.78 and 29.50 ± 1.65 in 2002 and 2003, respectively). The difference was more pronounced for 6-egg clutches (year x brood size $F = 4.12$ $df = 1, 33$, $p = 0.051$).

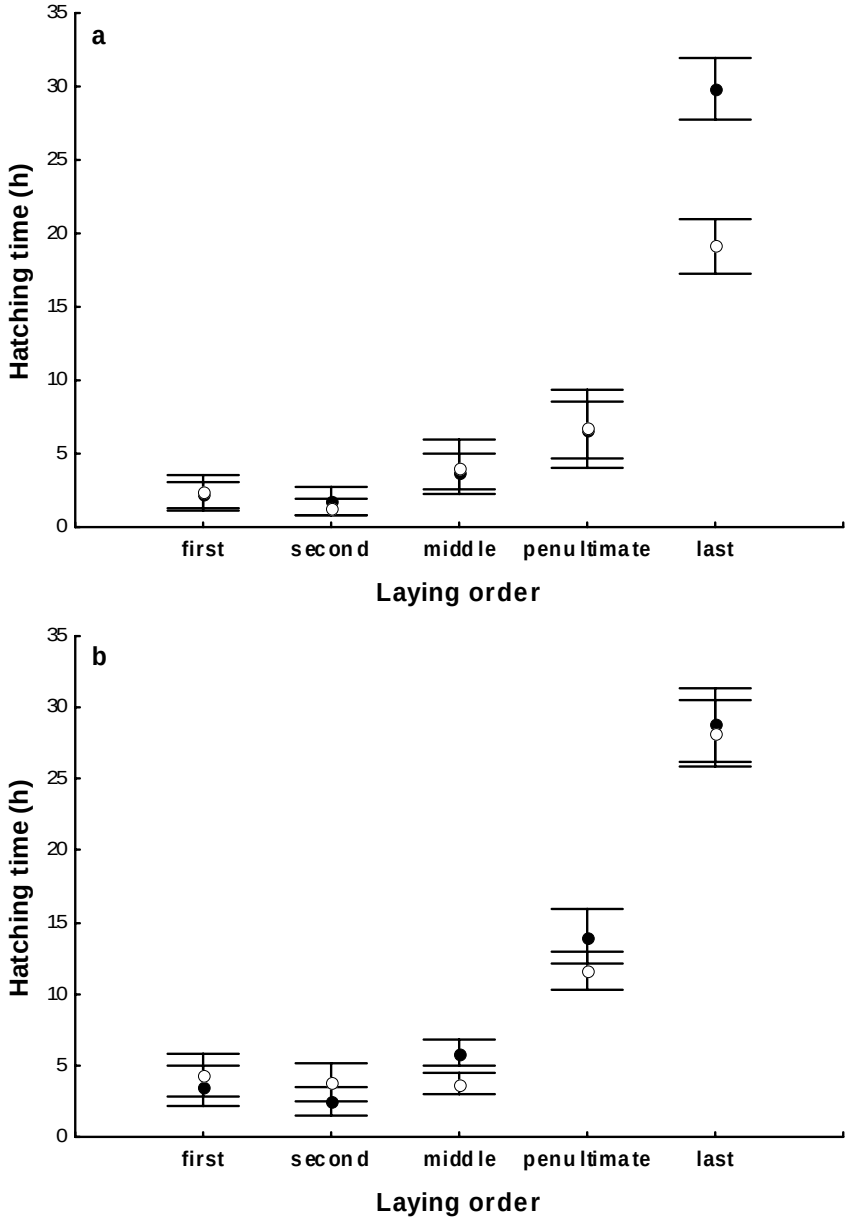

Figure 5. Hatching time (Mean ± SE) in relation to laying order in broods of six **(a)** and seven **(b)** eggs. Hatching time is the time elapsed between the first hatching in a brood and the hatching of the chick in question. Hatching time was therefore 0.0 h for the chicks hatched first in a brood. Open circles, 2002; filled circles 2003.

Though the laying order x brood size x year interaction was significant, there was no overall effect of laying order on the growth of primaries (Table 2). However, laying order affected the mass increase of the nestlings (Table 2, Figure 6).

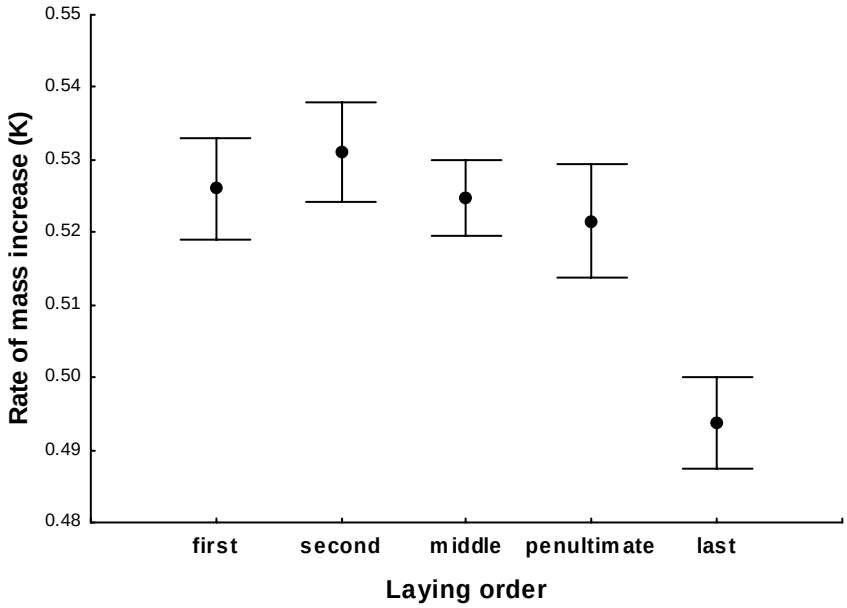

Figure 6. Mass growth in relation to laying order. K is the growth rate from the logistic growth function [$W=A/(1+\exp(-K*(t-t_i)))$], Means ± SE are shown.

Nestlings hatched from last laid eggs experienced lower growth rate (K). In spite of the lower mass increase, there was no relationship between laying order and body mass before fledging (day 14; see Table 2). However, the primaries of the chicks hatched from the last egg were shorter than those of their nestmates (Table 2; Figure 7). These results were consistent across years, even though in 2003 the length of the primaries was overall significantly shorter than in 2002.

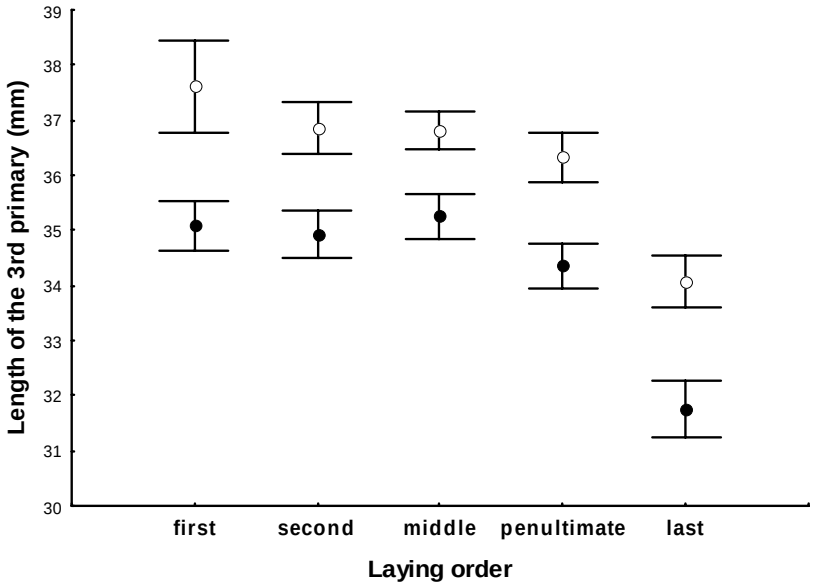

Figure 7. Length of the third primary (Mean ± SE) before fledging (day 14) in relation to laying order. Open circles, 2002; filled circles 2003.

The compensatory effect of larger last egg, can be tested by correlating the relative egg size of the last chicks (compared to their siblings) with their relative growth rate or fledging size. In the case of fledging mass, correlation with relative egg size is not expected, because in our broods, most chicks reached their maximal weight latest on day 14. However, the advantage of larger egg size was apparent, when we analysed its effect on body mass growth, while controlling for the effect of hatching asynchrony. In a multiple regression, relative hatching time (i.e. the difference between the hatching time of the last chick and the mean of its siblings) negatively affected the relative body mass growth rate of the last chick (ß = -0.37, $F = 6.11$, $df = 1, 32$, $p = 0.019$), while relative egg size had a significant positive effect (ß = 0.37, $F = 6.19$, $df = 1, 32$, $p = 0.018$). Relative egg size also tended to affect the feather growth rate (ß = 0.35, $F = 3.58$, $df = 1, 27$, $p = 0.069$), though it was not affected by the relative hatching time ($F = 1.14$, $df = 1, 27$, $p = 0.29$). Finally, the lag of the last chick before fledging, in terms of feather length, was affected by both relative hatching time (ß = -0.41, $F = 7.68$, $df = 1, 32$, $p = 0.009$) and relative egg size (ß = 0.32, $F = 4.73$, $df = 1, 32$, $p = 0.037$). The later the nestling hatched compared to its siblings, the larger its disadvantage was, however this disadvantage was decreasing with increasing egg size.

Table 2. The effect of laying order (first, second, middle, penultimate, last), brood size (6, 7) and study year (2002, 2003) on egg volume, hatching asynchrony (time since the first nestling in the focal brood hatched), nestling growth (K of the logistic mass growth curve and the slope of the linear feather growth) and fledgling size (body mass and length of the third outer primary on day 14). Displayed are F values with degrees of freedom in parentheses. Asterisks indicate the level of significance (* $p<0.05$, *** $p<0.005$).

	Egg volume	Hatching mass	Hatching time	Mass growth	Feather growth	14d mass	14d feather
Brood size	1.06 (1, 41.90)	1.17 (1, 36.19)	10.76 (1, 51.43)***	0.25 (1, 43.15)	0.02 (1, 34.63)	0.05 (1, 40.83)	0.03 (1, 38.81)
Year	1.02 (1, 41.90)	0.03 (1, 36.19)	2.35 (1, 51.43)	0.23 (1, 43.15)	0.33 (1, 34.63)	0.99 (1, 40.83)	22.99 (1, 38.81)***
Laying order	15.65 (4, 154.69)***	5.14 (4, 123.08)***	135.13 (4, 114.13)***	7.55 (4, 121.07)***	1.52 (4, 88.90)	0.24 (4, 124.35)	15.71 (4, 111.24)***
Brood size*year	1.29 (1, 41.90)	2.29 (1, 36.19)	0.64 (1, 51.43)	1.31 (1, 43.15)	0.17 (1, 34.63)	2.32 (1, 40.83)	1.82 (1, 38.81)
Brood size*laying order	1.081 (4, 154.69)	0.34 (4, 123.08)	1.85 (4, 114.13)	1.30 (4, 121.07)	1.68 (4, 88.90)	0.75 (4, 124.35)	1.62 (4, 111.24)
Year*laying order	1.18 (4, 154.69)	0.28 (4, 123.08)	2.02 (4, 114.13)	2.27 (4, 121.07)	0.62 (4, 88.90)	1.40 (4, 124.35)	0.33 (4, 111.24)
Brood size*year*laying order	0.68 (4, 154.69)	0.87 (4, 123.08)	2.49 (4, 114.13)*	0.72 (4, 121.07)	3.27 (4, 88.90)*	0.22 (4, 124.35)	0.18 (4, 111.24)

4.2. The effects of hatching asynchrony and rearing environment (study 2)

4.2.1. Growth and fledging size of individual nestlings

To investigate the possible fitness consequences of nestling size hierarchy under different rearing conditions, we analysed the effects of relative size of nestlings and brood size manipulation on body mass and feather growth rate and fledging sizes of the nestlings. The overall effects of these two variables and the interaction term were significant for all growth and fledging size parameters (Table 3, Table 4). Separate analyses of enlarged and reduced broods showed that initially smaller nestlings experienced slower body mass growth and smaller wing feather length at fledging in both manipulation categories (all $p < 0.001$; Figure 9a, Figure 8b) with the disadvantage being larger in enlarged broods. However, feather growth rate, as well as the tarsus length and body mass of fledglings was affected only in enlarged broods (enlarged broods: all $p < 0.006$, reduced broods all $p > 0.548$; Figure 8a, 8c).

Table 3. The effects of relative size (CD), brood size manipulation (enlarged, reduced) and age on body mass of individual nestlings. Displayed are F values with degrees of freedom in parentheses. Asterisks indicate the level of significance (*** p<0.005). Table shows the variables retained in the final model.

Effect	F (df)
Age	4922.78 (5, 1482)***
Manipulation	58.95 (1, 1482)***
CD	680.20 (1, 1482)***
Age*manipulation	83.32 (5, 1482)***
Age*CD	22.18 (5, 1482)***
Manipulation*CD	48.44 (1, 1482)***
Age*manipulation*CD	6.49 (5, 1482)***

Table 4. The effects of relative size (CD) and brood size manipulation (enlarged, reduced) on feather growth rate and fledgling size (body mass, length of the third outer primary and tarsus length on day 14) of individual nestlings. Displayed are F values with degrees of freedom in parentheses. Asterisks indicate the level of significance (* p<0.05, *** p<0.005). Table shows the variables retained in the final model.

Effect	Feather growth	14d mass	14d feather	14d tarsus
Manipulation	29.93 (1, 192)***	17.30 (1, 180)***	8.74 (1, 180)***	11.00 (1, 177)***
CD	4.39 (1, 192)*	45.29 (1, 180)***	305.90 (1, 180)***	28.61 (1, 177)***
Manipulation*CD	8.84 (1, 192)***	52.08 (1, 180)***	43.61 (1, 180)***	24.54 (1, 177)***

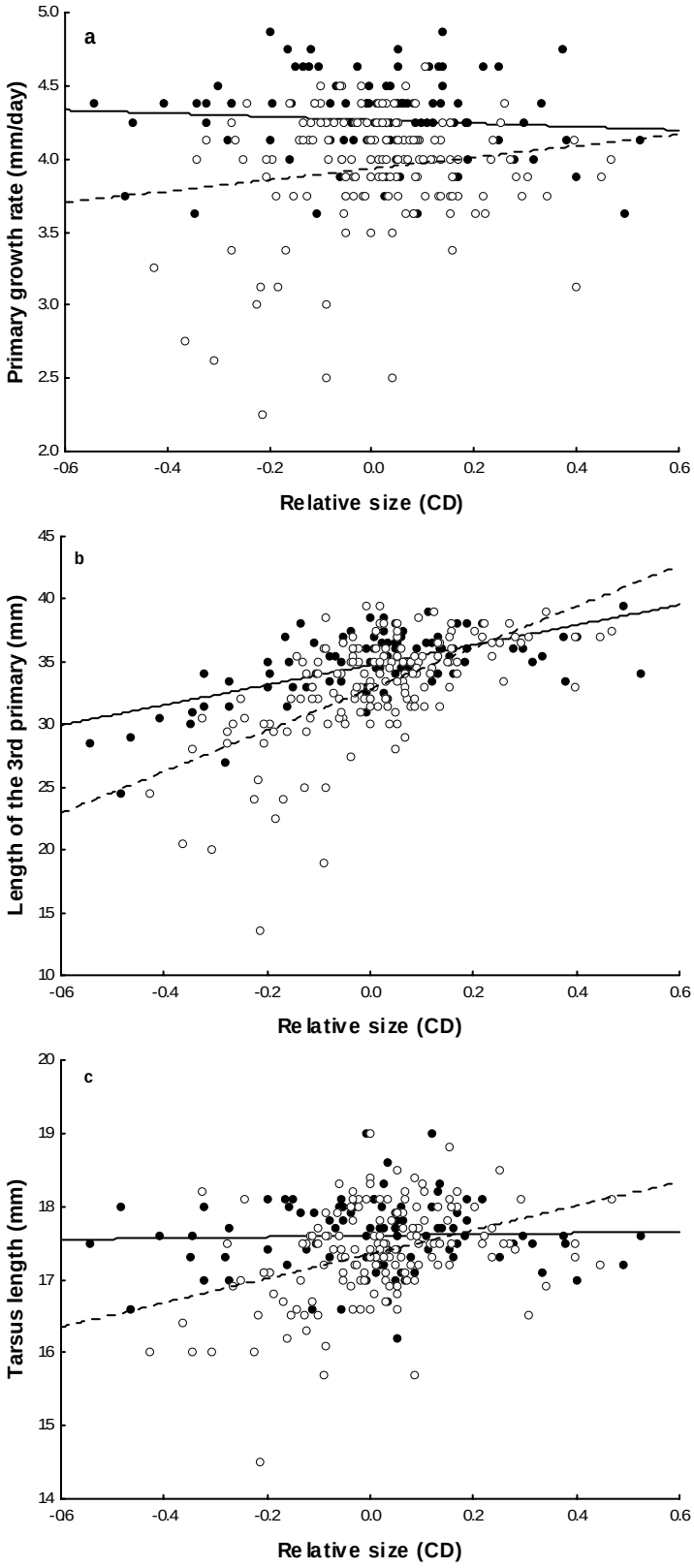

Figure 8. Feather growth and fledging size in relation to the relative size of individual nestlings (CD = (a - ā) / ā, where ā = mean body mass of the brood on day 2, a = the 2 day body mass of the chick in question). Open circles, dashed line: enlarged broods; filled circles, solid line: reduced broods. **a)** growth rate of the 3rd outer primary ; **b)** the length of the 3rd outer primary on day 14; **c)** tarsus length on day 14.

4.2.2. Average performance of the broods and survival of the parents

With respect to parental fitness brood performance may have a more important role than that of the individual nestlings. Therefore we performed similar analyses as above to evaluate the effects of estimated hatching asynchrony on average nestling growth and fledgling size. We also analysed how brood size manipulation and the magnitude of hatching asynchrony affected the survival of the parents.

Brood enlargement had an overall negative effect on body mass and feather growth rate and also on all measures of fledgling size (all $p < 0.019$). However, estimated hatching span of the brood affected only the average mass growth so that a higher initial size variation resulted in slower body mass growth (Figure 9b, Table 5). Neither initial size variation of the brood (for females: $p = 0.908$, for males: $p = 0.110$) nor brood size manipulation (for females $p = 0.259$, for males $p = 0.105$) affected survival of the parents.

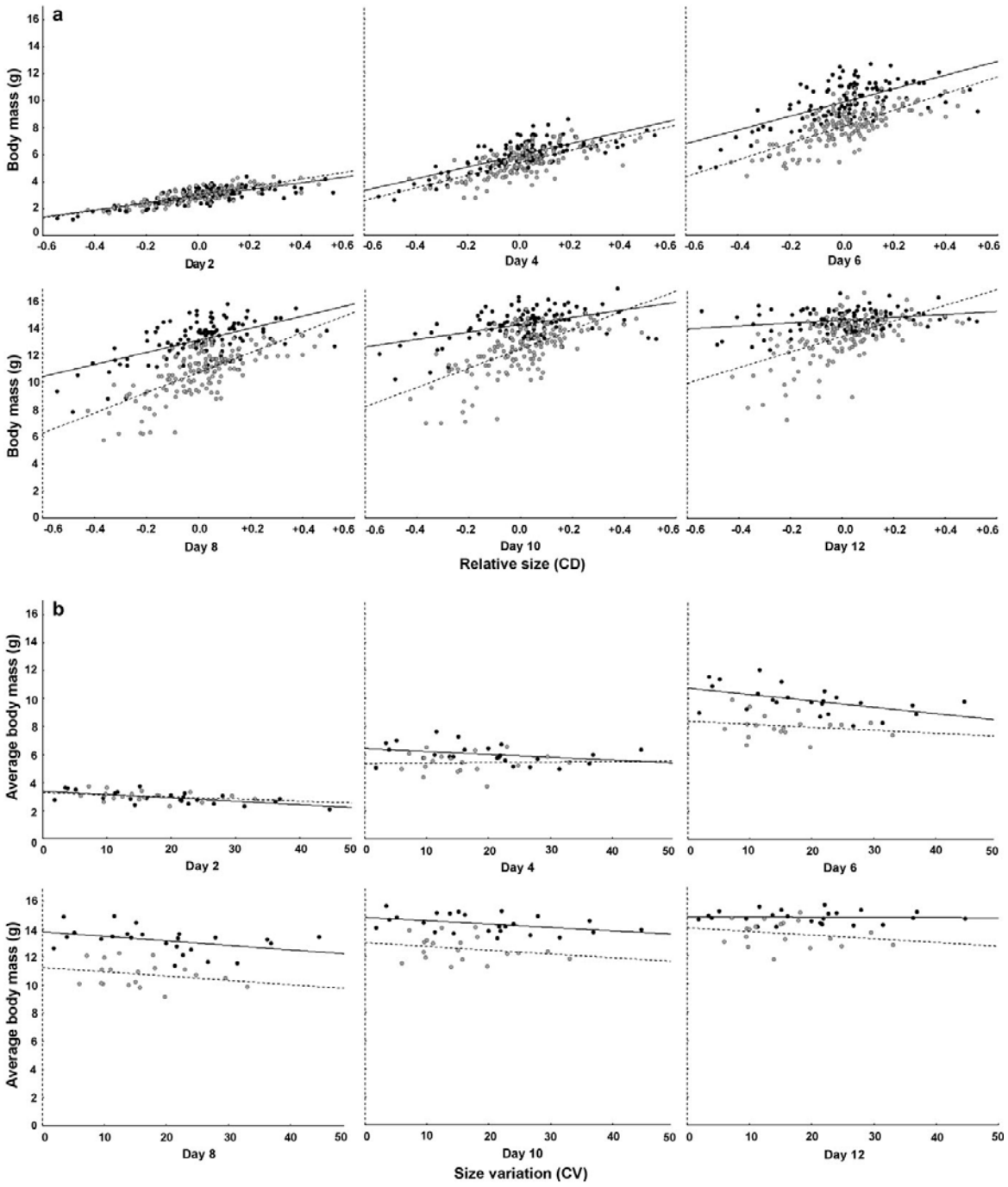

Figure 9. a) Body mass growth of individual nestlings in relation to their relative size (CD = (a - ā) / ā, where ā = mean body mass of the brood on day 2, a = the 2 day body mass of the chick in question). **b)** Average body mass growth in relation to initial size variation (CV) in the broods. Grey dots, dashed line: enlarged broods; black dots, solid line: reduced broods.

4.2.3. Average growth and fledging size of the initially heaviest chicks

According to some of the hypotheses proposed to explain the function of hatching asynchrony, it is also possible that the size hierarchy in the broods is beneficial only for nestlings with a competitive advantage. Therefore we aimed to examine the effect of hatching asynchrony on nestlings with a higher rank in the size hierarchy.

We found that body mass and wing feathers of the two largest chicks grew slower in enlarged broods than in reduced broods (for feather growth rate $F = 19.55$ $df = 1, 41$ $p < 0.001$; for body mass growth see Table 5) while brood size manipulation had no effect on the fledging sizes of these nestlings (all $p > 0.402$). The estimated hatching asynchrony did not affect any of the growth and fledging size parameters (all $p > 0.246$).

Table 5. The effects of size variation (CV), brood size manipulation and age on average mass of the broods and the two largest nestlings. Displayed are F values with degrees of freedom in parentheses. Asterisks indicate the level of significance (* $p<0.05$, *** $p<0.005$). Table shows the variables retained in the final model.

Effect	Mass growth of the broods	Mass growth of the largest nestlings
Age	646.38 (5, 200)***	3613.71 (5, 205)***
Manipulation	54.94 (1, 40)***	26.81 (1, 41)***
CV	6.18 (1, 40)*	Removed
Age*manipulation	30.43 (5, 200)***	20.91 (5, 205)***
Age*CV	3.76 (5, 200)***	Removed
Manipulation*CV	Removed	Removed
Age*Manipulation*CV	Removed	Removed

4.3. The effects of extra-pair paternity and sex on nestling performance (study 3)

A large proportion, 55.74% of the broods contained extra-pair young. Altogether 20.61% of the nestlings were sired by extra-pair fathers. None of the paternal traits (forehead patch size, wing patch size, body size and condition) predicted the females' participation in extra-pair copulations (Table 6). The timing of breeding and female body condition also showed no relationship with the paternity of the broods. However, the broods of large females were less likely to contain extra-pair young than that of small females (Table 6, Figure 10).

Table 6. The effect of parental traits on paternity of the broods. The significant variable retained in the final model is in bold. Values indicated for non-significant terms are derived from the last model, in which the given variable was included during the backward stepwise model selection.

parental trait	F	df	P
female tarsus length	**7.32**	**1,59**	**0.009**
male wing patch size	0.29	1,58	0.589
female body condition	0.30	1,56	0.583
male body condition	0.26	1,55	0.615
male forehead patch size	0.13	1,54	0.721
laying date	0.02	1,53	0.881
male tarsus length	0.00	1,52	0.967

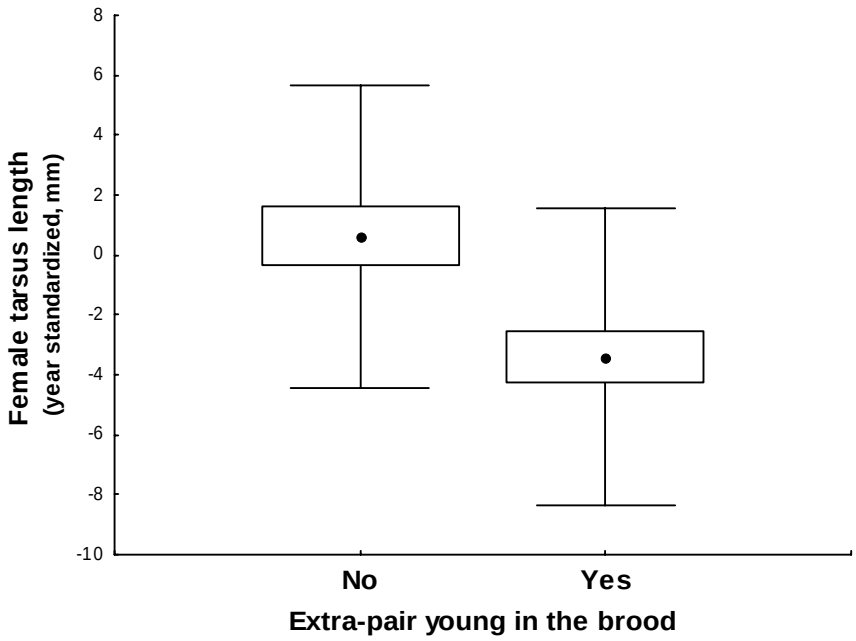

Figure 10. The relationship between female body size and paternity of the broods. Mean ± SD (whiskers) and SE (boxes) are indicated.

The embryonic development of extra-pair young was not faster than that of their half-sibs as indicated by the lack of difference in hatching time (Table 7a). They did not perform better after hatching either (Table 7a). Extra-pair young did not differ in body mass and feather growth from their half-sibs. Furthermore, nestlings fledged with the same size (body mass, tarsus length, feather length) and body condition independent of paternity. In broods with mixed paternity, the occurrence of extra-pair young was independent of laying order (χ^2 = 4.42, df = 6, p = 0.621). Sex of the extra- and within-pair young did not differ (proportion of males for extra-pair young = 0.453, for within-pair young = 0.441; $F_{1, 84.5} = 0.01$, p = 0.922).

As paternity did not affect any of the measures of offspring performance, we repeated all analyses with the inclusion of broods of genetically monogamous pairs when we analysed the effect of sex on nestling growth and size (Table 7b). Though we found no sex difference in the hatching time of male and female nestlings (indicating that they developed with the same speed until hatching), male nestlings gained body mass faster after hatching as indicated by the sex x age interaction effect on body mass (Figure 11, Table 7b). By the time of fledging, the sex difference in body mass had disappeared. However, the tarsus length was significantly longer in females than in males (Figure 12, Table 7b), although the mean difference was only 0.7%. Since we estimated nestling body condition as the residual of body mass on tarsus length, females seemed to be in worse body condition on day 14. Though males tended to

have faster feather growth than females, this difference was not significant and there was no sex difference in feather length at fledging (Table 7b). The sex of the young was independent of their place in the laying order ($\chi^2 = 3.41$, df = 4, p = 0.491).

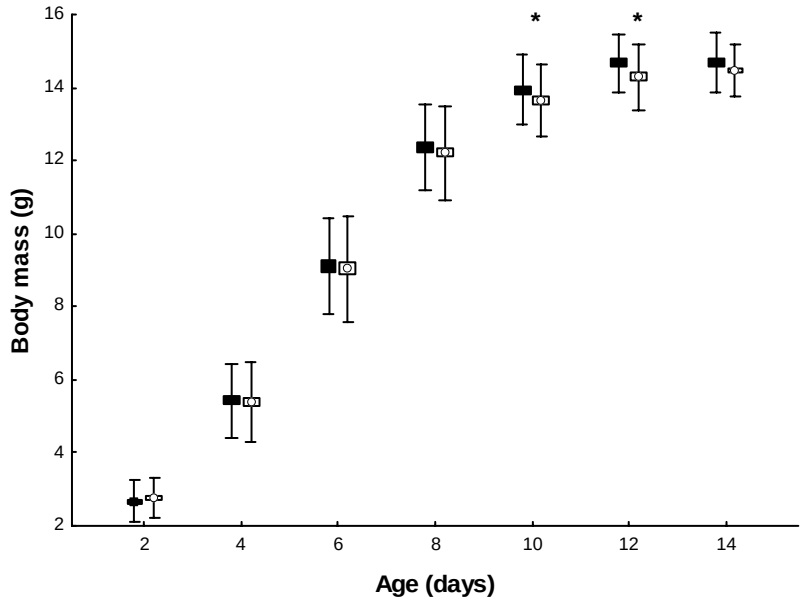

Figure 11. The effect of sex on nestling growth. Asterisks indicate significant difference in body mass between males (black box) and females (open box) when body mass was analysed separately in each age category. Mean ± SD (whiskers) and SE (boxes) are indicated.

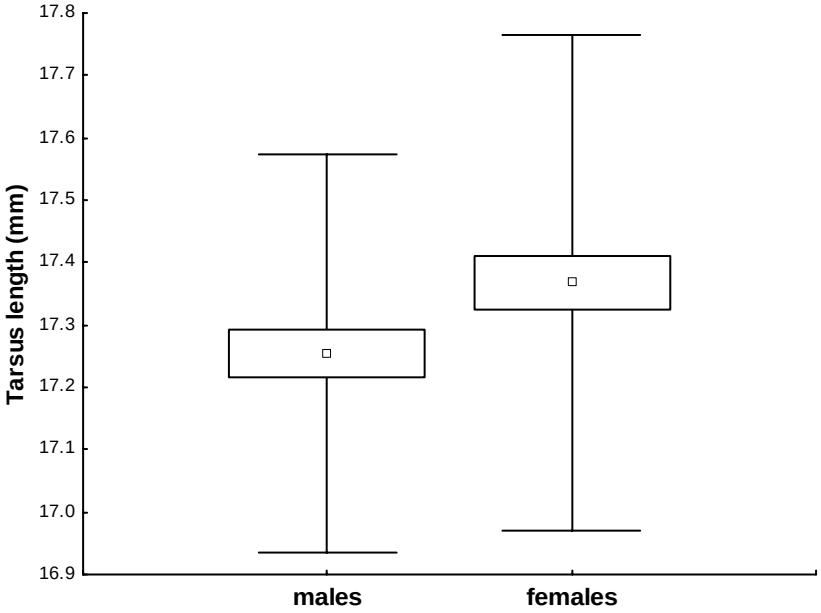

Figure 12. Sex difference in nestling tarsus length on day14. Mean ± SD (whiskers) and SE (boxes) are indicated.

Table 7. The effect of paternity and sex on nestling growth and size. Significant variables retained in the final model are in bold. Background variables other than laying order (see methods) are not indicated. Values indicated for non-significant terms are derived from the last model, in which the given variable was included during the backward stepwise model selection. Note that in case of mass growth where we used a repeated measure approach, all effects are interactions with age (e.g. "laying order" in the table refers to "laying order x age"), because these interactions show whether the given variable (e.g. "laying order") had an effect on mass growth.

	hatching time (~embryonic growth)			mass growth			14d mass			14d tarsus length			14d condition			feather growth			14d feather length		
	F	df	p	F	df	p	F	df	p	F	Df	p	F	df	p	F	df	p	F	df	p
a) mixed paternity broods																					
laying order	**65.80**	**4, 56**	**<0.001**	**2.79**	**20, 467**	**<0.001**	0.22	4, 64	0.925	1.63	4, 65	0.177	0.90	4, 64	0.467	1.57	4, 58	0.196	**13.34**	**4, 66**	**<0.001**
sex	0.83	1, 54	0.366	1.93	5, 460	0.088	0.78	1, 68	0.382	3.94	1, 69	0.051	3.40	1, 69	0.069	0.23	1, 57	0.631	0.78	1, 64	0.380
laying order x sex	1.59	4, 50	0.191	0.70	20, 407	0.828	1.53	4, 60	0.204	0.09	4, 56	0.984	1.52	4, 60	0.208	0.19	4, 48	0.943	1.38	4, 60	0.251
paternity	1.32	1, 55	0.255	0.74	5, 447	0.594	0.96	1, 69	0.331	0.20	1, 64	0.658	1.60	1, 68	0.210	0.01	1, 56	0.917	1.47	1, 65	0.230
laying order x paternity	0.72	4, 46	0.580	1.44	20, 427	0.101	0.97	4, 56	0.433	0.59	4, 60	0.671	1.56	4, 56	0.199	0.54	4, 52	0.709	0.43	4, 56	0.788
b) all broods																					
laying order	**96.55**	**4, 122**	**<0.001**	**7.89**	**20, 939**	**<0.001**	1.14	4, 128	0.340	2.15	4, 120	0.079	0.19	4, 119	0.943	1.52	4, 105	0.202	**28.89**	**4, 121**	**<0.001**
sex	0.57	1, 121	0.450	**3.41**	**5, 939**	**0.005**	1.57	1, 132	0.213	**5.64**	**1, 124**	**0.019**	**4.21**	**1, 123**	**0.042**	3.76	1, 109	0.055	1.24	1, 120	0.268
laying order x sex	0.27	4, 117	0.896	1.05	20, 915	0.404	0.17	4, 124	0.951	0.26	4, 116	0.903	0.15	4, 115	0.964	0.06	4, 101	0.993	1.80	4, 116	0.133

4.4. The effects of parental quality and malaria infection on nestling performance (study 4)

4.4.1. Male quality and malaria infection

We found no correlation between avian malaria infection and male wing patch size ($F = 0.74$, $df = 1, 39$, $p = 0.394$), forehead patch size ($F = 0.65$, $df = 1, 40$, $p = 0.426$) or tarsus length ($F = 0.40$, $df = 1, 41$, $p = 0.531$).

4.4.2. Quality of the putative parents and nestling performance

We investigated how origin of the nestlings (i.e. early maternal investment and genetic quality of the parents) affected the growth and fledging size of the offspring under good and bad rearing conditions. Brood size enlargement had an overall negative effect on all growth rate and fledging size parameters (Table 8). However, none of the secondary sexual characters of the putative fathers correlated with any of the nestling growth and fledging size parameters (Table 8). Tarsus length of the putative fathers showed a positive relationship with wing feather length before fledging (Table 8, Figure 13a). Tarsus length of the mothers, however, did not correlate with any of the nestling traits. Avian malaria infection in the parents did not correlate with any of the nestling growth and fledgling size parameters (Table 8). None of the interactions between parental traits and rearing conditions were significant (Table 8).

When we restricted our analyses only to those nestlings that were proved to be genetically related to their putative parents (i.e. extra pair young were omitted) the results did not change qualitatively (results not shown). The only difference was that the relationship between the tarsus length of the genetic father and the 14-day wing feather length of their offspring became weaker ($F = 3.87$, $df = 1, 121$, $p = 0.052$) probably due to the lower sample size.

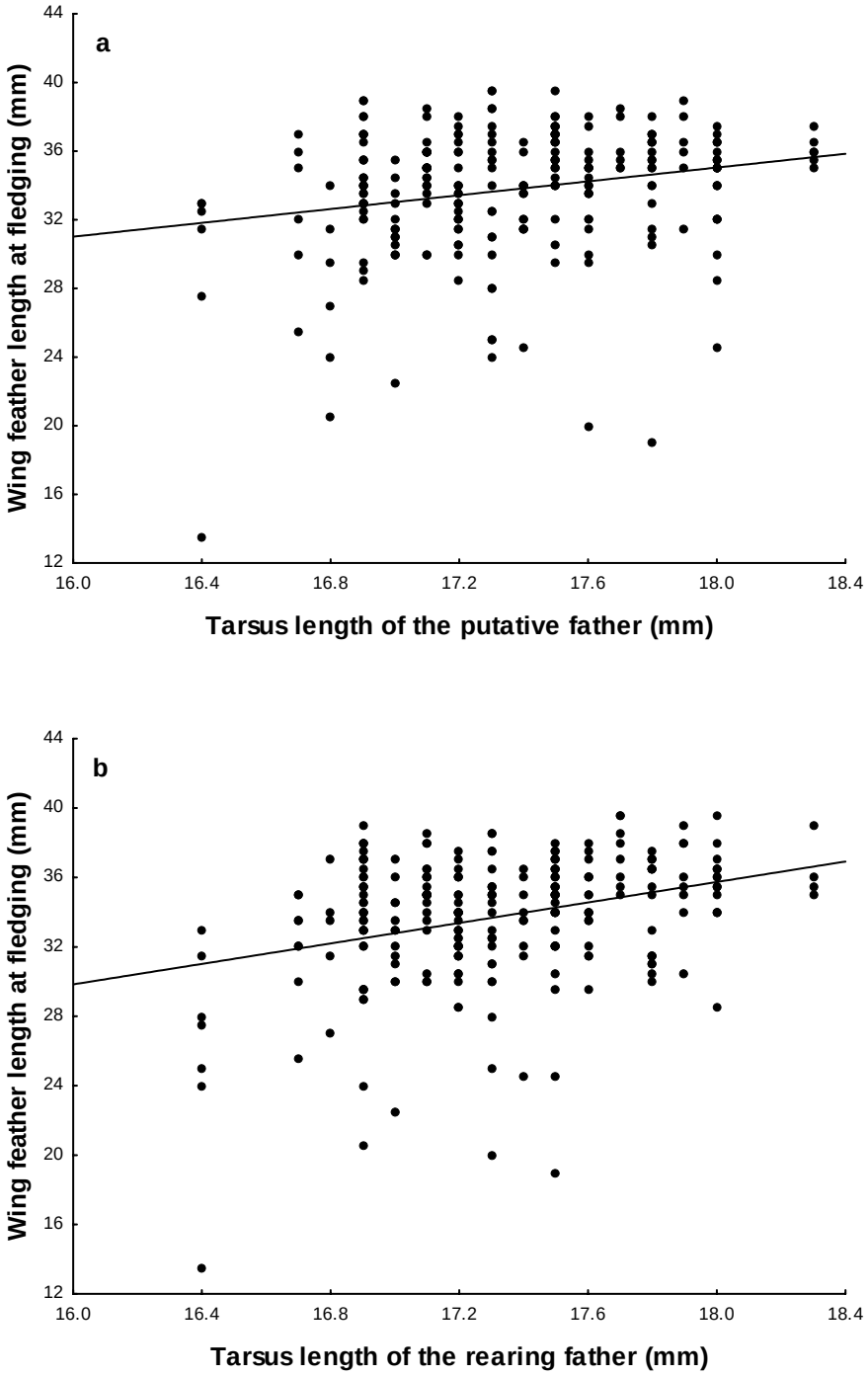

Figure 13. Offspring wing feather length at fledging (day 14) in relation to **a)** the tarsus length of the putative fathers; **b)** the tarsus length of the rearing fathers.

Table 8. The effect of morphology and malaria infection in putative parents on offspring performance. Significant variables retained in the final model are in bold. Values indicated for non-significant terms are derived from the last model, in which the given variable was included during the backward stepwise model selection. FPS means forehead patch size, WPS means wing patch size.

	Mass growth rate			Feather growth rate			Body mass on day14			Feather length on day14		
	F	Df	p	F	Df	p	F	df	p	F	df	p
Manipulation	**123.63**	**1, 187**	**< 0.001**	**26.34**	**1, 187**	**< 0.001**	**14.27**	**1, 176**	**< 0.001**	**17.32**	**1, 175**	**< 0.001**
FPS	0.10	1, 186	0.749	1.23	1, 187	0.269	0.39	1, 175	0.534	0.20	1, 175	0.652
WPS	2.26	1, 187	0.134	0.19	1, 186	0.665	0.05	1, 175	0.822	0.14	1, 175	0.710
Male tarsus	2.71	1, 187	0.101	0.36	1, 187	0.550	2.36	1, 175	0.126	**4.86**	**1, 175**	**0.029**
Female tarsus	1.43	1, 187	0.233	1.86	1, 187	0.174	0.97	1, 175	0.325	0.59	1, 175	0.444
Male malaria	1.76	1, 187	0.186	0.02	1, 186	0.875	0.41	1, 175	0.524	0.62	1, 175	0.434
Female malaria	1.45	1, 187	0.230	1.06	1, 187	0.304	2.71	1, 175	0.102	1.65	1, 175	0.200
FPS*manipulation	1.39	1, 185	0.240	0.02	1, 180	0.889	1.68	1, 173	0.197	0.57	1, 170	0.451
WPS*manipulation	1.60	1, 183	0.207	0.30	1, 182	0.582	0.24	1, 171	0.623	0.12	1, 169	0.726
Male tarsus*manipulation	2.34	1, 184	0.128	0.07	1, 181	0.785	0.13	1, 170	0.716	0.97	1, 173	0.326
Female tarsus*manipulation	0.62	1, 181	0.431	1.57	1, 184	0.212	0.01	1, 169	0.928	0.42	1, 171	0.520
Male malaria*manipulation	0.03	1, 180	0.855	1.30	1, 185	0.257	1.97	1, 174	0.162	1.87	1, 174	0.173
Female malaria*manipulation	0.64	1, 182	0.426	0.86	1, 183	0.356	0.89	1, 172	0.346	1.01	1, 172	0.317

4.4.3. Quality of the rearing parents and nestling performance

Malaria infection and morphological traits of the parents may be related to their parental investment during chick rearing and may in turn affect the growth and size of their offspring independent of early maternal and genetic effects. Therefore we performed the same analyses as above using the traits of the rearing parents as independent variables. Similarly to the above results, brood enlargement had an overall negative effect on all measures of nestling growth and fledging size (Table 9). Out of the two secondary sexual characters of male Collared Flycatchers only the size of the forehead patch showed a positive relationship with nestling growth in a way that wing feathers of nestlings reared by large patched males grew at a higher rate (Table 9, Figure 14). Neither forehead patch nor wing patch size was related to fledging size. Male tarsus length correlated positively with the 14-day wing feather length of the nestlings (Figure 13b), while female tarsus length did not show any relationship with the growth and fledging size parameters (Table 9). Avian malaria infection in rearing females and males did not show any relationship with the measures of nestling performance, and none of the parental traits × rearing condition interactions was significant (Table 9).

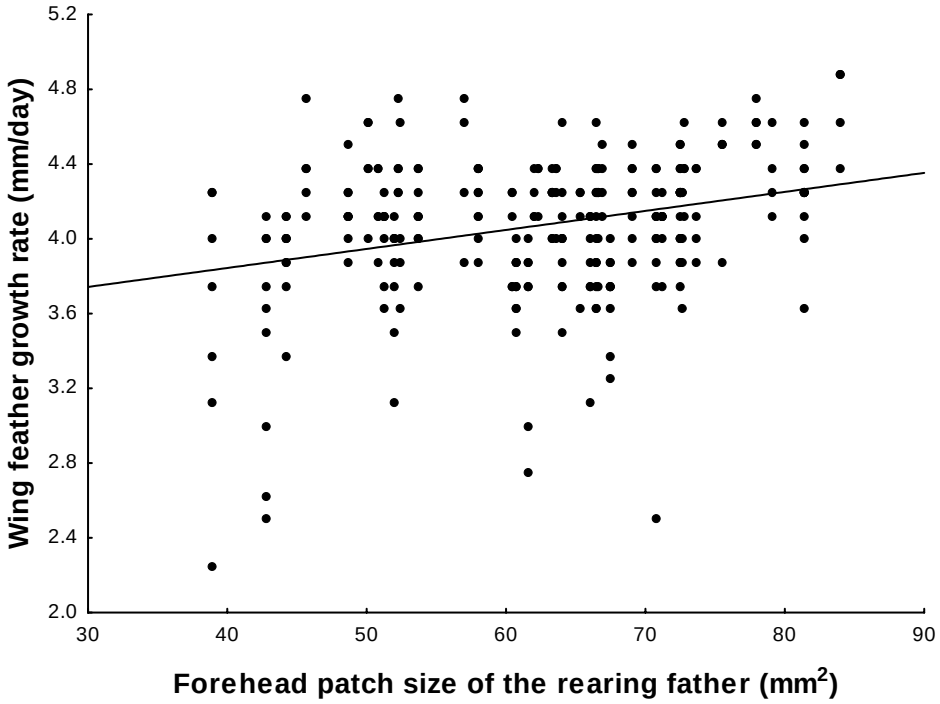

Figure 14. Wing feather growth rate of the nestlings in relation to forehead patch size of the rearing fathers.

Table 9. The effect of morphology and malaria infection in rearing parents on offspring performance. Significant variables retained in the final model are in bold. Values indicated for non-significant terms are derived from the last model, in which the given variable was included during the backward stepwise model selection. FPS means forehead patch size, WPS means wing patch size.

	Mass growth rate			Feather growth rate			Body mass on day14			Feather length on day14		
	F	df	p	F	Df	p	F	df	p	F	df	p
Manipulation	**122.80**	**1, 194**	**< 0.001**	**26.78**	**1, 194**	**< 0.001**	**16.25**	**1, 182**	**< 0.001**	**9.09**	**1, 182**	**0.003**
FPS	0.03	1, 193	0.864	**7.46**	**1, 194**	**0.007**	0.13	1, 181	0.719	0.79	1, 180	0.374
WPS	0.62	1, 193	0.433	0.19	1, 193	0.664	0.14	1, 181	0.705	0.15	1, 181	0.699
Male tarsus	1.76	1, 193	0.186	0.15	1, 193	0.699	1.88	1, 182	0.172	**7.26**	**1, 182**	**0.008**
Female tarsus	0.03	1, 192	0.859	1.35	1, 193	0.247	0.06	1, 180	0.803	1.90	1, 180	0.170
Male malaria	0.19	1, 193	0.664	0.01	1, 192	0.906	0.37	1, 181	0.544	2.04	1, 182	0.155
Female malaria	1.08	1, 193	0.300	0.15	1, 193	0.701	0.01	1, 182	0.941	1.91	1, 181	0.168
FPS*manipulation	0.64	1, 192	0.425	0.75	1, 192	0.389	3.73	1, 181	0.055	2.45	1, 180	0.119
WPS*manipulation	1.12	1, 192	0.291	2.30	1, 191	0.131	0.70	1, 181	0.404	0.35	1, 181	0.557
Male tarsus*manipulation	1.11	1, 192	0.294	1.01	1, 192	0.316	0.49	1, 180	0.486	3.36	1, 181	0.068
Female tarsus*manipulation	0.33	1, 192	0.564	2.34	1, 192	0.128	0.56	1, 181	0.457	0.09	1, 181	0.766
Male malaria*manipulation	0.94	1, 193	0.333	1.64	1, 192	0.202	1.08	1, 181	0.300	0.18	1, 181	0.672
Female malaria*manipulation	0.16	1, 192	0.688	1.12	1, 192	0.291	1.69	1, 181	0.195	3.53	1, 180	0.062

4.5. Between-year dynamics of Haemosporidian parasites (study 5)

As a part of our long-term study we regularly collect blood samples from adults during the chick rearing period each year. I have analysed some of these samples for avian malaria from year 2002 (N=144), 2003 (N=88) and 2004 (N=113). The distribution of parasite lineages is shown in Table 10. Our data clearly demonstrate that two *Haemoproteus* lineages (H-Coll2, and H-Coll3) are the most abundant and show more or less stable prevalence in each year. The prevalence of different *Plasmodium* lineages is low compared to that of *Haemoproteus* species and varies from year to year. Samples were collected also from 10-12 day-old nestlings in order to analyse circulating avian malaria parasites from their blood and to assess the fitness consequences of infection on chicks. Both female and male parents of these nestlings were infected with either *Haemoproteus* or *Plasmodium* species thus the probability for nestlings to be infected in the nest was higher compared to the population average (overall prevalence of malaria in adults was 29.2%, 34.1% and 30.1% in 2002, 2003 and 2004, respectively). However, none of the 23 nestling samples showed positive amplification for these parasites.

Table 10. Distribution of different avian malaria lineages between years in samples collected from 2002, 2003, 2004. "H" means *Haemoproteus* and "P" means *Plasmodium* in lineage names. Two lineages in the heading mean that the individual was infected with two different parasite lineages at the time of sampling.

	Year		
Lineages	2002	2003	2004
Haemoproteus			
H-COLL2	13	4	11
H-COLL3	15	9	9
Plasmodium			
P-AEMO01			1
P-BT8	1	1	
P-COLL1	1		
P-COLL4	1		
P-COLL6		1	1
P-COLL7	1		
P-COLL8	1		
P-COLL9			1
P-COLL10		1	1
P-COLL11		1	
P-GRW4			1
P-GRW9	1	6	3
P-GRW11		1	
P-RTSR1	1		
P-SGS1	2	2	1
P-WW4	3	2	1
Mixed infection			
H-COLL2+H-COLL3			1
H-COLL2+P-GRW9			1
P-COLL8+P-GRW9			1
P-GRW14+P-GRW9			1
Unresolved mixed	2	2	
Not infected	102	58	79
Screened individuals	144	88	113

4.6. A methodological note (study 6)

A subsample (N=186) of adult Collared Flycatchers screened for avian malaria was also tested for *Leucocytozoon* infection. However, out of the screened samples only four showed a positive amplification for *Leucocytozoon* species. Because of the low prevalence of *Leucocytozoon* species I did not investigate the effects of these parasites in any of my studies.

However, 10 of the screened samples showed "false" amplifications for *Leucocytozoon* parasites. In a larger dataset (N=495), including European migrant and African resident bird species (for details on screened bird species see Appendix) I also faced this problem since 123 of the screened samples produced a PCR product, however, in 23 cases the fragments seen on agarose gels were slightly longer than the usual 526-basepair-long *Leucocytozoon* specific fragment including primers (Figure 15). Despite several trials I was not able to sequence these fragments with the primers that are designed for the direct sequencing of *Leucocytozoon* species. New extractions and re-running of the PCRs under sterile conditions gave the same result excluding the possibility of contamination during the reactions. Interestingly, these longer fragments were amplified only if the birds were infected with either *Haemoproteus* or *Plasmodium*. Sequencing with the first primer pair (which is designed for the simultaneous detection of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* species) showed that this 617-basepair-long fragment (including primers) was a *Haemoproteus* or *Plasmodium* sequence indicating „false" amplification during the reactions specific for *Leucocytozoon* parasites. Therefore, next I investigated how these *Haemoproteus* or *Plasmodium* parasite sequences can be amplified in a reaction, which was designed to detect only *Leucocytozoon* species.

I supposed that the reason for this „false" amplification was not that the *Leucocytozoon*-specific primers in some cases amplify avian malaria parasites but instead that certain malaria lineages were amplified somehow better in the first PCR and the result of this amplification was seen also after the second PCR. To test this, I diluted the PCR products from the first reaction to the same concentration as I used in the second reaction and visualized the samples on 2% agarose gel. These tests never resulted in any PCR product visible on the gel suggesting that the first reaction in itself is not enough to result in a visible 617-basepair-long band on the agarose gel after the second PCR.

I then performed a special nested PCR. In the first step I used the same conditions as described by Hellgren et al. (2004). However, in the second PCR specific to *Leucocytozoon* species all reagents except the second primer pair were added to the samples and the reaction was performed. After visualizing the products from the second PCR on an agarose gel I

obtained a 617-basepair-long band that contained the same 570-basepair-long *Haemoproteus* or *Plasmodium* sequence as I got when running the nested PCR with the *Leucocytozoon* specific primers. This means that the primers from the first PCR continued the amplification of the 570-basepair-long fragment from the first reaction also in the second reaction, though no additional primers were added and a 12.5 times dilution was applied.

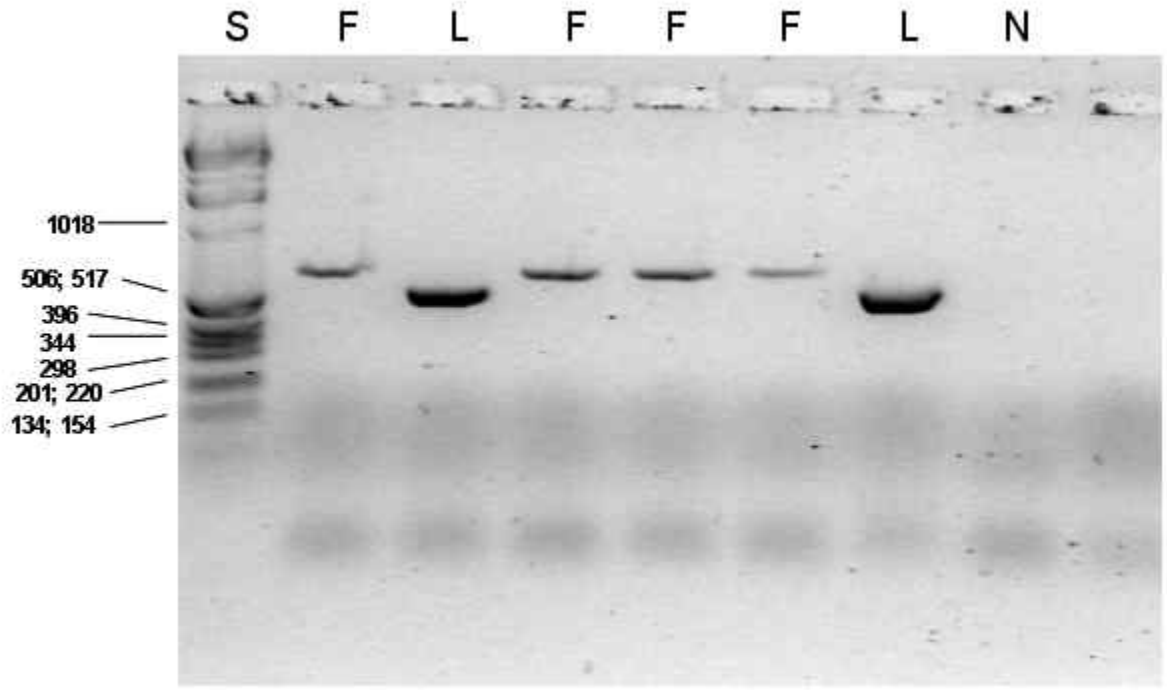

Figure 15. Products from the nested PCR specific to *Leucocytozoon* species after running in 2% agarose gel (for 30 min with 5 V/cm). S: 1 kb molecular standard, L: normal *Leucocytozoon* bands, F: false *Leucocytozoon* bands, N: negative control. Length of different fragments are shown on the left side of the size standard.

5. DISCUSSION

5.1. The role of egg size and hatching asynchrony in nestling growth and performance

I aimed to investigate whether in the asynchronously hatching Collared Flycatcher there exists a compensatory mechanism for the detrimental effects of hatching asynchrony in terms of egg size or the parents magnify the disadvantage of the last chick. From this viewpoint, hypotheses concerning hatching asynchrony (reviewed in Nilsson 1993, Stenning 1996) can be divided into two groups. Some of the hypotheses assume that parents start to incubate before clutch completion in order to enlarge the competitive disparities among nestlings. The *brood reduction hypothesis* (Lack 1954) predicts that under good food supply all young can be fledged independent of hatching order, while in case of food-shortage the smallest nestlings may die but the rest of the brood is fledged in better condition. The pre-determined size hierarchy among nestlings can reduce sibling competition and thus energy expenditure of the chicks (*sibling rivalry reduction hypothesis*: Hahn 1981) resulting in better nestling performance than in broods where all young are of the same size. The pronounced age hierarchy among siblings may also reduce parental energetic costs during feeding because nestlings reach their highest food demand at different times (*peak load reduction hypothesis*: Hussell 1972).

On the other hand a group of the hypotheses assumes that starting the incubation before clutch completion is adaptive for reasons other than establishing sibling size asymmetry. By hatching the nestlings asynchronously a part of the brood can be fledged earlier which is advantageous if there is heavy nest predation or food resources are strongly declining during the chick-rearing period (*nest failure hypothesis*: Clark and Wilson 1981; *hurry-up hypothesis*: Hussell 1972). The early start of incubation may also be adaptive in protecting the viability of eggs (*egg viability hypothesis*: Arnold et al. 1987, Veiga 1992).

If females start to incubate before clutch completion to enhance the competitive disparities among nestlings, they are not expected to compensate for the disadvantages of the last chick moreover they may even reduce the investment into the later laid eggs (Heeb 1994, Schwabl et al. 1997, Viñuela 1997). In other cases compensation for the disadvantages of the last hatched chick may increase the fitness of the females. In species with no conspicuous physical competition but with remarkable hatching asynchrony among nestlings, most hypotheses predict that establishing competitive disparities and reducing investment into later laid eggs has probably no adaptive value (but see *insurance egg hypothesis*: Clifford and

Anderson 2001). Indeed, in the Collared Flycatcher where there is no direct aggression between the nestlings (personal observation), we found that females increased the egg size in relation to laying order, thus probably providing nutritional help for the later hatching young (Figure 4, ***study 1***).

Some previous studies have also found similar egg size increase in relation to laying order (Howe 1976, Cichoń 1997) and hypothesized that it might adaptively reduce the detrimental effects of hatching asynchrony. In ***study 1***, we investigated the growth and fledging size of individual nestlings. We found that body mass before fledging was not related to laying order, which might be the effect of the logistic nature of the growth curve (i.e. older siblings finished their growth few days before fledging, thus last chicks had time to catch up), or the joint effects of logistic mass growth and larger last eggs. The fact that independent of the relative size of the eggs most of the last chicks reached their maximum weight latest on day 14, might indicate that larger egg size is not needed to reach the same size as their older offspring. But it is hard to draw conclusions, because we do not exactly know how nestlings would have grown if they had hatched from smaller eggs. However, nestlings from last laid eggs experienced slower body mass growth (Figure 6, ***study 1***), and had shorter primaries before fledging than their siblings (Figure 7, ***study 1***). These disadvantages were increasing with increasing hatching asynchrony but were partially counterbalanced by the larger egg size.

The hatching asynchrony and nestling development patterns within broods were consistent across years in ***study 1***. The only difference between the two subsequent years was that hatching asynchrony was higher and penultimate eggs hatched relatively later in 2003 (Figure 5), which probably means that in this year females started to incubate their broods earlier. This phenomenon is presumably the consequence of the higher mean temperature in 2003. The daily maximum temperature exceeded 25˚C some days and 20˚C every day during the egg-laying period of the studied broods (Török et al. unpublished data). Such temperatures may cause lower egg viability if eggs are not incubated (von Schalkwyk et al. 1999, Viñuela 2000, Sahan et al. 2003). Thus it may explain why females started to incubate relatively earlier in this year. Alternatively, higher mean temperatures may result in better food supply, which may also cause increased hatching asynchrony (Nilsson 1993).

Similarly to our findings Hargitai and her colleagues (2005) also showed that females laid larger eggs at the end of the laying sequence, however, this pattern was apparent only in good quality years but not in poor years. Therefore I assumed that the size hierarchy might have different effects on parental and offspring fitness depending on environmental conditions so that size hierarchy is adaptive or neutral in bad years but disadvantageous in good years. To

test this we applied a brood size manipulation experiment in ***study 2*** in order to simulate good and bad quality years keeping hatching asynchrony in the natural range.

However, nestlings with a relatively smaller size early in life suffered from reduced performance both in enlarged and reduced broods. They gained body mass more slowly and had shorter wing feathers before fledging (Figure 8b, Figure 9a, ***study 2***). The negative effects of small initial size were even more pronounced in enlarged broods where even feather growth rate and fledgling size (body mass and tarsus length) were correlated with relative size on day 2 (Figure 8, ***study 2***). Reduced growth or bad body condition early in life was reported to have negative impact, for example, on immune responsiveness, intensity of parasite infection, adult condition, elaboration of secondary sexual characters, time of sexual maturation and long-term survival in vertebrates, even if they could catch up later in size (Birkhead et al. 1999, Morgan and Metcalfe 2001, Blount et al. 2003, Stjernman et al. 2004a). Despite these negative effects on individual nestlings it is still possible that size hierarchy has beneficial effects at the brood level or for the parents. However this study showed that average body mass growth of the brood was negatively affected by the initial size variation (Figure 9b, ***study 2***), and even nestlings with a competitive advantage did not benefit from hatching asynchrony.

I conclude that our results on nestling growth and fledgling size in ***study 2*** do not support the predictions of the hypotheses that assume that hatching asynchrony is advantageous because of the pre-determined sibling size asymmetry in the broods. According to the *sibling rivalry reduction hypothesis* (Hahn 1981), nestlings should have experienced a better growth rate or should have fledged in better condition in asynchronous broods, since in these broods chicks could allocate saved energy into their maintenance.

The *peak load reduction hypothesis* (Hussell 1972) also predicts either the nestlings or the parents to benefit from a size hierarchy because in situations of food shortage (poor years or enlarged broods) parents could more easily meet the requirements of their progeny if nestlings reach their maximum energy demand at different times. Thus at least in enlarged broods, nestlings in more asynchronous broods should have grown faster (because they should receive enough food during their rapid growing period) or parents should have had higher survival probability (because they could save time for foraging for themselves in the most demanding phase of chick rearing) than those in synchronous broods. However, parents did not benefit from the increased size variation in the broods since parental survival was independent of the estimated hatching asynchrony under both conditions (for similar results see Stoleson and Beissinger 1997).

Finally, the *brood reduction hypothesis* (Lack 1954) is probably not applicable in Collared Flycatchers, because this hypothesis assumes that parents cannot predict the environment in which they will rear their offspring. In this case we should have found similar investments into the eggs independent of the quality of the year. Furthermore, in this study nestling mortality was very low (altogether 6 out of 162 and 2 out of 110 nestlings died in enlarged and reduced broods, respectively) suggesting that direct aggression is weak between nestlings, disabling efficient brood reduction.

Based on our results on nestling growth and parental survival I conclude that pronounced nestling size hierarchy is not beneficial in the Collared Flycatcher and parents would benefit from a compensatory investment into the last laid eggs. Our results also show that this compensatory investment would be even more beneficial in poor years than it was found to be in good years. This is because asynchronous broods suffered more in enlarged than in reduced broods. The fact that female Collared Flycatchers did not lay larger final eggs in cold years (Hargitai et al. 2005) suggests that they were not able to invest preferentially into those eggs. This was probably because of their poor energetic conditions due to ambient temperatures affecting both the size of insect populations and the activity of flying insects (Taylor 1963, Bryant 1975), thus the food availability to the parents.

Our results on the growth of the nestlings (***study 1***, ***study 2***) are in concordance with previous findings in other species. In the Marsh Tit (*Parus palustris*) mass growth was found to be depressed in the last hatched nestlings as a consequence of hatching asynchrony (Nilsson and Svensson 1996, Nilsson and Gårdmark 2001). On the other hand, the disadvantage of those chicks did not manifest in low feather growth rates (Zach 1982, Nilsson and Svensson 1996). In the Great Tit, parents were found to preferentially feed already fledged young if these and their siblings in the nests were begging simultaneously (Lemel 1989). This suggested that the priority of feather growth to mass growth reflects the importance of synchronized fledging, which in turn may have effects on the survival prospects of the nestlings. We got similar results under natural conditions (***study 1***) and in experimentally simulated "good quality years" (***study 2***), that is nestlings experiencing relatively smaller size early in life showed slower mass but not slower feather growth rates. However, small chicks in enlarged broods (***study 2***) were not able to keep up with their siblings even in feather growth. In this manipulation category, small nestlings also experienced reduced mass growth and smaller size at fledging.

5.2. The role of extra-pair copulations and sex in nestling performance

In addition to egg size and hatching asynchrony, females have further possibilities to alter the survival probabilities of their young within a clutch e.g. by choosing extra-pair mates. In our study population of Collared Flycatchers a previous study showed that forehead patch play an important role in extra-pair mate choice (Michl et al. 2002). By preventing sperm transfer by the social mate and analysing the sperm numbers on the perivitelline layer of the eggs, Michl et al. (2002) came to the conclusion that female Collared Flycatchers mated to males with a large forehead patch were faithful, whereas females mated to small patched males engaged in extra-pair copulations. Contrary to these results, in ***study 3***, forehead patch size of the males was not related to the paternity in their broods. Neither was paternity related to another secondary sexual character, the wing patch of the males and it was also independent of male body size and body condition. Though we have no information on song characteristics of the males, which may also indicate their quality and may be correlated with paternity (Garamszegi et al. 2004a), the observed pattern suggests that participation of females in extra-pair copulations was independent of the quality of their mates. This suggestion is also supported by the fact that extra-pair young did not differ from their half-sibs in any measures of performance. Nestlings grew with the same rate (both before and after hatching) and fledged with the same size and body condition independent of their genetic origin. If only those females which had poor quality social mates engaged in extra-pair copulations, we would have expected to find differences in offspring performance, as reported previously in a Swedish population of Collared Flycatchers. In the Swedish population, extra-pair young fledged in better condition than their within-pair half sibs (Sheldon et al. 1997). It has to be noted, however, that we cannot completely exclude the possibility that females obtained good genes for their young, because good genes may show their effect during e.g. an immune challenge or later in life.

The question arises why two studies conducted in the same study plot came to different conclusions. Though the sample size was moderate in the earlier study due to methodological constraints (Michl et al. 2002) and thus statistical artefact as an explanation cannot be excluded, methodological differences between the studies raise an exciting possibility. By preventing sperm transfer by the social mate and analysing the sperm numbers on the perivitelline layer of the eggs, Michl et al. (2002) could detect extra-pair copulations only during egg laying. However, it is clear from our data that extra-pair young occurred already in the first egg (Figure 16), thus females had to copulate with non-pair males also before egg

laying. Pre-laying copulations with non-pair, non-neighbour males have been suggested in other species too (Dunn et al. 1994). These copulations could have happened before mate choice (when females were visiting multiple male territories) or after mate choice. While in the second case mate quality dependent extra-pair copulation is reasonable to expect, in the first case, females have no knowledge about their future mate, so mate quality independent "extra-pair" copulations are expected. Participation of females in copulations before mate choice could be explained by multiple reasons: a) males may be able to force females to copulate with them because they are not yet guarded; b) females may actively solicit copulations to gain different benefits (see introduction and Griffith et al. (2002) for a review) without risking reduced paternal care. Anyway, if many females participate in male quality independent extra-pair copulation before mate choice but only those females continue the pursuit of extra-pair copulations after mate choice, which finally end up with a low quality male, different investigation methods may come to different conclusions. By counting the sperms on the perivitelline layers, only those extra-pair copulations can be detected which happened after the start of egg laying, so the investigators are expected to find male-quality dependent extra-pair copulation pattern like Michl et al. (2002). However, sperms from premating copulations may survive until egg laying and result in extra-pair young, so paternity analysis of nestlings may show that extra-pair copulations are independent of male quality.

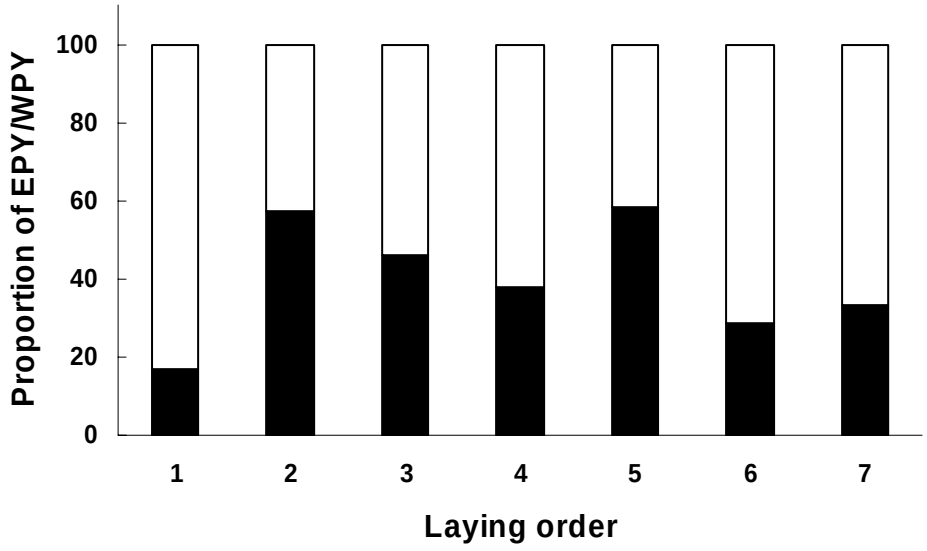

Figure 16. The proportion of extra-pair (black) and within-pair (white) young in relation to laying order in mixed-paternity broods.

While the above hypothesis may explain the difference found within the same study plot using different methods, differences among studies using the same method (Sheldon and Ellegren 1999, Garamszegi et al. 2004a, Krist et al. 2005 and ***study 3***) are hard to explain. However, I would like to highlight an interesting point that is male quality was suggested to have a role in extra-pair copulations in studies with low proportion of mixed paternity broods (Sheldon and Ellegren 1999, Garamszegi et al. 2004a; 32.9% and 30.6%, respectively), while studies which found high proportion of mixed paternity broods (Krist et al. 2005 and ***study 3***, 51.9% and 55.74%, respectively) came to the opposite conclusion. This is not surprising given that it is unlikely that more than half of the females are mated to low quality males.

The role of female characteristics in extra-pair copulations has received little attention so far. The constrained female hypothesis (Mulder et al. 1994, Gowaty 1996) suggests that females, which need less help from the social mate in rearing the chicks are more likely to risk reduced care as a cost of extra-pair matings. As a consequence, one may expect that good quality females are more likely to have extra-pair young. Male preference for high quality females in extra-pair matings would also result in such a pattern. However, our data show that larger females (suggested to be of better quality; Garamszegi et al. 2004c) were less likely to participate in extra-pair copulations (Figure 10, ***study 3***). If extra-pair copulations (or premating-copulations) are mainly due to males forcing unguarded females to copulate (Clutton-Brock and Parker 1995), the pattern could be explained by large females being more likely to successfully counteract these attempts. Alternatively, small, low quality females may benefit more from extra-pair copulations, however, it is unclear how, because extra-pair young did not perform better than within-pair young.

Though the primary goal of ***study 3*** was to investigate factors, which determine paternity in Collared Flycatcher broods and the effect of paternity on offspring performance, we also included offspring sex in the latter analyses as it may confound the observed growth patterns for the following reasons. First, females may manipulate the sex of their offspring in relation to paternity, if paternity of an offspring is predictable for example on the basis of laying order. Second, even in sexually size monomorphic species males and females may grow at different rates (Martins 2004). We found that the paternity of the young was not related to laying order, and similarly to the results of a previous study on a Swedish population (Sheldon and Ellegren 1996), the sex ratio of extra- and within-pair young did not differ. However, male nestlings had a faster body mass growth than females, even if females had caught up in body mass by day 14 (Figure 11, ***study 3***). Surprisingly, on day 14 the tarsus of the females was longer than that of males (Figure 12, ***study 3***). This apparent contradiction may be explained

by the different developmental states of the sexes. Body mass growth data imply that male nestlings grew faster, suggesting that on day 14 sons were more developed. Tarsus measurements of the nestlings show a slight decrease after the tarsus reaches its maximal length, probably due to water loss from the tissues. If males reach this phase of development faster, their tarsus size may appear to be smaller and consequently the estimate of female condition become systematically lower than that of males (similar sex difference in body condition was found in Krist et al. 2004). Indeed, in a small subset of the chicks where we measured tarsus length also during development, the change in tarsus length between day 14 and the day when the largest tarsus was measured prior to day 14 was more negative in males than in females (males = -0.604%, n = 23; females = -0.178%, n = 26). The difference (0.426%) is rather close to the difference observed between the tarsus length of males and females on day 14 (0.7%). This result clearly shows that caution has to be taken when using the residual body mass as an estimate of body condition in developing young, because it may primarily be determined by the developmental state of the offspring.

Our results on sex dependent growth rates have other implications too. In a previous study on sex ratio adjustment in Collared Flycatchers, Rosivall and his colleagues (2004) showed that females produced male-biased brood sex ratios late in the season. They then hypothesized that this pattern could be adaptive if male nestlings perform better late in the season. For example, faster development of males may be beneficial because this allows for earlier fledging. On the other hand faster development may require more resources and result in developmental failures when food is scarce. Further studies should clarify whether sexual differences in growth rate are dependent on rearing conditions and explain the observed sex ratio pattern like in the zebra finch (Kilner 1998, Martins 2004).

5.3. The role of parental quality in nestling performance

Reproductive allocation of birds is constrained also by their own health state. For example birds infected with avian malaria are often in poor nutritional condition and as a result their pre- and post-natal parental investments are reduced (Sanz et al. 2001, Marzal et al. 2005). This alter also the growth (Goodbred and Holmes 1996) and fledging success of the nestlings (Voltura et al. 2002). Unlike these studies we did not find any effect of malaria infection in the genetic mothers on the growth rate or fledging size of their nestlings (***study 4***), suggesting that presence of malaria in the blood did not alter maternal incubation behaviour or allocation of essential egg components. Though we did not directly measure parental care we suppose

that malaria parasites probably have limited effects on parental care, since malaria infection in the rearing parents did not correlate with the performance of their nestlings. I have to note, however, that because of the low prevalence of avian malaria in adult individuals (only 30.2% of them were infected), I was unable to separately analyse the effects of different malaria lineages on nestling performance. In addition, by using a PCR method I probably detected both acute and chronic avian malaria infections and as a previous study showed that chronic malaria infections may have no effect on the reproductive success of the birds (Kilpatrick et al. 2006).

Though in ***study 4*** we did not find negative effects of parental avian malaria infection on nestling performance, it can still be beneficial for the females to choose males that are free from blood parasites. If these males are resistant to malaria infections this mate choice would confer indirect benefits to the offspring through resistance genes (Barber et al. 2001, Langefors et al. 2001, Lohm et al. 2002). According to the Hamilton-Zuk hypothesis (1982), more elaborate secondary sexual characters may indicate resistance against blood parasites (Figuerola et al. 1999). In contrast to the predictions of this hypothesis we did not find any relationship between malaria infections and secondary sexual characters. This result is not surprising regarding that forehead patch is not a condition dependent secondary sexual signal in our population (Hegyi et al. 2002, 2006a), however, wing patch was expected to be linked to malaria infection via its condition dependence (Török et al. 2003). Alternatively, Stjernman et al. (2004b) proposed that stabilising selection may act on parasite resistance and the size of the ornaments is optimized to maintain a standard level of parasite burden. This is because both too low and too high defense against parasites would result in fitness related costs for the birds (Råberg et al. 1998). Furthermore we included only adult (i.e. more than 1-year-old) males in this study to control for the possible confounding effects of paternal age on offspring growth (Hegyi et al. 2006b). It is possible that these birds acquired the infection a year before in the breeding territory or at their wintering quarters in Africa and thus being in the chronic phase of the infection there was no detectable effect of parental malaria infection on nestling performance and on the expression of secondary sexual characters.

Though forehead patch size of the males was not related to malaria infection our data suggest that females may benefit from mating with males that have a large forehead patch because nestlings reared by such males had a faster wing feather growth (which has been previously reported to have important fitness consequences, see above) (Figure 14, ***study 4***). Fast feather growth of nestlings reared by large forehead patched males suggests that these nestlings were developing under better conditions and thus that they could allocate more

resources into feather growth than nestlings of smaller patched males. This result could be caused by several factors. It may indicate that more ornamented males are better fathers (Linville et al. 1998, Buchanan and Catchpole 2000), that they have better food sources on their territories (Keyser and Hill 2000) or that females mated to high-quality males invest more into their nestlings during chick rearing (Limbourg et al. 2004). Interestingly, the condition dependent wing patch size of the males was not related to nestling performance in Collared Flycatchers. One possible explanation is that large patched males are subjected to increased aggression during the mating period (Garamszegi et al. 2006) and cannot invest more in the chicks even if they are of superior quality.

One may argue that the lack of positive relationship between the size of the secondary sexual characters of the putative fathers and the growth of their nestlings can be explained by differential maternal allocation into eggs of less ornamented fathers which are of worse parental quality. However, previous studies on this species showed no differential maternal investments in relation to male ornamentation (for egg size see: Hargitai et al. 2005, for carotenoids see: Török et al. 2007) though paternal care was not assessed.

Tarsus length of the genetic fathers correlated positively with wing feather length of their 14 days old offspring (Figure 13a, ***study 4***). This may either reflect that larger males produce larger offspring or that larger males have better genes that are inherited by their offspring making them superior in utilizing resources or coping with stress allowing them to reach a larger size. Since nutrients, carotenoids, hormones and antibodies that females allocate into the eggs can have long lasting effects on nestling performance (Schwabl 1996, Biard et al. 2005, Reid et al. 2006, ***study 1***) it is also possible that the above relationship is the result of preferential maternal investment in the eggs and not of genetic effects. However, no preferential investment of egg components in relation to paternal size was found in our population of Collared Flycatchers (Hargitai et al. 2005, Török et al. 2007).

For several species it has been demonstrated that larger males are better fathers (Keyser and Hill 2000) or occupy better territories (Keyser and Hill 2000, Candolin and Voigt 2001) which in turn significantly affects the size of their offspring (Kruuk et al. 2001). Our results also suggest that body size indicates paternal quality, since also the wing feather length of fledglings correlated positively with the tarsus length of the rearing fathers (Figure 13b, ***study 4***).

5.4. Avian Haemosporidian parasites in the Collared Flycatcher

Acute avian malaria infections may have serious consequences on the hosts' health and reproductive success (Atkinson and van Riper III 1991, Valkiūnas 2005). However, distinguishing between the different infection stages (Figure 1) would require the thorough investigation of regularly collected blood samples and this is difficult for wild species without much disturbance. Therefore in ***study 5*** I intended to study the effects of avian malaria parasites on Collared Flycatcher nestlings because they can be infected only after hatching, so their infection stage should certainly be acute (Hasselquist et al. 2007). I used a PCR-based molecular method (Waldenström et al. 2004) which is more sensitive than the microscopical investigation of blood smears. It successfully and reliably detects infections in as low intensities as 1 parasite per 100.000 host blood cells and sometimes also in dilutions corresponding to 1 parasite per 1.000.000 host blood cells (Waldenström et al. 2004). Therefore I predicted that avian malaria could be detected soon after the prepatent period, i.e. when these parasites appear in the blood. However, none of the 23 samples collected from 10-12-day-old nestlings showed any signs of infection even though their parents were infected with either *Haemoproteus* or *Plasmodium*. The lack of *Haemoproteus* parasites in the blood of the nestlings is not surprising given that the prepatent period of these parasites varies between 11 days and three weeks. However, the prepatent period of *Plasmodium* spp. generally does not exceed five days (Valkiūnas 2005). To be able to detect these parasites from blood samples of 10-12-day-old nestlings, however, infection should have occurred early in life (i.e. until 5-6 days of age) when nestlings are still ectothermic. But actively brooded nestlings are difficult targets for mosquitoes. This fact may explain the lack of these parasites in nestlings' blood.

In addition, it is possible that out of the 19 avian malaria lineages found in adult birds (Table 10, ***study 5***) only a small proportion may be transmitted at the birds' breeding sites thus be detected also in nestlings. Though it has been demonstrated that migratory birds can be infected at their breeding sites in Europe, at stopover sites during migration and also at the wintering sites in Africa (Waldenström et al. 2002) it has also been shown that these parasites generally have highly restricted transmission areas and only a few species are transmitted both in Europe and Africa (Waldenström et al. 2002, Hellgren et al. 2007). Indeed, until now only three *Plasmodium* lineages are proved to be transmitted in Europe (P-COLL1, P-GRW11, P-SGS1; Hellgren et al., 2007, Szöllősi et al. unpublished data). Two out of them

were also shown to be transmitted at our study site (P-GRW11, P-SGS1; Szöllősi et al. unpublished data) because they were detected also from resident species.

The remaining 16 avian malaria lineages detected in adult Collared Flycatchers have been found only in migratory and in some African resident species until now. However, the lack of information on European transmission of these parasites does not mean that they are transmitted elsewhere, since until now only 450 bird species were sampled with various efforts from all over the world (http://mbio-serv4.mbioekol.lu.se/avianmalaria).

Differences between the prevalence of various parasite lineages in adults (i.e. *Haemoproteus* lineages are more common and do not fluctuate as much between years as *Plasmodium* lineages) might be explained by the fact that *Plasmodium* species are more pathogenic than *Haemoproteus* parasites (Atkinson and van Riper III 1991). Thus only those individuals, which have survived the infection, can breed and as a result sampled. In addition, different parasite species within the same genus may also have different pathogenicity. I suppose that those parasites are less virulent which can persist for several years within the same individual and thus cause chronic infections for the host. However, to be able to investigate this question the analysis of a larger dataset is needed in which the fates of different host individuals and/or different lineages are followed throughout years or the whole life.

5.5. Problems when using nested PCR for parasite screening

I applied a widely used nested PCR protocol (Hellgren et al. 2004) in the detection of avian Haemosporidian parasites and found that in some cases when the birds were infected with avian malaria the primers from the first PCR (which amplify a common sequence of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*) continued the amplification of the fragment from the first reaction also in the second reaction which is specific to *Leucocytozoon* parasites. Even though no additional primers were added and a 12.5 times dilution was applied (***study 6***). The fact that I did not always obtain these longer fragments when the birds were infected with avian malaria species suggests that there are a few lineages for which the amplification by the first primer pair is stronger. Indeed, out of the 63 avian malaria lineages that were found in our 181 malaria positive samples, only 9 produced these 617-basepair-long "false" *Leucocytozoon* bands (see Appendix). The 9 lineages that caused these amplifications were not particularly closely related (mean Jukes-Cantor distance: *Haemoproteus* spp.: 0.062 ± 0.008 S.E.; *Plasmodium* spp.: 0.059 ± 0.006 S.E.) compared to the mean genetic distance

between all lineages in our database (mean Jukes-Cantor distance: *Haemoproteus* spp.: 0.055 ± 0.006 S.E.; *Plasmodium* spp.: 0.058 ± 0.006 S.E.). This indicates that the 9 lineages do not group into a closely related clade of avian haemosporidians and the strong amplification might be a result of high parasite intensity, or similar differences in the primer binding sites that increases the amplification success. The latter notion is supported by the study of Valkiūnas et al. (2006) who found that in case of mixed infections of avian malaria parasites, some lineages were detected preferentially, but that this was not related to the level of parasitemia of different lineages in the blood. In addition, Sowmya et al. (2006) and Sipos et al. (2007) showed that differences in the primer-binding sites can affect the amplification success.

Based on these results, I suggest the necessity to apply molecular standards and positive controls in each gel during the detection of *Leucocytozoon* parasites and run PCR products in a well separating agarose gel for a period long enough to be able to detect differences between fragment lengths. If deviations from the standard *Leucocytozoon* fragment length are detected, then identification of the different fragments is essential to avoid the risk of considering "false" detections as normal *Leucocytozoon* infection. More generally, to avoid the problems that primer pairs from the first reaction continue to amplify also in the second PCR when using a nested approach, I suggest the following. First, primer pairs should be designed so that the optimal annealing temperatures for the first and second primer pairs, if possible, are different. Second, the amount of primers used in the first reaction should be optimized so that the amount of leftover is reduced without affecting the outcome of the results. Third, a cleaning step should be inserted after the first PCR and only the cleaned PCR product should be carried over in the second reaction.

6. FINAL REMARKS AND PERSPECTIVES

Results presented in this thesis (***study 1***) together with previous papers on the asynchronously hatching Collared Flycatcher (Hargitai et al. 2005, 2007, Török et al. 2007) suggest that this species follows a compensatory rather than a brood reduction strategy when they allocate egg components in relation to laying order. The amount of nutrients (***study 1***, Hargitai et al. 2005), carotenoids (Török et al. 2007) and immunoglobulins (Hargitai et al. 2007) increase in the eggs with laying order. The lack of relationship between egg size and laying order in bad years suggests that food supply probably acts as a constraint and laying

females are not able to compensate for the disadvantages of the last hatched nestlings (Hargitai et al. 2005). This is supported by the fact that hatching asynchrony is not adaptive in this species, since neither the broods on average nor parents benefited from the pre-determined size hierarchy. The negative effects of hatching asynchrony were even more pronounced in bad years (***study 2***).

Therefore the question arises why females still hatch their broods asynchronously and what factors initiate incubating behaviour. Though changes in hormonal levels in the females are the proximate determinants of the start of incubation behaviour (Mead and Morton 1985), from the evolutionary point of view, the identification of ultimate factors, such as environmental conditions is more interesting. High daily temperatures during laying cause lower egg viability if eggs are not incubated (von Schalkwyk et al. 1999, Viñuela 2000, Sahan et al. 2003) therefore starting to incubate in time is crucial from the viewpoint of brood survival. Indeed, in a year with warmer mean temperatures during laying, hatching asynchrony was more pronounced in the broods (***study 1***) suggesting that females started to incubate earlier than in colder years. However, to understand the findings of correlative studies experimental approaches are needed to demonstrate that nest temperature has indeed important role in the initiation of incubating behaviour of the females.

In ***study 3*** we found that secondary sexual characters of the social mate did not play an important role in extra-pair copulations and a high proportion of the females cuckolded their mates. In this study we only were able to identify whether a young was related to their social parents but the genetic fathers of the extra-pair young could not be determined. However, to understand the various benefits that arise from extra-pair copulations it would be important to identify the genetic fathers also of extra-pair young. This would require a more extensive laboratory work during which also samples from neighbouring males (with which females could have cuckolded their mates) should be analysed. In addition, more primers should be developed and optimised to identify the extra-pair fathers of these nestlings.

In a correlative study (***study 4***) I found that secondary sexual characters did not signal the ability of males to avoid malaria infections and nestlings of infected parents did not perform worse than young reared by malaria free parents. However, in this study chronic and acute infections could not be separated. To distinguish between chronic and acute infections blood samples should regularly be collected and analysed by microscopy or by quantitative PCR methods. However, regular collection of blood samples from wild species is difficult without much disturbance. In addition, the low prevalence of parasite lineages did not allow for the separate analysis of the effects of different avian malaria species though their pathogenicity

probably varies between lineages. To investigate this, sample size should be increased and the fate of different host individuals and/or different lineages should be followed throughout years or even the whole life.

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9. SUMMARY

One of the most important questions for iteroparous species is that how much to invest into current reproduction so that they have enough energy for self-maintenance, survival and future reproduction. Environmental and social conditions, furthermore the actual health status of the individuals can all have strong effects on the availability of resources which may affect the investment between and within reproductive events.

In my thesis I investigated how egg size affects the growth and fledging size of Collared Flycatcher nestlings and whether females reduce or enhance the size handicap of the asynchronously hatching last nestlings by differential allocation of nutrients into the last egg. We found that nestlings from the last laid eggs hatched later and experienced slower growth. However, the disadvantage of these nestlings was partially counterbalanced by the larger eggs from which they hatched. Since a previous study found that this compensatory mechanism was present only in years with good food supply but not in bad years, we tested whether hatching asynchrony also has different effects on fitness under different conditions. We found that hatching asynchrony has negative effects both under good and bad conditions and females would benefit from laying larger eggs at the end of the laying sequence independent of year quality.

I also investigated whether the expression of secondary sexual characters indicates the ability of males to avoid malaria infections and if malaria infection and quality of the parents affect the growth of their nestlings. Though secondary sexual signals did not predict resistance against avian malaria we found that wing feathers of nestlings reared by fathers with a large forehead patch grew faster. We further investigated the role of secondary sexual characters in extra-pair copulations. We predicted that the probability that females participate in extra-pair copulations depends on the quality (ornamentation) of their mates. However, secondary sexual signals of the mates did not predict the females' participation in extra-pair copulations and extra-pair nestlings did not grow better or fledge with a larger size. Independent of the paternity of the nestlings we found that male nestlings grew faster, however further studies should clarify the function of sex dependent growth rate in this species. In summary, the results presented in my thesis show that different constraints and adaptive decisions influence nestling performance and thus fitness of the Collared Flycatcher.

10. ÖSSZEFOGLALÓ

Az egyik legfontosabb kérdés iteropár fajok számára az, hogy hogyan osszák szét az elérhető forrásokat a különböző szaporodási események között úgy, hogy azokból önfenntartásukra és túlélésükre is maradjon. Miután az egyed környezete, szociális körülményei és aktuális egészségi állapota is komoly hatást gyakorolhat a források elérhetőségére és eloszthatóságára, ezért a különböző szaporodási események közötti, sőt az adott szaporodási eseményen belüli egyenletes forráselosztás sem feltétlenül maximalizálja az egyed rátermettségét.

Doktori dolgozatomban azt vizsgáltam, hogy a tojások mérete hogyan befolyásolja az örvös légykapó fiókák növekedését és kirepülési méretét és vajon a tojó madarak az utolsó tojásokba való differenciális tápanyag befektetéssel növelik, vagy csökkentik az utolsó fiókák kelési aszinkróniából eredő méréthátrányát. Azt találtuk, hogy az utolsó tojásokból származó fiókák később keltek és lassabban növekedtek mint testvéreik, azonban méréthátrányukat a tojók részben kompenzálni tudták azzal, hogy az utolsó tojásokba több tápanyagot juttattak. Egy korábbi tanulmány szerint azonban ez a kompenzációs mechanizmus csak jó táplálékellátottságú években volt kimutatható, míg rossz években a tojásméret nem nőtt a tojások lerakási sorrendjével. Ezért megvizsgáltuk azt is, hogy a kelési aszinkrónia rátermettségre gyakorolt hatása eltérő-e különböző évtípusok között. Azt találtuk, hogy a kelési aszinkrónia sem jó, sem pedig rossz körülmények között nem előnyös, és évtípustól függetlenül adaptív lenne az utolsó fiókák méréthátrányát kompenzálni.

Azt is vizsgáltuk, hogy a hím örvös légykapók másodlagos nemi jellegeinek kifejeződése jelzi-e a maláriás fertőzések elkerülésére/legyőzésére való képességüket, illetve hogy a szülők maláriával való fertőzöttsége és minősége hogyan befolyásolja a fiókák növekedését. A másodlagos nemi jellegek mérete ugyan nem jelezte a hímek maláriával szembeni rezisztenciáját, viszont a nagy homlokfolttal rendelkező hímek által nevelt fiókák szárnytollai gyorsabban növekedtek. A másodlagos nemi jellegek fontos szerepet játszhatnak a páron kívüli párzások során is, hiszen a tojók félrelépésének valószínűsége összefügghet szociális párjuk minőségével, ornamentáltságával. Azonban eredményeink szerint a tojók páron kívüli párzásokban való résztvételének valószínűsége nem függött szociális párjuk másodlagos nemi bélyegeinek kifejeződésétől. Így az sem meglepő, hogy a páron kívüli párzásokból származó fiókák nem növekedtek gyorsabban és nem értek el nagyobb kirepülési méretet, mint testvéreik. Függetlenül attól azonban, hogy a fiókák szociális vagy páron kívüli párzásokból származtak, azt találtuk, hogy a hím fiókák gyorsabban nőttek, mint tojó testvéreik, azonban

az ivarfüggő növekedés funkciójának felderítéséhez további tanulmányokra lenne szükség. Összefoglalva, az eredményeim azt mutatják, hogy különböző kényszerek és adaptív döntések együttesen határozzák meg a fiókák növekedését és így az örvös légykapó rátermettségét.

11. PUBLISHED PAPERS AND MANUSCRIPTS INCLUDED IN THE THESIS

study 1: Rosivall, B., **Szöllősi, E.** and Török, J. 2005. Maternal compensation for hatching asynchrony in the Collared Flycatcher (*Ficedula albicollis*). *Journal of Avian Biology* 36: 531-537**.**

study 2: Szöllősi, E., Rosivall, B. and Török, J. 2007. Is hatching asynchrony beneficial for the brood? *Behavioral Ecology* 18: 420-426

study 3: Rosivall, B., **Szöllősi, E.**, Hasselquist, D. and Török, J. (in press). Effects of extra-pair paternity and sex on nestling growth and condition in the collared flycatcher (*Ficedula albicollis*). *Animal Behaviour*

study 4: Szöllősi, E., Rosivall, B. Hasselquist, D. and Török, J. (in revision). The effect of parental quality and malaria infection on nestling performance in the collared flycatcher *Ficedula albicollis*. *Journal of Ornithology*

study 6: Szöllősi, E., Hellgren, O. and Hasselquist, D. 2008. A cautionary note on the use of nested PCR for parasite screening – An example from avian blood parasites. *Journal of Parasitology* 94: 562-564.

12. OTHER PUBLICATIONS

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13. APPENDIX

Species screened for the presence of avian malaria and *Leucocytozoon* in study 6. Malaria lineages which produced "false" *Leucocytozoon* bands are in bold. "H" means *Haemoproteus*, "P" means *Plasmodium* and "L" means *Leucocytozoon* in lineage names.

Common name	Scientific name	Family	Malaria lineage	Leucocytozoon lineage
African Paradise Flycatcher	Terpsiphone viridis	Monarchidae	P-AEMO01	
African Thrush	Turdus pelios	Turdidae	P-PLASM29	L-LEUCO40
			P-PLASM32	L-LEUCO41
			P-SYAT05	
African Yellow White-Eye	Zosterops senegalensis	Zosteropidae	H-HAEMO21	L-LEUCO59
			H-HAEMO22	
Bar-Breasted Firefinch	Lagonosticta rufopicta	Estrildidae		
Beautiful sunbird	Nectarinia pulchella	Nectarinidae	H-HAEMO13	
Black-Bellied Firefinch	Lagonosticta rara	Estrildidae		
Blackcap Babbler	Turdoides reinwardtii	Timaliidae	H-HAEMO14	
Black-Necked Weaver	Ploceus nigricollis	Ploceidae	H-HAEMO9	
			H-HIPOL1	
Black-Rumped Waxbill	Estrilda troglodytes	Estrildidae	H-HAEMO15	
Black-Winged Bishop	Euplectes hordeaceus	Ploceidae		L-LEUCO44
Bronze Manakin	Lonchura cucullata	Estrildidae		
Bush Petronia	Petronia dentata	Passeridae	H-HAEMO11	
Cinnamon-Breasted Bunting	Emberiza tahapisi	Emberizidae	P-SGS1	
			P-SYAT05	
Common Bulbul	Pycnonotus barbatus	Pycnonotidae	H-HAEMO23	
			H-HAEMO24	L-LEUCO43
			P-GRW9	
			P-WW3	
Collared Flycatcher	Ficedula albicollis	Muscicapidae	**H-COLL3**	
			H-COLL2+ H-COLL3	
Common Whitethroat	Sylvia communis	Sylviidae	H-HAEMO18	L-LEUCO49
			H-HAEMO19	
			H-HAEMO20	
Copper Sunbird	Cinnyris cupreus	Nectarinidae	P-PLASM39	L-LEUCO51
Dorst's Cisticola	Cisticola dorsti	Cisticolidae		L-LEUCO54
Eurasian Wryneck	Jynx torquilla	Picidae		
Garden Warbler	Sylvia borin	Sylviidae	H-SYBOR1	L-LEUCO46
			P-GRW2	L-S3
			P-GRW11	L-S5
			P-PLASM26	
			P-PLASM30	
			P-PLASM33	
			P-SGS1	
			P-TURDUS1	
Green-Headed Sunbird	Cyanomitra verticalis	Nectarinidae	H-HAEMO15	
Grey-Backed Camaroptera	Camaroptera brevicaudata	Cisticolidae	P-PLASM37	L-LEUCO59

Common name	Scientific name	Family	Malaria lineage	Leucocytozoon lineage
Icterine Warbler	Hippolais icterina	Sylviidae		
Lavender Waxbill	Estrilda caerulescens	Estrildidae	**H-HAEMO15**	
Little Weaver	Ploceus luteolus	Ploceidae		
Long-Billed Pipit	Anthus similis	Motacillidae	P-PLASM35	L-LEUCO59
Melodious Warbler	Hippolais polyglotta	Sylviidae	**H-HIPOL1**	
Mocking Cliff-Chat	Myrmecocichla cinnamomeiventris	Muscicapidae	P-BSR2	
Nightingale	Luscinia megarhynchos	Muscicapidae		
Northern Crombec	Sylvietta brachyura	Sylviidae		
Orange-Cheeked Waxbill	Estrilda melpoda	Estrildidae		
Pied Flycatcher	Ficedula hypoleuca	Muscicapidae	H-PFC1	L-LEUCO52
			P-SYBOR2	
Pin-Tailed Whydah	Vidua macroura	Viduidae		
Plain-Backed Pipit	Anthus leucophrys	Motacillidae	P-PLASM25	
Pygmy Sunbird	Hedydipna platura	Nectarinidae	P-PLASM38	
Quail Finch	Ortygospiza atricollis	Estrildidae		
Red Bishop	Euplectes orix	Ploceidae	**H-HAEMO1**	L-A12
			H-HAEMO2	L-LEUCO44
			H-HAEMO3	L-LEUCO53
			H-HAEMO4	L-LEUCO54
			H-HAEMO5	L-LEUCO57
			P-PLASM27	L-LEUCO58
			P-PLASM34	L-LEUCO59
				L-LEUCO69+ L-LEUCO70
Red-Billed Firefinch	Lagonosticta senegala	Estrildidae	P-PLASM28	
Red-Cheeked Cordon Bleu	Uraeginthus bengalus	Estrildidae	H-HAEMO16	L-LEUCO56
			H-HAEMO17	L-LEUCO59
			H-HAEMO16+ H-HAEMO17	
			H-WW1	
			P-PLASM28	
			P-PLASM34	
Red-Winged Warbler	Heliolais erythropterus	Cisticolidae	P-PLASM34	
Rock-Firefinch	Lagonisticta sanguinodorsalis	Estrildidae	P-PLASM34	
Scarlet-Chested Sunbird	Chalcomitra senegalensis	Nectarinidae	**P-PLASM28**	L-LEUCO55
				L-LEUCO59
Singing Cisticola	Cisticola cantans	Cisticolidae	P-PLASM34	
Snowy-Crowned Robin Chat	Cossypha niveicapilla	Muscicapidae		
Speckle-Fronted Weaver	Sporopipes frontalis	Ploceidae		L-LEUCO54
Sulphur-Breasted Bush-Shrike	Malaconotus sulfureopectus	Malaconotidae		L-LEUCO47
Sun Lark	Galerida modesta	Alaudidae	P-PLASM36	
Tawny-Flanked Prinia	Prinia subflava	Cisticolidae		
Tree Pipit	Anthus trivialis	Motacillidae	H-YWT2	L-LEUCO48
			P-PLASM25	L-LEUCO50
Variable Sunbird	Cinnyris venustus	Nectarinidae	H-HAEMO12	
			P-PLASM28	
			P-PLASM39	
Vieillot's Barbet	Lybius vieilloti	Capitonidae		

Common name	Scientific name	Family	Malaria lineage	Leucocytozoon lineage
Village Weaver	Plocues cucullatus	Ploceidae	H-HAEMO6	L-LEUCO44
			H-HAEMO7	L-LEUCO59
			H-HAEMO8	
Vitelline Masked Weaver	Ploceus velatus	Ploceidae	H-HAEMO8	L-LEUCO44
			H-HAEMO10	L-LEUCO59
			P-COLL7	L-LEUCO60
			P-WW3	
West African Thrush	Turdus pelios	Turdidae	**P-PLASM29**	L-LEUCO42
Whinchat	Saxicola rubetra	Muscicapidae	H-HIICT1	L-LEUCO53
			H-ROBIN1	
			P-SGS1	
White-Crowned Robin Chat	Cossypha albicapilla	Muscicapidae		
Willow Warbler	Phylloscopus trochilus	Sylviidae	H-HAEMO24	L-BT1
			H-WW1	L-LEUCO45
			P-PLASM31	
Yellow-Penduline Tit	Anthoscopus parvulus	Remizidae		
Yellow-Fronted Tinkerbird	Pogoniulus chrysoconus	Capitonidae		
Yellow-Mantled Widowbird	Euplectes macrourus	Ploceidae	H-HAEMO4	L-LEUCO54
			H-HAEMO22	L-LEUCO55
			H-WW1	L-LEUCO61
			P-PLASM27	
			P-PLASM34	
Zitting Cisticola	Cisticola juncidis	Cisticolidae		