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Causes and consequences of life- history variation

*The effects of parasites, glucocorticoids, and
environmental conditions in the collared flycatcher
(*Ficedula albicollis*)*

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Abstract

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Life-history is the study of all the different stages of life that affect reproductive success and survival between the birth and death of an organism. The reproductive output of an organism is constrained by many things including time, resource, disease agents and environmental conditions. In addition, lineage-specific traits and the limitations of the physiological systems can limit how an organism responds to ecological processes, and thus constrains the variation of life histories represented in nature. Central to the theory of life history are the trade-offs that organisms make during their lifetime to maximise their reproductive potential. In this thesis, I focus on the effect of haemosporidian blood parasites on host life history, in relation to the glucocorticoid response and environmental conditions. The host study species is a population of collared flycatchers (*Ficedula albicollis*), a species that provides bi-parental care, located in the south of Gotland. We show that nestling condition predicts parasite infection and that parasite-mediated selection can start early on in the birds' life. We also found a link between Lower levels of glucocorticoids and parasite infection, which might indicate a trade-off between immunity and reproductive effort. Adult birds' upregulated glucocorticoids in response to an increase in reproductive effort and a predictable change in energy demand during reproduction. I also show that glucocorticoids respond to changing environmental conditions. These results together accentuate the importance of the plasticity of the glucocorticoid response to reproductive success. Moreover, higher levels of hormone during reproduction predicted survival to the next breeding season. In nestlings, glucocorticoid levels increased as a consequence of parent infection status and an increase in reproductive effort. Overall, our results indicate that the glucocorticoid response is context dependent. Finally, female collared flycatchers might pay a fitness cost as a consequence of parasite infection, but can still reproduce successfully suggesting that they can tolerate the parasite. To further our understanding of costs related to parasite infection, we must understand better the mechanisms that enable the host to tolerate infection. This study indicates that glucocorticoids provide a useful tool to detect how wild birds respond to predictable and unpredictable challenges.

Keywords: Collard flycatcher, Haemosporidian blood parasite, glucocorticoid, life history

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To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. The pattern of haemosporidian blood parasite infection in collared flycatchers during their lifetime. *Manuscript.*
- II. Experimental brood size manipulation and parental infection status affect development stress as revealed by nestling feather corticosterone. *Resubmission.*
- III. Upregulation of baseline corticosterone in response to the life-history stage and a brood experiment detected in the droppings of a wild bird. *Submitted manuscript.*
- IV. Glucocorticoid level during reproduction predicts survival but is suppressed in response to parasite infection in collared flycatchers. *Manuscript.*
- V. Methods of glucocorticoid sampling in a natural population. *Manuscript.*

Papers written during my thesis but not included in my thesis:

Griesser, M., Mourocq, E., Barnaby, J., Bowgen, K. M., Eggers, S., Fletcher, K., ... Ekman, J. (2017). Experience buffers extrinsic mortality in a group-living bird species. *Oikos*, *126*(9), 1258–1268. doi:10.1111/oik.04098

Causes and consequences of life-history variation

The effects of parasites, glucocorticoids, and environmental conditions in the collared flycatcher (*Ficedula albicollis*).



Drawing by Juho Könönen 2015.

“We are the offspring of history, and must establish our own paths in this most diverse and interesting of conceivable universes—one indifferent to our suffering, and therefore offering us maximal freedom to thrive, or to fail, in our own chosen way.”

- Stephen J. Gould

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Abbreviations

GC

BCI

Cort

cort_r

Mcort

Glucocorticoid

Body condition index

Corticosterone

Corticosterone

Metabolised corticosterone

Introduction

Life history

Life history evolution, which bids to understand lifecycle patterns, such as birth, death, and reproduction in all living organisms is a fascinating and complex area of scientific study (Ricklefs & Wikelski, 2002; Williams, 1966). An organisms' life-history characterizes all the events that happen between birth and death that have a direct influence on survival and reproductive success. The approach of life history theory is to determine how the different life history traits combine to affect fitness and centrally important to the theory of life-history are trade-offs (van Noordwijk & de Jong 1986; Stearns, 1992; Williams, 1966). The most notable trade-offs involve current and future reproduction and current reproduction and later survival that are bound together by the said life history traits (Williams, 1966). Within the bird (*Aves*) class there are diverse life histories. For example, a Laysan albatross (*Phoebastria immutabilis*) from the order *Procellariidae* usually won't start to breed until they reach eight years old but can continue to reproduce beyond 60 years old. Laysan albatrosses lay only a single egg at each reproductive event, the incubation period is 62-66 days and the nestling period is 165 days. In contrast, collared flycatchers (*Ficedula albicollis*) from the order *Muscicapidae* commonly breed before they reach one year old and have been found breeding up to nine years old. Collared flycatchers will lay an average clutch size of 6 eggs, the incubation period is 12-14 days and the nestling period is 15 days. Although the life histories of these two species are diverse, their strategies are both linked to a common mechanism that produces a relationship between age to maturity and mortality, and reproductive output (Stearns, 1992). There is a considerable amount of variation in life-history traits within individuals, which is mostly a consequence of phenotypic plasticity in response to changing environmental conditions (reaction norm)(Van Noordwijk, & De Jong, 1986). The environment-phenotype associations may then be further amended by an evolutionary response. The diversity of phenotypes that yields selection, the underlying genetic component that allows a response to selection and the lineage-specific constraints that act with selection to produce the variety of phenotypes we see, is a good summation of the study of life history evolution (Stearns, 1992). Further, we can investigate how the variation in life history traits and how life history traits combine (i.e., life-history strategy) to affect fitness.

Physiological constraints and adaptation to environmental conditions

Physiological trade-offs are the differential allocation of a finite resource between two or more competing mechanisms. An example is producing fewer larger eggs from a limited resource. The underlying physiology governs these trade-offs, which has the potential to constrain life history diversity as a consequence of differential allocation, but also because the physiological mechanisms may only allow for certain combinations of behavioural, physiological and anatomical states to occur at any given time (Ricklefs & Wikelski, 2002). Perhaps the most studied physiological mechanism that mediates trade-offs is the endocrine system. The endocrine system controls physiological and behavioural responses to unpredictable environmental events, and also predictable events such as reproduction (Landys, Ramenofsky, & Wingfeld, 2006; Breuner, Wingfield, & Romero, 1999; Romero & Remage-Healey, 2000; Michael Romero, 2002). The hypothalamus-pituitary-adrenal axis regulates the level of glucocorticoids (GCs) (Figure 1), a steroid hormone, in the blood that is essential in the mobilization of stored non-carbohydrate energy, along with regulating glucose levels to fuel behaviours (Harvey, Phillips, Rees, & Hall, 1984). Allostasis is the maintenance of homeostasis (Sterling & Eyer, 1988) as an organism progresses through different stages of their lifecycle mediated by GCs (Landys et al., 2006). Furthermore, the plasticity of the GC response during reproduction is linked to reproductive success (Ouyang et al., 2015), while GCs have both permissive (Brown et al., 1982; Wiegers & Reul, 1998) and inhibitory (Besedovsky et al., 1991) effects on the immune system. Thus, GCs have the potential to provide important insights into the relationship between physiology and life history. Moreover, the role of GCs as immune inhibitors as well as being essential to reproductive success suggests that GCs are involved in important trade-offs (Martin, Gilliam, Han, Lee, & Wikelski, 2005; Miller et al., 1997). A further consideration to be made with regards to GC levels is the negative consequences associated with prolonged elevated levels of GCs, which can lead to the abandonment of reproduction, or if left unchecked can increase the risk of mortality (Wingfield et al., 1998).

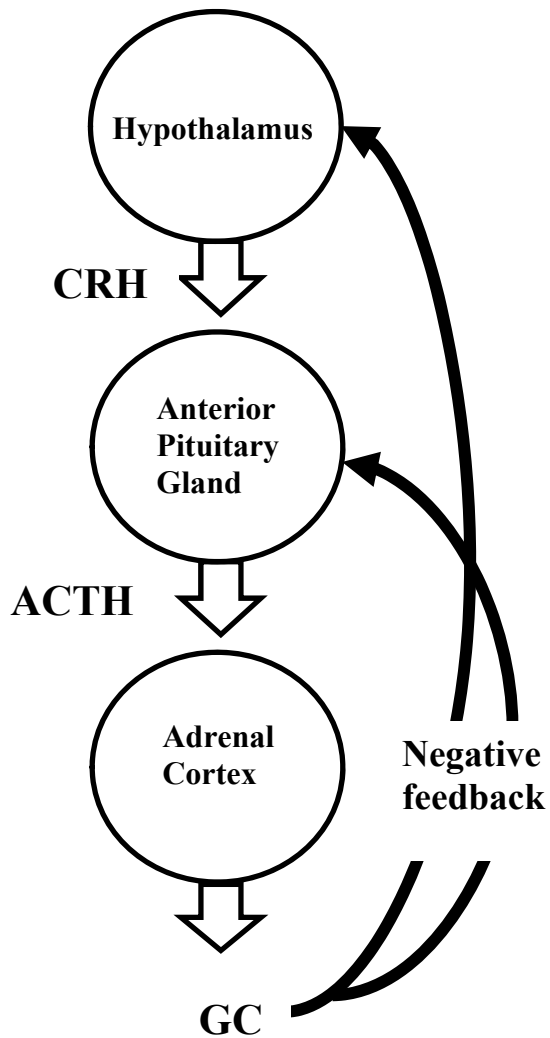


Figure 1. The Hypothalamus-pituitary-adrenal axis. Stimulation of the hypothalamus results in the production of corticotropin-releasing hormone (CRH) which stimulates the anterior pituitary gland to produce adrenocorticotropic hormone (ACTH). Then ACTH stimulates the adrenal cortex to produce glucocorticoids (GC). GCs initiate many metabolic pathways, while also functioning as a negative feedback system on the Hypothalamus and anterior pituitary gland, which prevents extended periods of elevated levels of GCs.

Selective pressures can influence life histories which include unpredictable conditions, disease-causing agents, seasonality, predation, nutrition, conspecifics and family dynamics. In the absence of these stressors, the reproductive performance of organisms would be largely a reflection of lineage-specific constraints. Parasites, an important agent of evolution (Haldane, 1949), are said to affect host life histories because they compete directly for the hosts' resource to fuel their reproduction. In response to the parasite, hosts have evolved a plethora of defence mechanisms mostly controlled by the innate and acquired immune system, all of which carry a cost to the host (Zuk & Stoehr, 2002). Therefore immunity and life-history traits are involved in trade-offs (Lee, 2006; Sheldon & Verhulst, 1996). Trade-offs involving the immune system termed 'the cost of immunity' includes costs related to the evolution, deployment, and maintenance of the immune system (Sheldon & Verhulst, 1996). It has been shown mainly in studies focusing on life histories in invertebrates, that an increase in resistance to parasites can affect lifespan (Ye, Chenoweth, & McGraw, 2009) and reproductive productivity (Konig & Schmid-Hempel, 1995; McKean, Yourth, Lazzaro, & Clark, 2008). There are also examples in wild birds of trade-offs between immune responsiveness and reproductive effort (Nordling, Andersson, Zohari, & Lars, 1998) and a costly secondary sexual ornament (Saino & Moller, 1996).

Haemosporidian blood parasites

Haemosporidian are single-celled blood parasites that belong to the Apicomplexan phylum and is a major parasite of many bird, mammal and reptile species. The parasite life cycle involves an invertebrate intermediate host that has a penchant for the blood of vertebrates, and transmit the parasite to a vertebrate host during a successful feeding session (Ross, 1898). It is during this initial phase of infection, known as the acute phase of infection that the host is most likely to relinquish to parasite-mediated mortality (Carter T Atkinson, Dusek, Woods, & Iko, 2000). During this period the parasite can negatively affect the host by blocking capillaries, causing tissue necrosis and the host destruction of blood cells and anemia. Hosts that survive this period then enter into the long-term chronic infection stage (Valkiūnas, 2005) when the parasite is controlled at low levels by the host immune system (Marm Kilpatrick et al., 2006; Zuk & Stoehr, 2002). At this point, the negative physiological effects caused by the parasite are reduced. It is considered that the majority of infected individuals that breed will be in the chronic infection phase, as those with an acute infection will favour self-maintenance over reproducing (Wingfield et al., 1998). Studies looking for fitness costs related to haemosporidian blood parasite infection, often find that individuals with chronic infections can

perform equally as well as healthy individuals (Bensch et al., 2007; Carter T Atkinson, Dusek, & Lease, 2001; Kulma, Low, Bensch, & Qvarnström, 2014; Podmokła et al., 2014). When hosts and parasites share a long evolutionary history, the relationship becomes complex. Hosts evolve tolerance to the parasite (Carter T. Atkinson, Sali, Utzurrum, & Jarvi, 2013). Moreover, tolerance and susceptibility to parasite infection might be involved in a trade-off (Cornet, Bichet, Larcombe, Faivre, & Sorci, 2014a).

The exposure of potential hosts to a parasite depends on the spatial and temporal dynamics of the parasite, host and parasite genetics and the condition of the potential host. In birds, it is often found that several common lineages and many rare lineages infect a single host population (Bensch et al., 2007; Ricklefs & Fallon, 2002). Although, there is very little information available of when or where these transmission events occur, which means that we have very little knowledge of when, during a birds lifetime, parasite-mediated selection can occur. Determining when an infection is transmitted, would bring further understanding of the parasites potential to affect evolutionary change on the host population. More detailed studies that determine where and when parasite transmission occurs would thus help to elucidate the relationship between the parasite and the hosts' fitness. Birds with chronic infections are often found to perform equally as well as uninfected individuals during the breeding season regarding their reproductive output. There is, however, the potential that birds infected with parasites have to make trade-offs between immunity and reproductive effort. Glucocorticoids are immune suppressors while also function to meet the energy demands of periods like reproduction. The level of glucocorticoid could then vary depending on infection status which would subsequently influence the level of care provided by the parents. Glucocorticoids help animals respond to changing conditions, and could, therefore, provide a good proxy for how individuals respond to the environment at different stages of their life.

Aims of the thesis

1. How does host condition influence the probability of becoming infected with haemosporidian blood parasites? Also, where and when in a hosts life cycle is there potential for parasite-mediated selection to occur? Furthermore, how does this influence their fitness and survival? (**Paper I**).
2. During the breeding season parents that suffer a cost to their condition can pass those costs on to their offspring. Do the condition and haemosporidian blood parasite infection status of a bird influence the glucocorticoid response in the nestlings? Is there a relationship between the level of glucocorticoid experienced during early development and the likelihood of recruitment back into the breeding population? (**Paper II**).
3. Glucocorticoids mediate homeostasis in response to changing energy demands during an animal's life. Is it possible to detect changes in glucocorticoid levels in response to changing energy demands, in bird droppings? More specifically, do female collared flycatchers regulate the level of glucocorticoids, as they progress through life history stages of the reproductive phase? Does the glucocorticoid level vary in response to weather and reproductive effort? (**Paper III**)
4. Glucocorticoids have roles in immune suppression and reproductive success. What is the relationship between the level of glucocorticoids expressed during the nestling feeding phase of reproduction and haemosporidian blood parasite infection? How does this relationship influence survival? (**Paper IV**)
5. Measuring glucocorticoids in natural populations is challenging. The most commonly used technique is to sample the hormone in the blood, although other hormone sampling techniques including in fecal matter and feathers are less invasive and can be easier to collect.

What is the relationship between these three glucocorticoid quantification techniques? How do they relate to nestling condition? (**Paper V**)

Methods

Study species

The collared flycatcher is a small to medium-sized flycatcher that exhibits sexual dimorphism. The Female during the breeding season has a dull brown colour (Figure 2), whereas males are jet black with an exuberant large white forehead patch, full white collar around the neck and also white on the outer halves of the tertial feathers, and at the base of the primary and secondary feathers (Figure 2). They also have white on the rump and sometimes tail feathers (Cramp & Perrins, 1993).



Figure 2: Left: collared flycatcher female. Right: collared flycatcher male.

Collared flycatchers' winter in sub-Saharan Africa. A recent study using data from geolocators, suggested that the birds probably migrate during nights, resting during daylight, but both during day and night when crossing the Mediterranean sea and the Sahara desert, and may even continue without stopping for up to >70 hours (Adamík et al., 2016). At least one individual ringed in Gotland has been found wintering in Angola and another in the Democratic Republic of the Congo (Cramp & Perrins, 1993). The birds arrive to breed in Gotland from the end of April when the males begin to compete for nesting sites. Collared flycatchers have a penchant for their natal nesting site and are often found breeding near it (Pärt, 1990). It is the females that decide on whether or not to accept a nesting site while the male attempts to attract her using distinctive mating calls and showing off his ornaments, which includes characteristic wing flicking behaviour (Field observation). When courtship ends both the male and the female will search for nest material although

it's the female that builds the nest. The female lays the eggs around the first week in May, which she solely incubates for 12-14 days, while the male partially supports the female with food during this time. The collared flycatcher is a bi-parental care species, and the nest is known to suffer if either bird abandons the nest, but the nest will almost certainly fail should the female abandon. Furthermore, males will occasionally have an extra brood, and extra-pair paternity is approximately 15% in this species (Sheldon & Ellegren, 1998). Nestlings typically leave the nest after 15 days but will stay in Gotland often until September before they embark on their maiden migration to the wintering grounds. Collared flycatchers are often found breeding when they return the following spring, but some individuals won't be found breeding until their second or even third year. They have been found breeding up to nine years old and are usually found breeding with a new partner each year. In fact, so rare are breeding events between the same two birds in successive breeding seasons that when they do occur it is probably a chance event.

We monitor approximately 2000 nest boxes in the south of Gotland (57°10' N, 18°20' E) some of which have been monitored since 1980 (Gustafsson & Nilsson, 1985). Starting in May, all the nest boxes are checked, and all breeding attempts recorded. We record all phenology data from each nest and took morphometric data from adults and nestlings as well as a sample of blood.

Haemosporidian blood parasite diagnosis (**Papers I, II & IV**)

DNA was extracted from the blood using the salt precipitation technique (Paxton, Thorén, Tengö, Estoup, & Pamilo, 1996). The birds were screened for haemosporidian blood parasites using a conserved region of the cytochrome *b* gene of the apicomplexan parasite mitochondrial genome. The presence or absence of haemosporidian blood parasites was determined using a nested-PCR protocol (Hellgren, Waldenström, & Bensch, 2004). The primers to determine *Haemoproteus* and *Plasmodium* parasites are listed in table 1. Positive and negative control reactions were included with each PCR run. *Haemoproteus* and *Plasmodium* infections were genetically typed by amplifying and sequencing a cytochrome *b* amplicon of 480 base pairs (bp). We mostly sequenced in one direction using the forward primer from the 2nd PCR run (Table 1.) The sequences were edited and aligned using the free software bioedit (Hall, 1999). We then identified the parasite lineages using the MalaVI database (Bensch, Hellgren, & Perez-Tris, 2009). Figure 3 depicts the phylogenetic relationship between the 31 parasite-lineages found in the collared flycatcher population. The tree was based on a 480 bp fragment of the mitochondrial cytochrome *b* gene and was produced in Beast (Drummond & Rambaut, 2007).

Table 1. The primers used to detect the presence or absence of haemosporidian parasites (*Haemoproteus* and *Plasmodium*).

Nested PCR run	Forward primer	Reverse primer
1	5'-CATATATTAAGA-GAAITATGGAG-3'	5'-ATAGAAAGATAAGAAA-TACCATTC-3'
2	5'-ATGGTGTTTTAGA-TACTTACATT-3'	5'-CATTATCTGGATGAGA-TAATGGIGC-3'

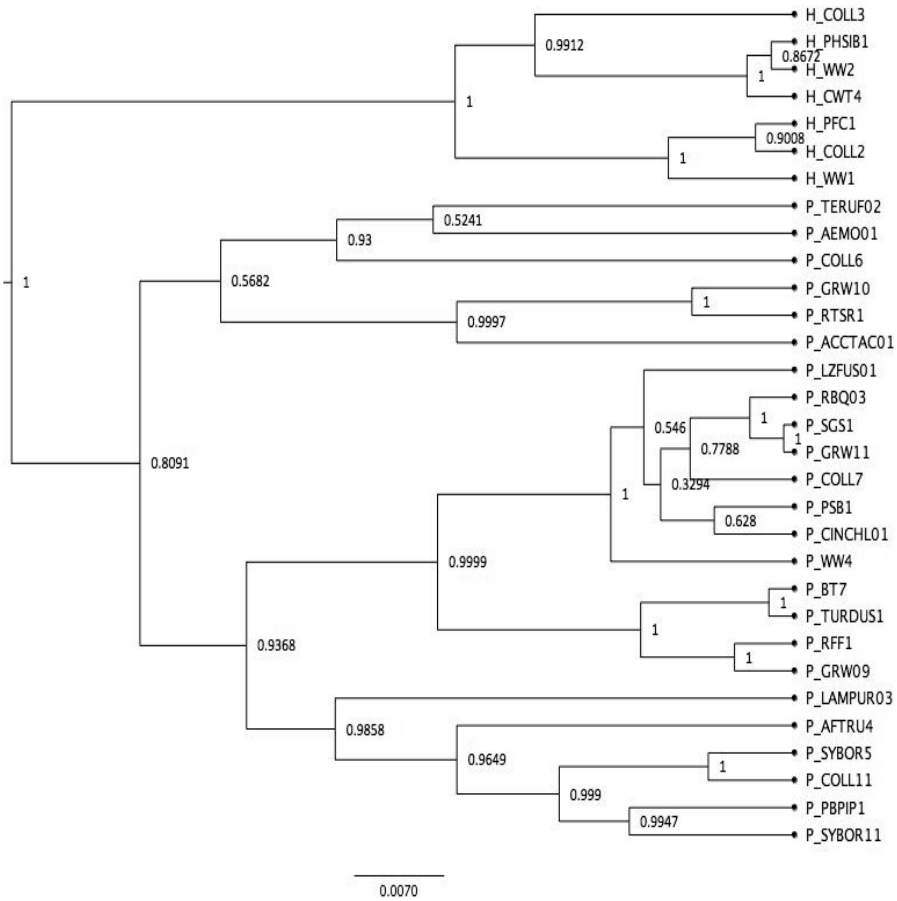


Figure 3. A phylogenetic tree of the 31 lineages of haemosporidian blood parasite found in the population of collared flycatchers studied. The tree was based on a 480 bp fragment of the mitochondrial cytochrome *b* gene. The lineages were either from the *Haemoproteus* (H_) or the *Plasmodium* (P_) genus. The tree was produced in Beast (Drummond & Rambaut, 2007) with a HKY substitution model. The node posterior probabilities are indicated on the tree. We found the lineages to be related to the following species: *Haemoproteus majoris* (H.PHSIB1; H.WW2; H.CWT4); *Haemoproteus pallidus* (H.PFC1; H.COL2); *Haemoproteus balmorali* (H.COL3); *Haemoproteus palloris* (H.WW1); *Plasmodium delichoni* (P.COL6); *Plasmodium relictum* (P.GRW11; P.LZFUS01; P.SGS1); *Plasmodium circumflexum* (P.TURDUS1). Lineages and the associated species were identified using the Malavi database (Bensch, Hellgren, & Perez-Tris, 2009).

Brood manipulation (**Papers II & III**)

We manipulated the brood size of collared flycatchers similar to Gustafsson & Sutherland 1988. We increased the number of nestlings in a brood by adding two randomly selected nestlings thus decreasing the brood from which we removed two nestlings. As a control, we swapped two chicks between broods. In **paper II**, we adjusted the brood size to test if costs related to reproduction, i.e., current reproduction, were transmitted from the parents to the offspring and could be detected as a change in the level of corticosterone detected in the feather tissue of the nestlings. In **paper III**, we changed the brood size to determine if we could detect a corticosterone response to a change in reproductive effort in bird droppings.

Body Condition Index (**Papers II, III & V**)

We use a linear relationship between body mass and the tarsus length of the bird as an index of body condition. The method we choose is the residual index where we used an ordinary least squares regression analysis to regress bird mass on the length of the tarsus and used the residuals as an index of body condition (BCI), more specifically a measure of stored fat, which is an essential energy source during reproduction. In **paper II**, we use the BCI as a representation of male and female condition independently and link this to the level of stress in the brood of nestlings as represented by the level of corticosterone in feather tissue. In **paper III**, we compare the BCI of adult collared flycatchers with the level of metabolized corticosterone produced during nestling feeding. Also, we compare how the mean BCI of male and female birds varies between years in response to environmental conditions. Finally, in **paper V**, we compare the individual nestling BCI with three different corticosterone sampling techniques.

Corticosterone extraction

Feather tissue (**Paper II & V**)

The third tertial feather taken from collared flycatcher nestlings was weighed and measured before removal of the calamus. After cutting the remaining feather tissue into small pieces ($<5\text{mm}^2$) in a vial, we added 10 ml of methanol (HPLC grade). The samples were then sonicated in a sonicating water bath for 30 minutes. Following that, the samples were placed in shaking water bath and incubated at 50°C overnight. The feather tissue was separated from the methanol by syringe filtering the methanol through synthetic polyester media; we washed the filter media with 2.5 ml of methanol to capture any remaining

corticosterone. We then evaporated off the methanol in a 50°C water bath under a fume hood. Feather hormone values are expressed as pg corticosterone per mm⁻¹ feather (Bortolotti, Marchant, Blas, & German, 2008, Bortolotti, Marchant, Blas, & Cabezas, 2009, López-Jiménez et al., 2016). The average corticosterone recovery was 90% ± (n=10)

Droppings (Paper II, IV &V)

We weighed out 0.1 g of bird droppings that had been previously homogenized using a spatula. The samples were dried out before adding them to a 1.5 ml Eppendorf tube with 1 ml of ethanol. The samples were then shaken vigorously for 30 minutes. Following that the samples were centrifuged for 15 minutes at 5,000 rpm. We then transferred 0.8 ml of the supernatant to another Eppendorf tube when the ethanol was evaporated to dryness in a SpeedVac. The average Corticosterone recovery was 91.4% ± 1.1% (n = 10).

Corticosterone quantification

CLIA (Paper II)

Total corticosterone concentrations of collared flycatcher nestlings were measured using chemiluminescent (CLIA) assay kits (ARBOR ASSAYS Detect X) 2015). This assay uses a sheep polyclonal antibody specific for corticosterone. The sensitivity of the assay was 17 pg/ml. We pooled five feather samples and produced a two-fold serial dilution of the extracted hormone. We found there to be no significant difference between the curve produced using the feather samples and the standard curve provided by the assay kit ($F_{1,7} = 0.4$, $P=0.5$; Figure. 4) The dried corticosterone samples were re-dissolved 1:10 with the provided assay buffer. We tested the extraction efficiency by running a known concentration of corticosterone through the extraction procedure. The average extraction efficiency based on two samples per plate was 90% ±. Once reconstituted 50 µl of each sample was immediately randomly added in duplicates to individual wells of the assay plate. The average inter-plate coefficient of variation was 10% ± 1.7 (based on two replicates per plate), and the average intra-plate coefficient was 8.8% ± 1.2 (n=10). We also tested the third tertial from opposite wings from several randomly selected nestlings and the average coefficient of variation between feathers was 11.3% (n=8).

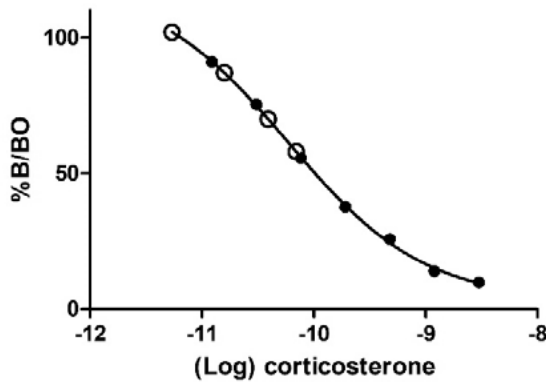


Figure 4. A comparison between the assay kit standard curve (solid circles) and a two-fold serial dilution of corticosterone extracted from feather tissue from 5 pooled samples, to test for parallelism (Open circles). There was no difference between the lines ($F_{1,7} = 0.4$, $P=0.5$).

EIA (Paper III, IV & V)

An immune enzyme assay (EIA) kit (ARBOR ASSAYS Detect X), was used to determine corticosterone concentrations in both Adult and Nestling collared flycatchers. The assay has been verified for use on fecal, feather tissue and blood plasma samples. The assay uses a sheep polyclonal antibody specific for corticosterone. The assay was tested for parallelism and was found to be suitable for use on collared flycatcher dropping extracts. We pooled ten dropping samples and produced a two-fold serial dilution of the extracted hormone, and found there to be no significant difference between the curve it produced and the standard curve provided by the assay kit ($F_{1,10} = 2.9$, $P=0.1$; Figure 5). The sensitivity of the assay was 31 pg/ml. Following the extraction procedure, the dried corticosterone samples (i.e., hormone extracted from droppings and feather tissue) were re-dissolved 1:10 with the provided assay buffer. Then the samples were randomly added to the plates in duplicates. For those birds tested for plasma levels of corticosterone. We added 5 μ l of dissociation reagent to 5 μ l of the plasma sample, and vortexed gently before incubating at room temperature for 5 minutes. The samples were diluted 1:100, which was adequate for the EIA kit, we added 50 μ l of each sample to the assay plate. We added all corresponding samples on the same assay plate (i.e., dropping samples from incubating female, feeding female, feeding male and any associating plasma samples). The average inter-plate coefficient of variation was 5.8% \pm 0.05 (based on two replicates per plate), and the average intra-plate coefficient was 3.25% \pm 0.04 (n=10).

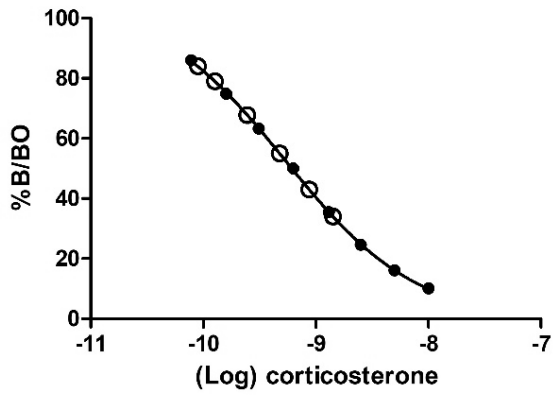


Figure 5. A comparison between the assay kit standard curve (solid circles) and a two-fold serial dilution of metabolized corticosterone pooled together from 10 birds to test for parallelism (Open circles). There was no difference between the lines ($F_{1, 10} = 2.9$, $P=0.11$).

Results and discussion

Paper I explores the pattern of haemosporidian blood parasite infection in the collared flycatcher population. Nestlings were followed through to adulthood to determine if and when they became infected and how this influenced their fitness (number of recruits) and survival. A survey of 700 juveniles sampled just before they migrated to the wintering grounds, found that 10% were already infected before leaving the breeding ground. The juveniles were infected with eight different parasite lineages, which included five from the *Haemoproteus* and three from the *Plasmodium* genus. Four of the lineages found are common in adult birds (HPSIB1, HCOLL3, HPFC1, and PGRW09), which indicates that parasite-mediated selection has the potential to act early on in the birds' life. In total, 28% of the nestlings became infected, and we found that nestlings in poorer condition were more susceptible to parasite infection (Fig. 1).

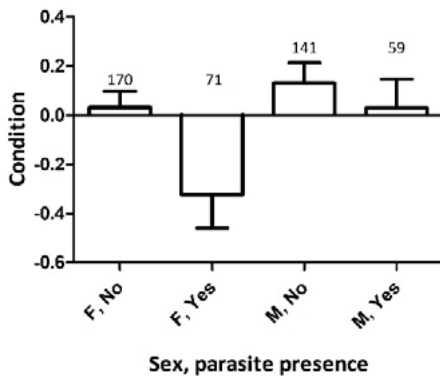


Fig. 1 The relationship between female (F) and male (M) nestling condition (BCI) and haemosporidian parasite presence (Yes) and absence (No) in adulthood.

There was, however, no apparent effect of parasite infection on future survival. These results indicate that there exists a trade-off between host susceptibility to parasites and the ability to tolerate the infection (Carter T. Atkinson, Saili, Utzurrum, & Jarvi, 2013; Cornet, Bichet, Larcombe, Faivre, & Sorci, 2014). Our results indicate that infected females may incur a parasite-mediated fitness cost. Furthermore, females that were infected in the second or third breeding season had significantly more recruits than those infected in the first breeding season. This pattern was not evident in male birds.

Paper II shows that changes in glucocorticoid levels are detectable in the feathers of developing nestlings. In response to a brood manipulation experiment, we found that nestlings from the enlarged brood treatment had higher levels of corticosterone in feather tissues (Fig. 1), indicating that the level of stress in the brood had been affected by the addition of nestlings.

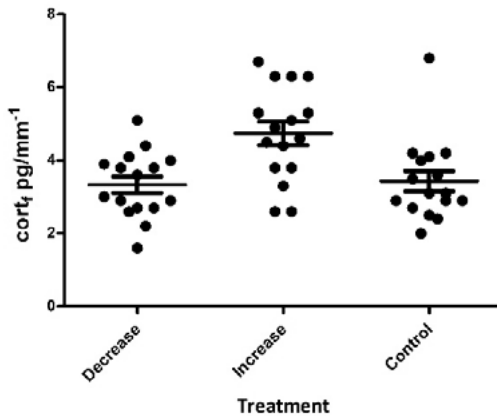


Fig.1. Influence of the experiment on nestling cort_f concentrations. For each of the three treatments, we randomly assigned 16 broods. Bars represent means (±SE).

Experimental and field studies have shown that during early development a reduction in nutrition and parental care can result in a stress-induced elevated cort response in altricial young (Astheimer, Buttemer, & Wingfield, 1992; Kitaysky, Wingfield, & Piatt, 1999). We suggest that the higher cort levels detected in nestlings from enlarged broods is a consequence of a reduction in nutritional intake. The cost of elevated corticosterone during early development can have both short- and long-term effects on the young, in the short-term it was linked to increased begging behaviour (Kitaysky, Kitaiskaia, Piatt, & Wingfield, 2003). Long-term effects included a reduction in cognitive ability and also influenced how individuals responded to future stressful events (Pravosudov & Kitaysky, 2006; Schoech, Rensel, & Heiss, 2011; Wada et al., 2008). Following the brood manipulation experiment, we found that it was more likely that individuals with lower development cort levels as nestlings would return although this result was marginally non-significant.

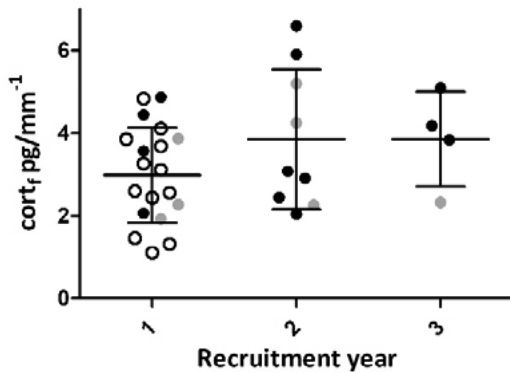


Fig.6. Nestling cort_f concentration and recruitment into the breeding population in the following 3 years after the brood manipulation experiment. Each data point is coloured depending on the experimental brood the recruit was randomly assigned to: decreased brood (empty circle); increased brood (closed circle) and control brood (grey circle). Horizontal lines denote the means (\pm SD).

Moreover, we found that the mean brood cort level was a good predictor of recruitment, with more recruits coming from broods with lower mean cort levels. However, individuals recruited from the full spectrum of cort levels detected, which indicates that there is unlikely to be a single cort phenotype that best adapts nestlings to unpredictable challenges (Fig. 6).

Paper III uses a less invasive technique of glucocorticoid quantification to ask questions related to allostasis, as collared flycatchers progress through life-history stages. The corticosterone -adaptation hypothesis predicts that animals will adapt their level of corticosterone in response to the level of energy demand (Bonier, Martin, Moore, & Wingfield, 2009; Bonier, Moore, & Robertson, 2011). Our results support this hypothesis as female collared flycatchers' upregulate corticosterone in response to the increased level of energy demand required during nestling feeding (Fig. 1). Further supporting this hypothesis, we found that female birds' upregulate corticosterone in response to experimentally increased broods (Bonier et al., 2011). Glucocorticoids such as corticosterone mediate physiological processes in response to predictable changes in energy demand (Ouyang, Hau, & Bonier, 2011; Breuner et al., 1999), but also in response to unpredictable challenges such as inclement weather (Ouyang et al., 2015). Our study was conducted over a two-year period that presented us with very different weather conditions. We found a relationship between air temperature and female corticosterone levels during incubation. In the colder year females had significantly lower levels of corticosterone during incubation, while during nestling feeding, in the year with significantly higher precipitation, male birds had significantly lower corticosterone responses. The main function of elevated corticosterone levels is to

mobilize stored energy(Harvey et al., 1984). If elevated corticosterone levels are left unchecked during poor weather conditions consequences can be reproductive abandonment or even death(J. C. Wingfield & Sapolsky, 2003). It is conceivable that the birds adjusted their level of corticosterone as a prevention mechanism against prolonged elevated corticosterone levels during difficult times. We also identified a potential direct role for corticosterone in reproductive investment. Higher cort levels during incubation were related to smaller clutch sizes.

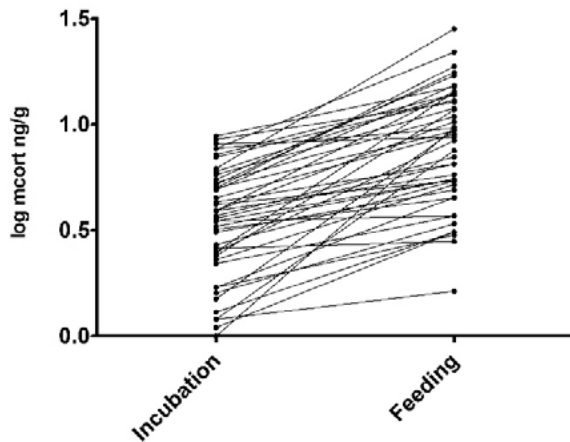


Fig. 1 The change in female log MCORT concentration between the incubating and nestling feeding stage. The samples were taken five days after the onset of incubation and five days after the nestlings hatched.

Paper IV looks at the relationship between the level of corticosterone produced during nestling feeding and the parasite status of the collared flycatchers. Glucocorticoids have both permissive and restrictive functions in immunity. They are essentially important in mediating the initial acute phase response to an infection (Brown et al., 1982; Wiegers & Reul, 1998), while equally important in dampening down the immune function to prevent negative consequences like autoimmunity if the infection persists (Besedovsky et al., 1991). Our results show that during the nestling feeding period collared flycatchers carrying a parasite have significantly lower corticosterone levels. This could be a consequence of a trade-off between immunity and reproductive effort. Hosts' with chronic haemosporidian parasite infection are said to maintain the infection through acquired immunity (Atkinson & Van Riper III, 1991; Carter T Atkinson et al., 2001). While during the nestling feeding period higher levels of glucocorticoids are required to meet the energy demand of feeding nestlings (Ouyang, Sharp, Quetting, & Hau, 2013). Parasitized birds might, therefore, maintain their corticosterone levels lower to prevent immune suppression effects that might interfere with controlling the infection. We also

found corticosterone level during the nestling feeding period to be a good predictor of survival, which might indicate that higher levels of cort during this period are an indicator of body condition or indeed quality. We didn't, however, find a relationship between the upregulation of corticosterone during reproduction and survival, or parasite infection in female collared flycatchers.

Paper V compares quantifying corticosterone in feathers and metabolized corticosterone in bird droppings with the most commonly used blood sampling technique. We didn't detect a significant relationship between the two alternative techniques and the blood sampling technique. Although, we did find a significant negative association between metabolized corticosterone in droppings and corticosterone stored in feather tissue (Fig. 3(C)).

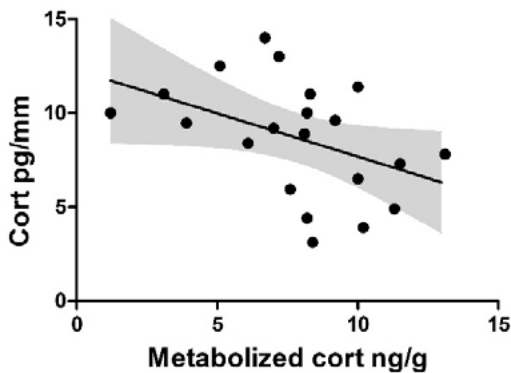


Fig. 3(C) The relationship between feather (pg/mm) and metabolized corticosterone (cort) (ng/g) ($n=21$; $R^2=0.19$, $P=0.049$). The graph includes the fitted line and 95% confidence intervals.

If, as is expected, the corticosterone bound in feather tissue is the unbound free corticosterone molecule then this result could indicate a relationship between circulating levels of free unbound corticosterone and metabolized corticosterone. Quantifying glucocorticoids in wild populations is a popular way of determining how an animal is responding to the environment (Schoech, Rensel, & Heiss, 2011). We compared the level of the three glucocorticoid techniques with the condition of the nestlings (BCI). There was a significant positive relationship between metabolized corticosterone and nestling condition (Fig. 4 (C)), which suggests that quantifying metabolized corticosterone is potentially a good technique for quantifying glucocorticoids in wild populations.

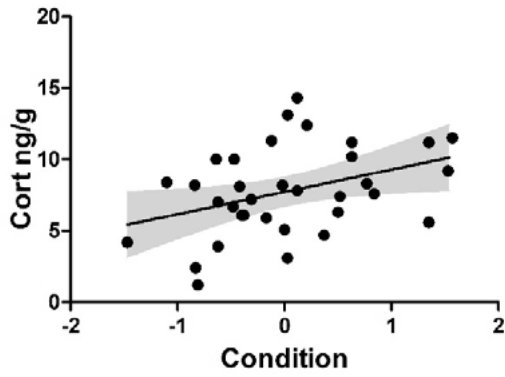


Fig. 4 (C) the level of metabolized corticosterone detected in the droppings of nestlings regressed on nestling condition ($n=34$; $R^2=0.14$, $P=0.02$). The graph includes fitted line and 95% confidence intervals.

Conclusions and future directions

In this thesis, I provided some preliminary results with regards to the pattern of haemosporidian blood parasite infection, in the population of collared flycatchers that breed in the south of Gotland. This study supports the use of physiological mechanisms to determine how a host might cope with chronic infection in light of reproduction and survival. In **paper I**, we show that the collared flycatcher (host) and haemosporidian blood parasites come in to contact early during the birds' life, which has implications for parasite-mediated selection. The presence of four of the most common lineages found in adult birds, detected in juvenile birds (<3 months), suggests that these lineages are important parasites of the collared flycatcher. It is probable that those individuals that return and breed successfully are sorted from the individuals unable to maintain an immune response to control the parasite, while successfully navigating life-history stages. We also found that nestling body condition predicted the probability of becoming infected, which could indicate a cost to immunity, potentially in response to poor nutrition, which increases the susceptibility of the host to the parasite. Our results indicate that longitudinal studies would help to determine the factors that increase the likelihood of the host's exposure to parasites. An important area of research is ecological immunology which bids to understand the relationship between immunity and life history. Studies such as those focusing on the role of the major histocompatibility complex (MHC), which is an important arm of acquired immunity, could further our understanding of how some hosts and not others, can maintain infection while successfully progressing through life-history stages, i.e., tolerating infection. In **paper II**, we found that changes in glucocorticoid levels could be detected as a consequence of increased reproductive effort and in response to parasite infection. The increase in glucocorticoids is most likely to be due to a below optimal energy level in the nestlings, as a consequence of insufficient parental care. In **paper I**, we found that infected birds still reproduced successfully, but females produced fewer recruited offspring over their lifetime. If providing quality parental care is involved in a trade-off with immunity. Then we might expect that infected birds might provide lower quality parental care because of the cost of immunity and consequently produce fewer offspring that recruit. Furthermore, in **paper IV**, we found that infected birds reduced glucocorticoid level during nestling feeding maybe to prevent immune suppression, while higher glucocorticoid levels predicted survival.

This might indicate that infected birds' trade-off higher glucocorticoid levels in favor immunity controlling the infection. Future studies are required that experimentally test the role of glucocorticoids as a mediator of trade-offs between immunity and reproductive effort. In **paper III**, we found that changes in energy demand were linked to an increase in glucocorticoid level, which could be detected as metabolized corticosterone in bird droppings. In **paper V** we found that metabolized corticosterone had a linear relationship with nestling condition. It was, therefore, concluded that quantifying metabolized glucocorticoid in bird droppings provides a viable alternative to the commonly used blood sampling technique. Particularly when using glucocorticoids to determine how wild birds are responding to their environment.

Svensk sammanfattning

I naturen använder djur många olika strategier för att föröka sig. Dock har alla samma mål att under sin livstid producera så många avkommor som möjligt, som i sin tur överlever för att föröka sig och därmed vidarebefordra sina gener till kommande generationer. Reproduktionsstrategier varierar eftersom djur har olika anatomi, lever i varierande miljöer och uppvisar skilda beteenden. Till exempel gräver laxhonor ett hål på älvens botten där de lägger hundratals små ägg och täcker äggen med stenar. De dör sedan själva och lämnar äggen för att klara sig själva. När ynglen kommer ut från äggen, kommer de att vara självförsörjande och börja jaga föda omedelbart, men väldigt få kommer att överleva. I motsats till detta kommer en albatross att lägga ett enda stort ägg som de ruvar i två månader. Efter kläckning kommer albatrossungen att vara beroende av föräldrarna i minst ytterligare fem månader, vilket är den ungefärliga tiden när de lämnar boet. För en del laxar kommer de att reproducera sig endast en gång under sin livstid, medan en albatross kan leva mer än ett halvt sekel och kan göra så många som 20 häckningsförsök under den tiden. Anledningen till att en del laxar bara har ett reproduktionstillfälle är att förflyttningen från havet tillbaka till reproduktionsplatser i älvarna är en välkänt svår resa. Därför satsar de all energi på ett enda reproduktionsförsök, eftersom chansen att överleva och kunna återvända en andra gång är minimal. Albatrosser, å andra sidan, kommer ofta att överge ägg eller ungar när det blir svåra förhållanden på häckningsplatsen, eftersom det sannolikt kommer att finnas många fler chanser till häckningsförsök i framtiden. Dåligt väder, parasitinfektioner och otillräcklig tillgång till mat åt deras unge är olika skäl till att avbryta en häckning.

Det endokrina systemet reglerar djurens respons till både förutsägbara händelser som reproduktion och oförutsägbara händelser som parasitinfektioner. Glukokortikoid hormoner produceras när hypotalamus stimuleras. De har blivit ett populärt hormon att studera på grund av deras roll i responsen till stressfaktorer. Dessa hormoner är viktiga hos djuren som svar på förändringar i energibehovet när ett djur går in i reproduktionscykeln. Glukokortikoid nivåer ökar som en följd av en ökning av energibehovet, men en långvarig period av höga nivåer av glukokortikoider kan ha negativa effekter på djuret och är ofta ett tecken på ett stressat djur. Vidare är glukokortikoider involverade i reglering av immunsystemet, vilket innebär att de är inblandade i försvaret mot parasiter. Djur reproducerar sig ofta även när de är infekterade. Reproduktiv

framgång i närvaro av en parasit kräver att glukokortikoider balanserar reglering av immunsystemet i förhållande till reproduktionskraven.

För att svara på frågor som rör hur parasitinfektioner påverkar reproduktionsbeteendet, i relation till glukocorticoidnivån, studerade vi en population av halsbandsflugsnappare som häckar på södra Gotland, en ö som ligger utanför Sveriges fastland mitt i Östersjön. Glukokortikoidhormoner, även kända som stresshormoner, är viktiga för att mobilisera energiresurser och reglera immunsystemet.

Över en tredjedel av halsbandsflugsnapparna reproducerar sig samtidigt som de hyser en hemosporidisk blodparasit. Hemosporidiska parasiter är välkända för att de inkluderar malariaparasiter, som fortfarande har förödande effekter på mänskliga populationer. Över 2 miljoner fall av malaria och över 400 000 malariarelaterade dödsfall hos människor bekräftades 2015. Effekterna av hemosporidiska blodparasiter på vilda fågelpopulationer har i olika studier producerat motstridiga resultat. I denna studie tittar vi på hur parasitinfektion påverkar halsbandsflugsnapparna under olika stadier av deras liv.

I artikel I undersökte vi mönstret av parasitinfektion i halsbandsflugsnapparpopulationen genom att bestämma på vilket stadium av livscykeln fåglarna smittades och hur detta påverkade deras reproduktiva framgång och överlevnad. Vi fann att halsbandsflugsnapparna potentiellt kan infekteras av hemosporidiska blodparasiter under de första 2 månaderna av deras liv. Vi fann också att ungar som redan i boet har dåligt kondition, låg kroppsvikt, är mer benägna att smittas. Vid vuxen ålder kan infekterade individer reproducera sig framgångsrikt, men de avkommer de producerar kan drabbas av dålig kondition och har sämre chanser att överleva för att själva reproducera sig.

Halsbandsflugsnapparungar är helt beroende av sina föräldrar tills de är ungefär 15 dagar gamla, vilket de är när de lämnar boet, men även delvis tre veckor efter detta. Det betyder att om föräldrarna häckar i dåliga miljöförhållanden, så kommer ungarna drabbas negativt av detta.

I artikel II manipulerade vi reproduktiva ansträngningar genom att öka antalet ungar i vissa kullar och minska antalet i andra kullar för att bestämma hur reproduktiva ansträngningar påverkar glukokortikoidreaktionen i ungarna i boet. Vi visade att ökad reproduktiv ansträngning ökade glukokortikoidreaktionen i ungarna, potentiellt som ett svar på ökad konkurrens mellan ungarna. Ökat glukokortikoidreaktion hos unga fåglar har kopplats till ökat tigande av mat. Vi fann också att när båda föräldrar hade en parasitinfektion producerade ungarna en högre glukokortikoidreaktion. Det endokrina systemet fungerar för att förbereda organismer för att hantera förändringar i energibehovet. Hos fåglar som matar sina ungar i boet betraktas den perioden som den mest energikrävande perioden av häckningen.

I artikel III utvärderade vi förändringar i glukokortikoidreaktionen i förhållande till energibehov och klimatförhållanden. Vi fann högre halter av hormon

under ungmättningsperioden än under ruvningstiden hos honor, vilket indikerar att ungmättningsperioden är den mer energikrävande perioden. Vi fann också att det endokrina systemet svarade på miljöförhållandena, vilket tyder på att glukokortikoider har en roll för att anpassa fåglarna till det aktuella vädret.

I artikel IV undersökte vi förhållandet mellan parasitinfektion och glukokortikoidrespons under ungmättningsperioden. Det viktigaste resultatet var att smittade fåglar hade lägre glukokortikoidnivåer, medan högre nivåer av glukokortikoider var kopplade till överlevnad.

Slutligen, i artikel V, jämförde vi olika metoder för hormonkvantifiering för att bestämma hur de fungerar i en vild population av fåglar som våra halsbandsflugsnappare. Vi fann att provtagning av glukokortikoider i fågelfekalier var ett bra alternativ till provtagning från blod.

Sammanfattningsvis ger glukokortikoider oss ett fönster för att observera hur ett djur svarar på miljöförhållanden och även hur detta förändras under olika stadier i deras liv. Vidare ger studier av fysiologiska processer oss möjlighet att lära oss mer om värd-parasitrelationer. Om man undersöker när och var djuren smittas under sin livstid kan det ge viktiga insikter om värdparasitdynamiken.

Translated by Lars Gustafsson

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