Eurasian Blackbirds (*Turdus merula*) and their gastrointestinal parasites:

A role for parasites in life-history decisions?

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General introduction

Parasites have fascinated men since thousands of years but still wrap themselves in deep mysteries (Bush et al. 2001). This is not really surprising if one bears in mind that the essence of parasitism rests with the nature of parasite-host relationships. Studying these relationships means that the ecology and the biology of the parasite and of the host, as a habitat for the parasite, have to be considered simultaneously. Moreover, since hosts are alive and responding physiologically, immunologically and behaviourally to their parasites, physiological, immunological and behavioural interactions have to be considered as well. Finally, it is a common phenomenon that a single host harbours parasite communities instead of just one parasite taxon (Bush et al. 2001). These parasites probably all interact with each other and with the host, such that extremely complex relationships arise. Thus, even for only partly understanding parasite-host relationships skills and knowledge from various disciplines are required. Only in the last 25 years have parasitologists, immunologists, physiologists, population biologists, ecologists, behavioural ecologists and evolutionary biologists begun to overcome interdisciplinary barriers and have, since then, created many fascinating new hypotheses about parasite-host associations (Clayton and Moore 1997; Bush et al. 2001). Much debate has been on the recognition of parasites as important agents in the evolution of host sex (Hamilton 1980; Hamilton et al. 1990), host life history (Stearns 1992; Møller 1997) and host sexual selection (Hamilton and Zuk 1982; Read 1989; Møller 1990; Clayton 1991). Bird-parasite models have been very popular in testing hypotheses about the role of parasites in sexual selection and in life-history evolution, because birds belong to one of the ecologically best studied vertebrate groups. Our knowledge of parasites, however, especially of

their ecology is still punctual as with regard to the enormous diversity and complexity of their life cycles. After the publication of the seminating paper of Hamilton and Zuk in 1982, haematozoan parasites, such as *Haemoproteus, Leucocytozoon, Hepatozoon, Plasmodium* and *Trypanosoma* became the most popular parasites in the fields of behavioural ecology and evolution, probably because of the relative ease with which these parasites can be sampled from live birds. They were used for interspecfic and intraspecific tests of the hypothesis that extravagant secondary sexual characters were honest indicators of a birds health and ability to resist parasitic infection (Hamilton and Zuk 1982; Read 1987; Read and Harvey 1989; Gibson 1990; Pruett-Jones et al. 1990; Weatherhead 1990; Johnson and Boyce 1991; Kirkpatrick et al. 1991; Zuk 1991). Ten years later haematozoans not only enjoyed great popularity in the field of sexual selection but also in life history theory (Norris et al. 1994; Richner et al. 1995 a; Oppliger et al. 1996; Ots and Hõrak 1996; Allander 1997; Oppliger et al. 1997; Nordling et al. 1998; Wiehn and Korpimäki 1998; Fargallo and Merino 1999; Merino et al. 2000). Although first testings of the hypotheses have been done without much knowledge about these parasites (Cox 1989; Weatherhead and Bennett 1991, 1992) a growing body of literature is now accumulating about the biology, physiology, immunology and ecology of these parasites and their relationship to their hosts (van Riper et al. 1986; Hayworth and Weathers 1987; Kirkpatrick and Suthers 1988; Atkinson and Riper 1991; Bennett et al. 1993; Desser and Bennett 1993; Merilä et al. 1995; Dufva 1996; Ots et al. 1998; Hatchwell et al. 2000; Schrader et al. 2003; Scheuerlein and Ricklefs 2004). At the same time arthropod ectoparasites also moved into the minds of behavioural ecologists and evolutionary biologists. These conspicious parasites are relatively easy to collect from live animals and can be counted directly. Therefore, they also became popular in studies testing the role of parasites in sexual selection (Borgia 1986; Borgia and Collis 1989; Clayton 1990; Møller 1991; Spurrier et al. 1991) and in studies investigating the impact of parasites on various fitness aspects of their hosts (Johnson and Albrecht 1993; Richner et al. 1993; Young 1993; Møller et al. 1994; Oppliger at al. 1994; Merino and Potti 1995; Richner et al. 1995 b; Bauchau 1997; Allander 1998; Heeb et al. 1998; Merino and Potti 1998; Wesolowski 2001; Saino et al. 2002;

Nilsson 2003; Walker et al. 2003; Fitze et al. 2004 a, b). A considerable part of these investigations was conducted on the host-parasite system of Great tits (*Parus major*) and their common ectoparasite, the hen flea (*Ceratophyllus gallinae*) (for a review see Richner 1998) which can be considered one of the best studied in the fields of behavioural ecology and evolution.

Although there is a vast body of literature on birds as hosts of helminth and coccidian parasites (for reviews see Rausch 1983; Janovy 1997) only a few studies considered these parasites in their role on host sexual selection and on host life history (Rausch 1983; Hudson 1986; Saumier et al. 1986; Hillgarth 1990; Zuk et al. 1990; Connors and Nickol 1991; Buchholz 1995; John 1995; Delahay and Moss 1996; Brawner et al. 2000; Piersma et al 2001). In view of the enormous number and variety of endoparasite species, belonging to as different taxa as protozoans and arthropods, this parasitic group deserves much more attention than it is given at time. One aim of the present thesis is to add to the still scarce knowledge about gastrointestinal parasites in their potential role as mediators of various fitness aspects in birds.

The second aim is to critically elucidate methodological aspects in the study of parasite-host relationships. All studies on parasite-host systems require a reliable method to assess a host's infection status (the presence or absence of an infection) and/or intensity of an infection. If the host of concern is required to stay alive, which is usually the case in ecological, behavioural and evolutionary studies, only indirect methods can be applied for the assay of endoparasitic infections, i.e. infections of the blood, digestive tract and inner organs. All indirect methods, however, involve the danger, that parasites, although present, will not be detected. This problem arises due to several biological aspects of the host, of the parasites and of their mutual relationship. It will be dealt with in this thesis from an exemplary and theoretical point of view.

A very common passerine bird, the Eurasian blackbird (*Turdus merula* Linne, 1758), which' biology is well studied (Snow 1958; Greenwood and Harvey 1976; Haffer 1988; Desrochers 1992; Desrochers and Magrath 1993a, 1993b; Tomialojc 1993, 1994; Kentish et al. 1995; Ludvig et al. 1995; Stephan 1997; Creighton 2001) was examined for gastrointestinal parasites by the examination of fecal samples. The occurrence and distribution of the parasites

in the wild blackbird population was investigated and the findings discussed with respect to fruitful future research on this parasite-host system (Chapter 2). Questions about the costs of parasitism as the crucial factor in life-history considerations are treated in Chapter 3 and Chapter 4. I test the hypothesis predicted by life-history theory that an investment in one costly trait can only occur at the detriment of another costly investment. I investigate whether parental feeding is payed-off in terms of a higher probability of gastrointestinal infection (Chapter 3). In Chapter 4 the costs of parasitism on nestling blackbirds are examined, by investigating the association between aspects of growth and coccidian infection. In Chapter 5 a specific property of the *Isospora*-host system is examined, namely the diurnal fluctuation of oocyst shedding in the feces of blackbirds. The results are discussed with respect to the practical implications on the planning and interpretation of parasitic assays. Finally, a model is developed to give future investigators a tool to estimate the quality of indirect parasitic assays, on which most ecological, behavioural and evolutionary studies on parasite-host systems rely (Chapter 6).

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2

The occurence and distribution of endoparasitic infections in a population of Eurasian Blackbirds (*Turdus merula*)

Abstract - In a wild, urban population of Eurasian blackbirds (*Turdus merula*) the occurrence and the distribution of gastrointestinal endoparasites was studied by the examination of fecal samples. The recent incresed interest in parasites has focused mainly on haematozoan parasites and on arthropod ectoparasites, although parasitic surveys in wild birds have shown that almost every species harbours endoparasites of various taxa. In this study mainly four parasite taxa, sporozoans of the genus *Isospora*, nematodes of the genus *Capillaria*, cestodes and acanthocephalans were determined from oocysts and eggs passed in feces of 68 adult and 188 nestling birds, sampled during the breeding seasons of 1996-1998. Mean infection prevalence over the three years was high (88 %), with multiple infections being as common as single infections. *Isospora* spp. and *Capillaria* spp. were the most frequent parasites, infecting 53 % and 56% of birds, respectively. These results markedly differ from a similar study conducted 35 years ago (Binder 1971), in which cestodes and the nematode *Porrocaecum ensicaudatum* were the most prevalent parasites. The underlying causes for such differences of the parasite community and distribution in two host populations may lie 1) in differences of local environmental conditions affecting the availability and/or the abundance of intermediate hosts, or 2) in locally adapted immunocompetences of hosts. Females and males did not differ in overall infection prevalence, but females were significantly more often affected by multiple, infections. Furthermore, they were infected with *Capillaria* spp. more frequently and more severily than males. These results suggest that females are more than males susceptible for infections, probably due to the strenuous activities of nest building, egg-laying and incubating for which they are alone responsible. In nestlings only infections with *Isospora* spp. were considered. Nestlings were infected as frequently as adults, but at higher intensities, which may be due to the still immature immune system of young birds.

2.1 Introduction

Over the last two decades parasites have gained increased interest in ecological, evolutionary and behavioural studies. The seminating paper of Hamilton and Zuk (1982) in which a new “good genes” model of parasite mediated sexual selection was presented, brought haematozoan endoparasites into the focus of interest. They became the most popular parasites in testing the Hamilton and Zuk hypothesis (Read 1987; Read and Harvey 1989; Gibson 1990; Pruett-Jones 1990; Weatherhead 1990; Clayton 1991; Johnson and Boyce 1991; Kirkpatrick et al. 1991; Zuk 1991), which assumes that females base their mate choice on extravagant secondary sexual characters, such as brightly coloured plumage, to assess a male’s genetic ability to resist parasites and diseases. Since it was unequivocally demonstrated that parasites often have severe negative effects on a wide range of fitness components of their hosts (Møller 1997) parasites were also incorporated into models of life history theory (Stearns 1992). Again, haematozoan endoparasites were frequently considered, but arthropod ectoparasites also became a very popular parasitic group in testing predictions of life-history theory (review: Møller 1997). A parasitic group till now greatly underrepresented in ecological, evolutionary and behavioural studies are parasites of the gastrointestinal tract and of other inner organs. This fact is surprising, since oocysts, eggs and larvae of parasites infecting the digestive tract and other inner organs leave the hosts' body by the feces. The examination of fecal samples for parasitic stages presents an indirect method to detect infections without sacrifycing hosts. Furthermore, and in contrast to the commonly used method of examinating blood smears for the presence of haematozoan endoparasites, this method does not even require capturing of birds.

Nevertheless, only a few ecological, evolutionary and behavioural studies devoted interest to gastrointestinal endoparasites in birds (Rausch 1983; Hudson 1986; Hillgarth 1990; Zuk et al. 1990; Connors and Nickol 1991; John 1995; Delahay and Moss 1996; Goater et al. 1995; Brawner et al. 2000). Most information on gastrointestinal parasites in wild birds came, so far, from parasite

surveys (Boyd 1951; Frank 1980; Rausch 1983; Ching 1993; Macko and Stefancikova 1996; Zlatka et al. 1997; Hanzelova and Rysavy 1999).

In the present study, fecal samples from 256 adult and nestling blackbirds are examined for parasitic oocysts and eggs, and the occurrence and the distribution of intestinal endoparasites in the population are described. Eurasian Blackbirds are a species suitable for parasite related studies because they are one of the most common urban songbirds in central Europe and their biology is well studied (Snow 1958; Greenwood and Harvey 1976; Haffer 1988; Desrochers 1992; Desrochers and Magrath 1993a, 1993b; Tomialojc 1993, 1994; Kentish et al. 1995; Ludvig et al. 1995; Stephan 1997; Creighton 2001). Blackbirds have already been shown to harbour ectoparasites (Hicks 1974; Doby 1998; Behnke et al. 1999; Gregoire et al. 2002) and haematozoan endoparasites (Hatchwell et al. 2000). The occurrence of isosporan parasites in blackbirds has also been reported before (Scholtyseck 1956; Pellerdy 1974) but their distribution in a population has never been described. Apart from reports on the occurrence of this or that helminth parasite, all information on helminths in blackbirds came from a study of Binder (1971), who examined the helminth fauna of blackbirds caught within the rural area of Gießen (Germany) and its surrounding forest habitats.

The findings of the present study are compared to those of Binder (1971) 35 years ago, and are discussed in the frame of worthful future investigations.

2.2 Methods

2.2.1 *Study area and study species*

This study was conducted throughout the breeding seasons of 1996, 1997 and 1998 on a resident urban population of individually marked Eurasian Blackbirds (*Turdus merula* Linne´ 1758) in the Botanical Garden of Bonn (Germany). The Botanical Garden is situated in the centre of the city and comprises an area of 8.5 ha of evergreen and deciduous vegetation, large patches of low undergrowth and open grass areas. As part of an ongoing

project, all adult blackbirds were captured in mist nets prior to the breeding season and marked with aluminium rings and a combination of colored rings for easy field identification**.** The number of breeding pairs varied from 34 - 36 pairs per year. Eurasian Blackbirds are socially monogamous thrushes which hold long-term territories maintained by both sexes throughout the year (Snow 1956). In our population breeding started in mid March, and the last young fledged by early August, the breeding season extending beyond that typical for southern England (Desrochers and Magrath 1993a, Snow 1958). Blackbirds start breeding when a year old. Clutch size in our population ranged from one to five eggs. Usually two, but sometimes even up to four broods are raised in a single breeding season. Only the female engages in nest building and incubating, but both parents feed nestlings until they fledge 13-14 days after hatching. Thereafter, fledglings are fed for about twenty more days, or by both parents or by the male only if the female is already busy with the next breeding attempt.

From March to August nests were located by systematic searches and were then inspected on a regular basis to record egg production and loss, onset of incubation, day of hatching, nestling number and day of fledging**.** Nestlings were ringed, weighed and measured at age 8d to 10d.

2.2.2 *Fecal collection method*

For adult birds a purely observational collection method was chosen in order to minimize disturbance to birds. Birds were observed in their daily activities with a binocular, mainly in those activities which take place on the ground (feeding, collecting nesting material and collecting food for nestlings) and were followed until they defecated. If the position and the identity of the fecal dropping could be determined unambiguously the sample was collected in a labeled plastic vial. With this observational method it was possible to gather several samples from most adult birds without disturbing breeding activities.

Fecal samples from nestlings were collected during ringing which took place 8 to 10 days after hatching. Samples for which an unambiguous individual assignment was not possible, and for which mutual contamination of droppings

could not be excluded were discharged. From nestlings only one sample per individual was collected.

2.2.3 *Analysis of fecal samples*

Upon arrival in the laboratory samples were transferred into glass vials and stored at 4°C in a 2% potassium bichromate solution up to further analysis. Prior to quantitative analysis, samples were thoroughly vortexed and a droplet of each sample was then qualitatively screened under the microscope at a magnification up to 400 X. By this means, the quality of the sample was assessed, the presence of parasitic stages recorded and photographs taken for further identification of parasites. In order to detect also small numbers of oocysts and eggs, and to assess infections quantitatively a modified flotation method with a saturated $NaCl/ZnCl_2$ solution (specific density of 1.3) was used (Bürger & Stoye 1983). Oocysts and eggs of most parasites have a specific density <1.3 (Bürger and Stoye 1983). Thus, they flotate on the surface of the flotation medium were they can be detected and counted.

Large particles (seeds, grass, pebbles) were separated out from the initial suspension and washed with water twice to remove any adhering eggs. These washes were combined with the initial suspension in a 15 ml centrifuge tube, and centrifuged for 10 minutes at 300 g. The supernatant was then removed, and the pellet weighed to the nearest of 0.01. Only samples weighing 0.06 g or more were included in the analysis. 6 ml of flotation medium were added and carefully mixed to avoid production of air bubbles which can render detection of eggs tiresome. Four compartments of McMaster counting chambers, each with a volume of 0.15 ml, were then filled. The floating oocysts and eggs of each chamber were counted microscopically and a mean oocyst/egg count per sample was then calculated. This mean oocyst/egg count was divided by the mass of the fecal pellet to obtain standardized counts.

2.2.4 *Infection status and infection intensity of birds*

The terms “infected“ and “not infected“ used in this study refer to the success or failure to detect parasitic oocysts or eggs in faecal samples. If parasitic oocysts/eggs were found in at least one faecal sample collected from

the respective individual, was the bird scored “infected“. Only if no eggs were found in all the samples was a bird scored “not infected“. Owing to several factors of the host (gastro-intestinal anatomy, consistency of feces, resistance to parasites) and of the parasites (age, sex, size, developmental and reproductive cycle, susceptibility to crowding effect) endoparasitic infections will not be detected with the same probability and with the same intensity at all times by means of indirect methods. Thus, the inspection of multiple samples, as applicated for adult birds in this study, and as is usual in human medical parasitology, decreases the probability to miss infections and improves the accuracy of infection intensity estimates. Nevertheless, the relationship between fecal egg- or oocyst-counts and infection intensity is notoriously difficult to interpret, even in well-studied systems (Thienpont et al. 1979; Keymer and Hiorns 1986; Doster and Goater 1997). For this reason, infection intensity estimates are always to be treated with caution, although they probably principally present the more powerful tool in studying parasite dependent processes. In this study they should be referred to as for the sake of completedness. The main attention is to be devoted to the presence/ absence data.

2.2.5 *Samples available for the study*

Over the whole study period 905 fecal samples were collected, from which 551 have been examined for parasitic oocysts and eggs. Out of these 551 samples, 352 samples appertained to 84 adult birds. Birds, for which only a single sample had been collected were excluded from the analysis. Furthermore, for each bird only samples from one year were considered. If fecal samples had been collected in two or all three of the study years, only the year in which most samples of the respective individual had been collected was considered. Finally, 278 fecal samples from 68 adult blackbirds (39 males and 29 females) were used for this investigation: 71 samples from 17 birds in 1996, 51 samples from 14 birds in 1997 and 156 samples from 37 birds in 1998. An average of 4,09 samples per adult bird was considered.

Out of the 551 analyzed fecal samples, 191 appertained to 188 nestlings (85 males and 84 females), for which only one sample per indidividual was

available. Since in adults the assay of infection prevalence due to multiple samples is probably more accurate, infection prevalence in the nestling population may be underestimated in this study.

2.2.6 *Data analysis*

Parasite numbers of macro- as well as of microparasites show a highly variable distribution in the host population, which is never normally distributed (Goater and Holmes 1997). For this reason only nonparametric testing was used. All tests were performed two-tailed and α was set 0.05.

2.3 Results

2.3.1 *Parasitic eggs and oocysts in blackbird feces*

The analysis of 551 fecal samples from blackbirds unequivocally prooved the occurrence of protozoans from the family Eimeriidae, representants of the class cestoda, of three nematode families and of the order acanthocephala. Since parasites were identified from eggs only, they were classified within high taxonomic groups.

2.3.1.1 *Class Sporozoasida*

Family: Eimeriidae: Oocysts found in blackbird feces are of 18 – 25 µm in size, and probably belong to different species of the genus *Isospora*. Species of *Isopora* have two sporocysts per oocyst and four sporozoites in each sporocyst (Fig. 1). Distinct sized oocysts cooccured in one and the same sample, suggesting that multiple infections with different *Isospora* species were common. In the literature *Isospora lacazei* and *Isospora turdi* (Scholtyseck 1956; Pellerdy 1974) have been reported for Eurasian Blackbirds.

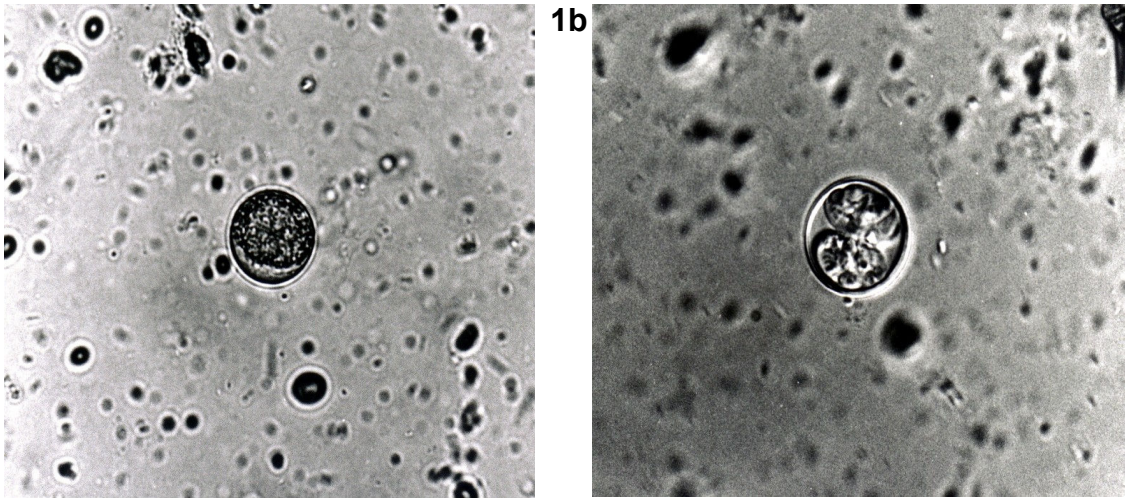

Fig.1 Oocysts of the protozoans *Isospora* spp. Oocysts are excreted with feces in the unsporulated form (1a). Under humid, oxygenated conditions they sporulate to the infective stage, in which two sporocysts per oocyst are visible (1b).

2.3.1.2 *Class Trematoda*

Sporadically found eggs sized 16-22 µm x 26-38 µm were classed within the trematoda, but since determination was not unambiguously possible they will not further be considered here.

2.3.1.3 *Class Cestoda*

Cestode eggs from our blackbird samples varied in size from 41-66 x 37-58 µm, containing hooked larvae with three pairs of hooks (Fig. 2). We confined our diagnosis to class level. In the literature for blackbirds the occurrence of representants from the families Davaineidae (*Fernandezia spinosissima)*, Dilepididae (*Dilepis undula, Anomotaenia verulamii, Liga passerum, Choanotaenia spp.*), and Hymenolepididae (*Variolepsis farciminosa, Haploparaxis spec*.) have been reported (Binder 1971, Lübcke and Furrer 1985, Index catalogue 1968).

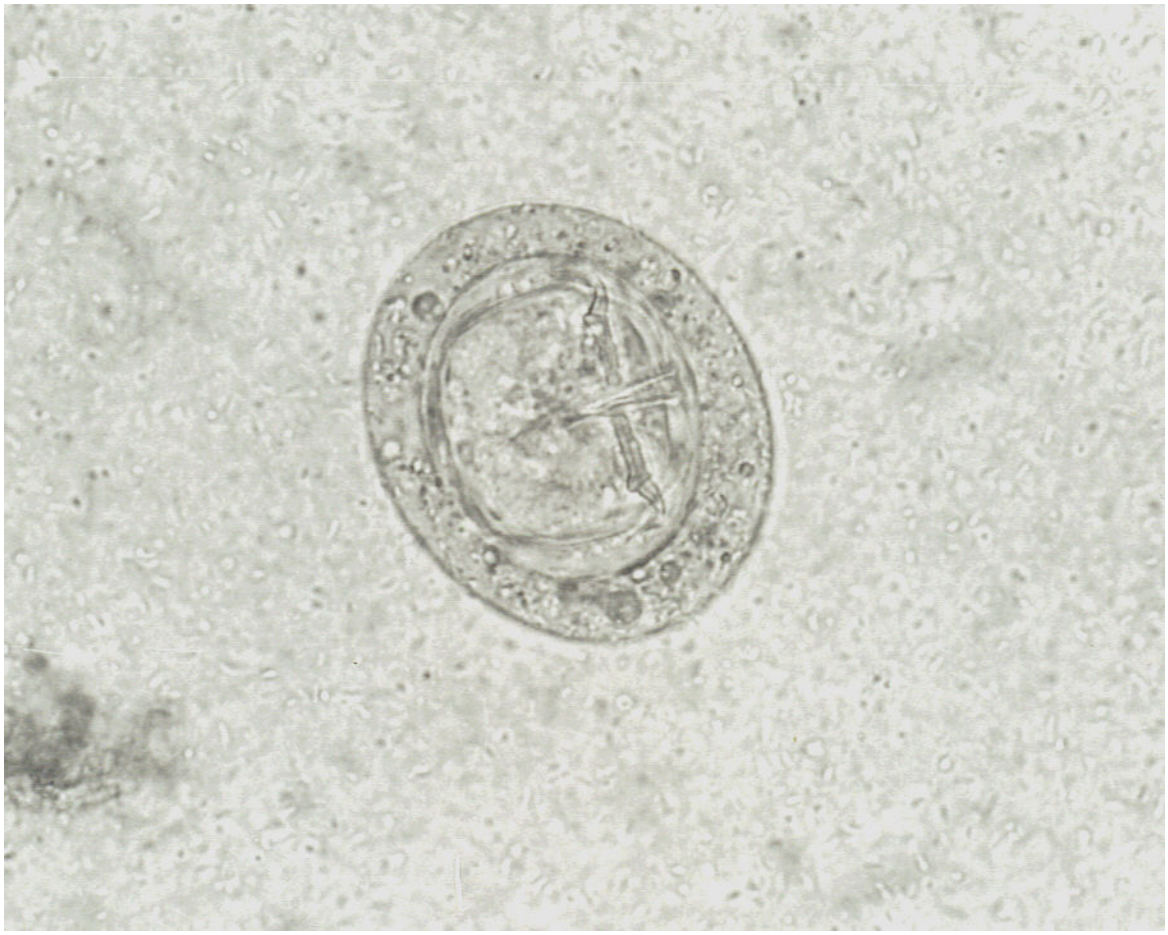

Fig. 2 Cestode egg from blackbird feces. Eggs found in blackbird feces vary in size from 41-66 x 37-58 µm and contain a hooked larva.

2.3.1.4 *Phylum Nematoda*

Three members from the families Syngamidae, Trichuridae and Oxyuridae were found in feces, whereby Oxyuridae and Syngamidae were only sporadically found. From the literature also the occurrence of species from the families Ascarididae (*Porrocaecum ensicaudatum, P. skrjabinensis*), Spiruridae (*Habronema spp*.) and Acuariidae (*Acuaria spiralis*) has been reported (Binder 1971, Index catalogue 1968).

Family Trichuridae: *Capillaria* spp.: Eggs excreted with blackbirds' feces are typically asymmetric, lemonshaped and of 60–70 x 25-33 µm in size. Two

distinct polecaps are always visible (Fig.3a). *Capillaria longicollis* (Binder 1971), *C. similis, C. exilis* and *C. ovopunctata* have all been reported for blackbirds (Wakelin 1966; Index catalogue 1968).

Family Syngamidae: *Syngamus* spp.: Eggs from this genus were found only in a few samples of nestling birds (Fig. 3b). They measure 85-95 x 45-46 µm and probably belong to the species *Syngamus merulae,* which is the only species so far described for blackbirds (Binder 1970, Lübcke and Furrer 1985).

Family Oxyuridae: Eggs were found in a few samples from nestlings only. They are 77-85 x 32-36 µm in size. From the literature, to my knowledge, no reports of Oxyuridae from blackbirds are known.

3a **3b**

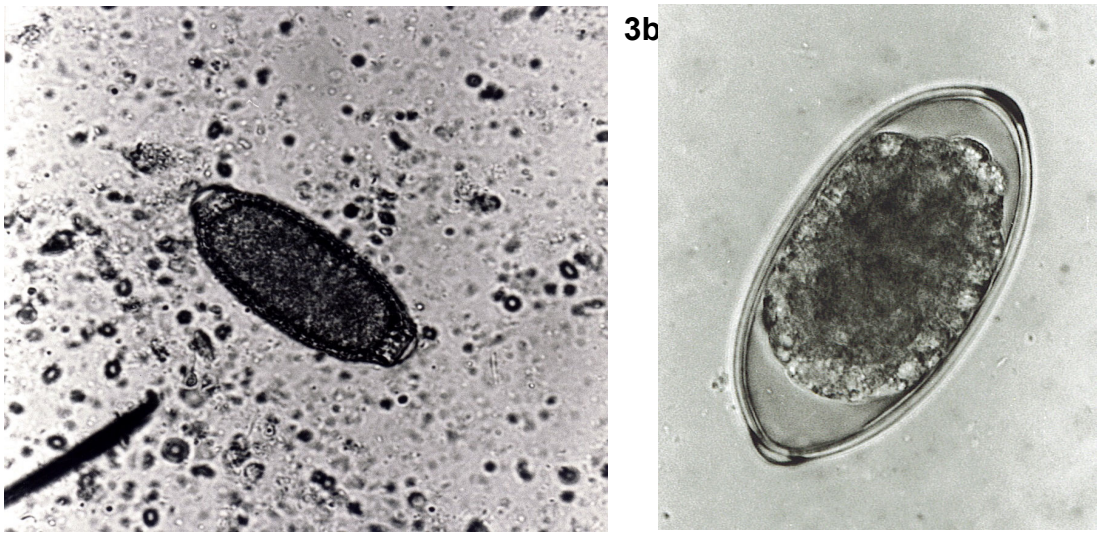

Fig. 3 Nematode eggs from feces of blackbirds. The lemonshaped asymmetric egg of *Capillaria* spp. (3a) is 60x 25 µm in size. It just contains a granulated mass. The egg of *Syngamus* spp.(3b) is 92 x 48 µm in size and contains a morula with blastomeres.

2.3.1.5 *Phylum Acanthocephala*

Acanthocephalan eggs found in blackbird feces varied in size from 73-80 x 30-35 µm containing a hooked acanthor larva. Eggs are lemonshaped with two distinct polecaps (Fig. 4). The occurrence of *Prostorhynchus cylindraceus* (Binder 1971)*, P. transversus, Centrorhynchus scanensis* (Index catalogue 1968) and of *Sphaerirostris scanensis* (Lundström 1942) has been reported for Blackbirds.

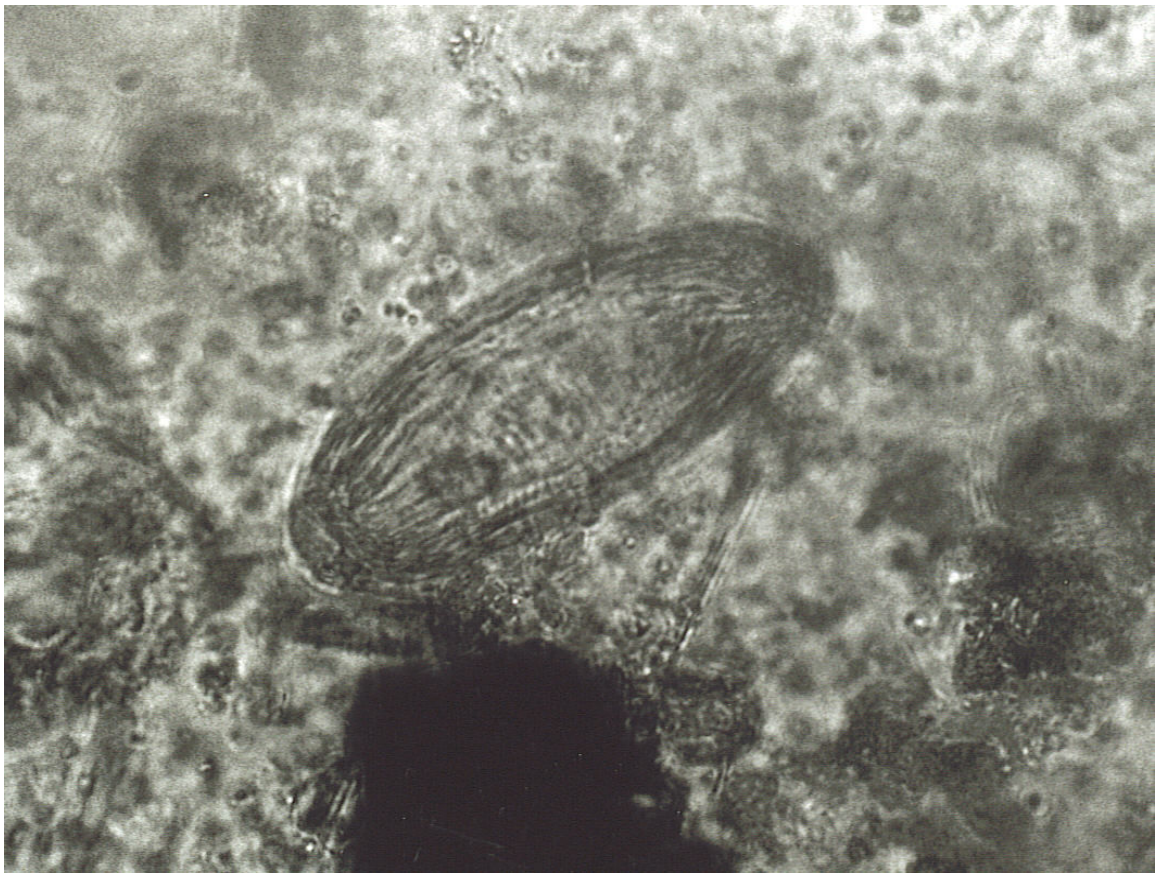

Fig.4 Acanthocephalan egg from blackbird feces. The lemonshaped, thick-shelled egg sizes 77 x 30 µm and contains a hooked acanthor larva.

2.3.1.6. *The four most prevalent parasites*

From the above listed parasites only four were regularily found in blackbird feces. Although acanthocephalan eggs did not flotate and, thus, could not be concentrated and quantitatively assessed by the flotation method, they were regularily found by the qualitative inspection of the samples. In the following analyses the four most common parasite types, all parasitizing sections of the gastrointestinal tract, will be considered: 1) protozoans of the genus *Isospora*, 2) nematodes of the genus *Capillaria,* 3) cestodes and 4) acanthocephalans.

Isospora spp. are sporozoan parasites usually parasitizing cells of the intestinal tract, but also other tissues in a variety of vertebrate species (Bush et al. 2001). Species of *Isospora* have so far been reported to have a direct life cycle, similar to that of *Eimeria*. Recent findings,

however, suggest, that the life cycle of *Isospora* includes two hosts (Bush et al. 2001). In any case, ingestion of infective stages is followed by merogony which is accompanied by extensive destruction of the intestinal tissue. Merogony is followed by gametogony which ends with the production of oocysts. Oocysts are released with feces where they sporulate to the infective stage under oxygenated, humid conditions. The prepatent period of *Isospora* is six to seven days (Mehlhorn et al. 1986).

Capillaria spp. are nematodes parasitizing various sections of the gut. In many species, earthworms, a major component of blackbirds' diet, serve as intermediate hosts. After ingestion of infected intermediate hosts, in which development to invasive larvae has occurred, larvae develop to sexually mature worms within about three weeks (Mehlhorn et al. 1986).

Cestodes also parasitize various parts of the intestinal tract. Their life cycles are diverse and complicated, but all require at least one intermediate host for development, some require two or even three. Oligochaets (*Lumbricus terrestris*), diplopods (*Julus* spp.), coleopterans and snails have been reported as intermediate hosts (Binder 1971; Mehlhorn et al. 1986). The prepatent period is usually 2-3 weeks but varies with species (Mehlhorn et al. 1986).

Acanthocephalans parasitize the intestines of all vertebrate groups. Their life cycles include an arthropod intermediate host, normally a crustacean, a myriapod or an insect. The intestinal wall of the final host is mechanically destroyed by the hooked proboscis of the adult worm. The prepatent period lasts 1-2 months (Mehlhorn et al. 1986; Bush et al. 2001).

2.3.2 *Infection prevalence in adult birds*

2.3.2.1 *Inter- and intraannual variation*

Considering an individual as “infected” if oocysts or eggs of any of the four parasites were found in the fecal material, infection prevalence in the adult blackbird population ranged from 82% in 1996 to 91% in 1998, with a mean infection prevalence over the study period of 88%. Infection prevalence did not differ significantly between the three study years (G-test: G= 1.110, df=2, p=0.574). The comparison of infection prevalences of each parasite type between years (Fig. 5) did also not result in significant differences (Tab.1), although prevalence with *Capillaria* spp. was lower in 1997 than in 1996 and 1998. A comparison of infection prevalences between months is complicated by the fact, that sample collection in 1996 mainly took place in May and June, in 1997 in July and in 1998 from April to August. Thus, infection prevalences between months were compared only in 1996 (May and June) and 1998 (May,

June, July and August). In 1996 data (at least two samples/bird) from 21 birds (10 birds in May, 11 birds in June) and in 1998 data (at least two samples/bird) from 31 birds (11 in May, 6 in June, 7 in July and 7 in August) were analyzed. The results are summarized in table 2. In both years overall infection prevalences remained constant between months. The separate consideration of the four parasites, however, yielded differences for *Isospora* spp. in both years, with lower infection prevalences in May compared to June (1996), and

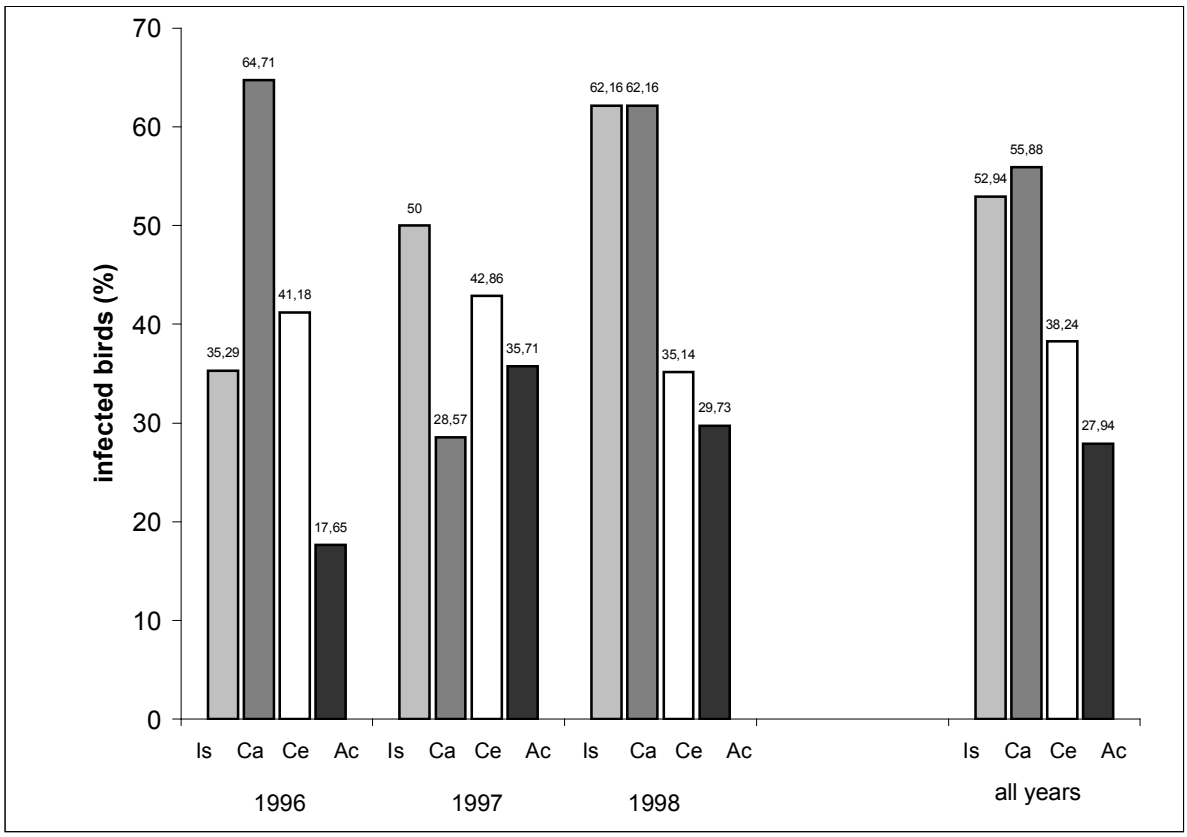

Fig. 5 Infection prevalences with the four parasite types in the three study years with sample sizes n=17 (1996), n=14 (1997), n=37 (1998) and n= 68 (all years). Abbreviations denote *Isospora* spp. (Is), *Capillaria* spp. (Ca), cestodes (Ce) and acanthocephalans (Ac).

compared to June, July and August (1998). Similarily, in 1996 cestode infection prevalence was higher in June than in May, however no difference between months was found in 1998. Infection prevalences of *Capillaria spp*., and of acanthocephalans remained constant between months in both years.

2.3.2.2 *Infection prevalences with the four parasite types*

Considering the distribution of the four parasite types in the population (Fig. 5), only in 1997 parasite prevalence was similar for all four parasites (Cochran's Q-test: Q=6.00, df=3, p=0.112), whereas in 1996 *Capillaria* spp. were the most prevalent parasites (Cochrans Q-test: Q=15.720, df=3, p=0.001), infecting 65% of birds. In 1998 *Isospora* spp. and *Capillaria* spp. were the most prevalent parasites in the adult blackbird population (Cochran's Q-test: Q=32.087, df=3, p<0.001), each infecting 62% of birds. Thus, considering the whole study period, a significant part of infections could be attributed to *Isospora* spp. and *Capillaria* spp. (Cochran's Q-test: Q=42.403, df=3, p<0.001), with a mean infection prevalence of 53% and 56%, respectively. Mean cestode and acanthocephalan infection prevalences were 38% and 28%, respectively.

Tab.1 Comparison of infection prevalences with the four parasite types between the three study years. Percentage of infected birds in parentheses.

	1996	1997	1998	All years	G	*P*
Isospora spp.						
birds sampled	17	14	37	68		
birds infected	6 (35 %)	7 (50%)	23 (62%)	36 (53%)	3.468	0.177
Capillaria spp.						
birds sampled	17	14	37	68		
birds infected	11 (65%)	4 (29%)	23 (62%)	38 (56%)	5.417	0.067
Cestodes						
birds sampled	17	14	37	68		
birds infected	7 (41%)	6 (43%)	13 (35%)	26 (38%)	0.339	0.844
Acanthocephalans						
birds sampled	17	14	37	68		
birds infected	3 (18%)	5 (36%)	11 (30%)	19 (28%)	1.440	0.487

2.3.2.3 *Multiple infections*

Multiple infections in adult birds were very common. From all infected birds (n=60), 38 birds (63%) were infected with more than one parasite type. 22 birds (37%) were infected with only one parasite type (Binomial test, p= 0.052), whereby two thirds of these single infections could be attributed to *Isospora* spp. and to *Capillaria* spp.. Dual infections were found in 19 birds (32%), triple

infections in 16 birds (27%) and 3 birds (5%) were infected with all four parasite types. In the case of dual infections only one out of six possible associations did not occur, namely the association between cestodes and acanthocephalans. In the case of triple infections one out of four possible associations did not occur. *Capillaria* spp., cestodes and acanthocephalans never co-occurred, whereas Isospora, *Capillaria* spp. and cestodes co-occurred in 9 birds.

2.3.2.4. *Infections in males and females*

The overall infection prevalence of males (n=39) and females (n=29) did not differ significantly in any of the three study years (Tab.2), although in 1998 the difference was close to significance. In that year all females have been infected. However, if infection prevalences with the four parasite types were considered separately (Fig. 6), females showed up to be infected with *Capillaria* spp. significantly more frequently than males (G-test: G= 4.391, p=0.036), whereas no significant differences were found in infection prevalences with the three other parasite types (*Isospora* spp.: G=0.657, p=0.418; cestodes: G=0.211, p=0.646; acanthocephalans: G=3.473, p=0.062).

Tab. 2 Comparison (G-tests) of overall infection prevalences between males and females in the three study years. Percentage of infected birds in parentheses.

	males	females	G	*P*
1996				
birds sampled	9	8		
birds infected	7 (78%)	7 (88%)	0.281	0.596
1997				
birds sampled	9	5		
birds infected	8 (89%)	4 (80%)	0.200	0.655
1998				
birds sampled	21	16		
birds infected	18 (86%)	16 (100%)	3.599	0.058
All years				
birds sampled	39	29		
birds infected	33 (85%)	27 (93%)	1.218	0.270

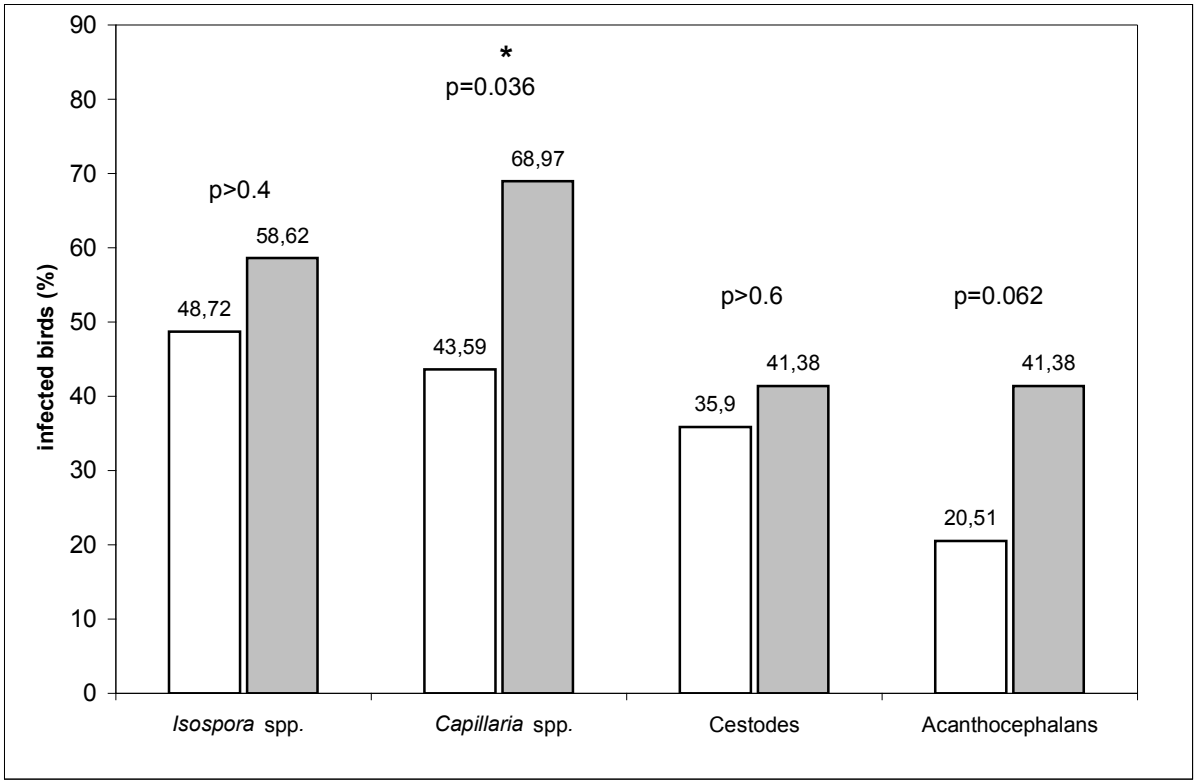

Fig. 6 Infection prevalences with the four parasite types for males n= 39 (white bars) and females n= 29 (shaded bars).

Furthermore, the analysis of infection intensities showed, that females were infected with *Capillaria* spp. not only more frequently than males, but also at higher intensities (Mann-Whitney U-test: n1=38, n2=29, z=-2.474, p=0.013). Infection intensities with *Isospora* spp. and cestodes were similarily high in males and females (Mann- Whitney U-tests: Isospora:n1=38, n2=29, z=-0.427, p=0.670; cestodes: n1=39, n2=29, z=-0.333, p=0.739).

Finally, males and females were compared as with regard to the frequency of multiple infections (Fig. 7). Although males and females were infected at similar frequency (see above), females were significantly more often affected by multiple infections than males (G-test: G=4.537, df=1, p=0.033). In females 78% of infected birds (21 out of 27) had multiple infections, whereas in males single infections were as frequent as multiple infections (52% and 48% of infected male birds, respectively).

2.3.3 *Isospora* spp*. infections in nestlings*

Due to the more extended prepatent periods of *Capillaria* spp., cestodes and acanthocephalans, only infections with *Isospora* spp. could be considered in nestling's feces at an age of 8 to 10 days.

From 188 nestlings, 60% (112 birds) were found infected at the age of 8 to 10 days. Infection prevalence with *Isospora* spp. did not differ significantly between years (Tab. 3), although in 1997 prevalence was higher than in the two other years. Furthermore, infection prevalence remained constant over the main sampling period (n=172) from May to July (G-test: G=3.968, df=2, p=0.138).

Tab. 3 Comparison of isosporan infection prevalences in nestlings between the three study years. Percentage of infected nestlings in parentheses.

	1996	1997	1998	All years	G	*P*
Isospora spp.						
nestlings sampled	57	68	63	188		
nestlings infected	30 (53 %)	48 (71%)	34 (54%)	112 (60%)	5.498	0.064

2.3.3.1 *Infections in male and female nestlings*

With 60 % of male nestlings (n=85) and 62 % of female nestlings (n=84) being infected with *Isospora* spp*.* no difference in infection prevalence between the sexes could be recorded (G-test: G=0.064, df=1, p=0.8). Furthermore, male and female nestlings were infected at the same intensity, with a mean (median) oocyst count per unit feces of 3.25 and 4.00, respectively (Mann Whitney U-test: n1=84, n2=84, z=-0.777, p=0.437).

2.3.3.2 *Distribution of infections within and among nests*

The 188 nestlings in this study originated from 82 nests. In 27 nests only from a single chick a sample could be obtained, in the remaining 55 nests a fecal sample was obtained from two up to five chicks. Only in 6 nests all chicks were free of infection, whereas in 17 nests all chicks were found to be infected. In as many as 32 nests as well as infected as not infected chicks were present,

indicating that infection probability was not bound on the nest as a unit, but on individuals.

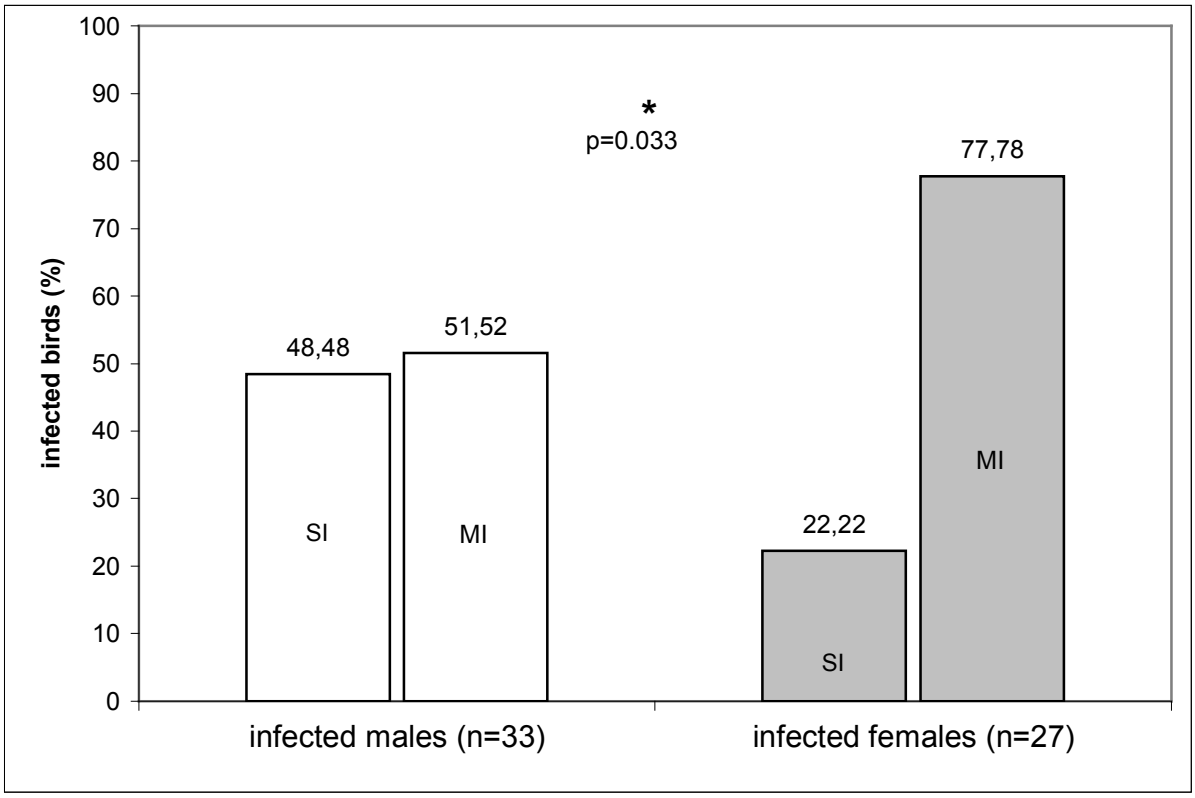

Fig. 7 Comparison of single infection frequencies (SI) and multiple infection frequencies (MI) between males and females.

2.3.4 *Comparison of adults and nestlings*

Nestling and adult blackbirds were infected with *Isospora* spp. at similar frequency, with 60% and 53% of birds being infected, respectively (G-test: G=0.896, p=0.344). Prevalence in nestlings, however, may be underestimated due to the examination of only a single sample per individual (see methods). Although being similar frequent, infections in nestlings were significantly more severe than in adults, with a mean (median) infection intensity (eggs per unit feces) of 3.25 compared to 0.21 in adult birds (Mann Whitney U-test: n1= 187, n2=67, z=-2.402, p=0.016).

2.4. Discussion

2.4.1 *Differences to the helminth fauna in the study of Binder* (1971)

Overall infection prevalence in adult blackbirds was high. 88 % of birds harboured at least one taxon of the four considered intestinal parasites. Most information on the occurrence and distribution of helminths in Eurasian blackbirds has, so far, come from a study of Binder (1971), conducted within the rural area of Gießen (Germany). He found an even higher overall infection prevalence of 98,7 % in his blackbird populations, although he did not consider infections with coccidians. However, he used a direct method for the assay of endoparasitic infections (dissection of birds) and, thus, also considered a higher number of species. Furthermore, he considered the whole calendar year, whereas in the present study only the breeding season from late April to early August was considered. Cestodes were the most prevalent parasites in his study, parasitizing 90,5 % of birds, whereas they parasitized only 38 % of birds in the present study. Two cestode species which regularily parasitized blackbirds in Binders study, *Anomotaenia verulami* and *Fernandeza spinosissima* both showed high infection prevalences in fall, a season which was not considered in my study. Thus, the lower overall cestode infection prevalence in the present study can partly be explained by this fact. Nevertheless, several differences in the parasite species composition and distribution seem to exist between both studies, which are not necessarily due to methodological differences.

Binder does not mention the occurence of Oxyurids in his birds, but, in contrast to the present study, trematodes seemed to play a much more important role in his bird populations. Two species of trematodes regularily parasitized about 30 % of his blackbirds, whereas in the present study trematode eggs were only sporadically detected. Infection prevalences in my study were highest for *Isospora* spp. and *Capillaria* spp., which were present in 53% and 56% of birds, respectively. Binder did not consider coccidians in his

study and *Capillaria* only sporadically parasitized his blackbirds. The most prevalent nematode in his study was an ascarid (*Porrocaecum ensicaudatum),* which' eggs did not occur in fecal samples of my study. More or less comparable in both studies are the infection prevalences of acanthocephalans, which were 28 % in my study compared to 20,8 % in Binder's study.

It is known from other host species, that the occurrence and prevalence of parasites vary among hosts of the same species inhabiting different localities. This phenomenon has been described for haemoparasites (vanRiper 1991; Allander and Bennett 1994; Merilä et al. 1995), for ectoparasites (Gregoire et al. 2002) and for gastro-intestinal parasites (Goater et al. 1995; Müller-Graf et al. 1996; McQuistion 2000). The most frequent cited causes of such variation are 1) variation in a host 's exposure to infective stages and 2) variation in host susceptibility and (or) immune responsiveness (Goater et al. 1995). If parasites rely on intermediate hosts (like most helminths) or on vector species (like most haematozoans) for successful transmission, the availability and/or abundance of intermediate hosts and vectors is one aspect, potentially influencing the composition, prevalence and abundance of parasites in the host population. Binder (1971) found high temporal coincidences between the availability of invasive larvae in intermediate hosts and feeding preferences of blackbirds on these hosts. The availability and abundance of intermediate hosts and vectors, on turn, depends on various environmental conditions, such as temperature, moisture, vegetational structure of the habitat, presence or absence of wet areas, etc.. The absence of running water in a swedish study area, for example, was responsible for the absence of haematozoans from the genus Leucocytozoon in a great tit population, since successful transmission of these parasites relies on vector species reproducing in running water (Allander and Bennett 1994). Similarily, infection prevalence with *Ixodes* ticks varied markedly between a rural and an urban population of Eurasian Blackbirds in eastern France, with the rural population being infected more frequently (Gregoire et al. 2002). The life cycle of ticks requires at least three different hosts to be completed. Gregoire et al. suggest that final hosts, which are often medium sized to large mammals, may have been absent, or been present only at low densities, in the urban habitat. Another explanation they offer is that ticks

experienced higher mortality in urban habitats due to unsuitable vegetation, microclimatic conditions and possibly pesticide use. The two most prevalent parasites in Binders study, the cestode *Dilepis undula* and the nematode *Porrocaecum ensicaudatum*, which parasitized 71 % and 54,3 % of birds, respectively, both use *Lumbricus terrestris* as intermediate host. According to the literature *L. terrestris* presents a main part of the blackbirds diet in spring and summer (Stephan 1997). After ingestion of *L. terrestris* infected with invasive larvae, hosts pass parasitic eggs from *P. ensicaudatum* in feces about 2 months later, i.e. as early as mid May (Binder 1971). Developmental periods of *D. undula* are much shorter, such that parasitic eggs can be passed in feces as early as April (Binder 1971). However, eggs of *P. ensicaudatum* were not detected in my study and cestodes parasitized blackbirds in my population only to 38 %. No definitive explanation can be given for these differences at the moment. Blackbirds in the present study may have fed less on *L. terrestris* because weather conditions may have favoured other food sources. The food spectrum of blackbirds varies markedly between populations (Stephan 1997). In the Wolga-Kama area, e.g. *L. terrestris* presents only 8% of the blackbirds diet, whereas coleopterans (mainly Cuculionidae) present 45% of their diet. It is plausible that variations in main food sources can lead to markedly different exposure risks to parasites, first, because potential intermediate hosts or infective parasitic stages are ingested at different rates and, second, because the respective foraging technique differentially exposes birds to parasites. McQuistion (2000) showed that birds feeding on fruit had significantly lower infection prevalences with coccidians than birds feeding on insects. Furthermore, it is known that granivourous and omnivourous birds, which spend more time foraging on the ground are more likely to be infected with coccidians than insectivorous birds, because oocysts are more likely to be ingested from contaminated soil (Scholtyseck 1956; McQuistion 2000; Zinke et al. 2004).

Alternatively, lower susceptibility or higher resistance to local parasites may lead to altered infestation levels. Walker et al. (2003) in a study on great tits showed that early exposure of nestlings to hen fleas (*Ceratophyllus gallinae*) reduced the reproductive rate of fleas later in the nestling cycle through a parasite induced immunological response of the chicks. Other studies have

documented a transfer of maternal immunity to offspring as an induced response to the local parasites (Smith et al. 1994; Gasparini et al. 2001; Buechler et al. 2002). High local parasitic pressure may, thus, produce genetic resistances against certain parasites (e.g. to *P. ensicaudatum*) while at the same time weakening resistances to other parasites (e.g. to *Capillaria*).

I want to emphasize that the differences in the occurrence and prevalence of parasitic helminths found between the two above discussed studies on blackbirds, living in relatively similar habitats (as compared to their broad, almost worldwide distribution), explicitly demonstrate, that the parasite communities and prevalences assessed from single populations can not be taken as representative for the whole species. This fact has to be considered in most parasite related studies, but most importantly in comparative studies, such as Hamilton and Zuk's (1982) study on bright birds.

2.4.2 *Infections with Isospora* spp.

2.4.2.1 *Infections in adults*

Infection prevalence with *Isospora* spp. was relatively high (52%) in this study compared to the infection prevalence of 17% reported for wild blackbirds in 1956 (Scholtyseck). The relative high population density in this study population (8.2 birds per ha), which is typically higher than in comparable rural populations of blackbirds (Haffer 1988), may have promoted the high infestation rate in this study. It is known that in parasites with horizontal transmission parasite prevalence is a function of host population density (Rausch 1983; Kruszewicz 1995). The lower prevalence of *Isospora* spp. in May, compared to June, July and August may be due to environmental conditions in May still being suboptimal for the oocysts' development to the infective stage, which requires oxygenated, warm, humid conditions. Alternatively, increased parasite prevalence may be the result of reduced immunocompetence. If resources are increasingly allocated to reproduction (mating, nest-building, incubating, egg-laying, feeding nestlings), allocation to immune function may be compromised

(Stearns 1992). This explanation receives further support by the result that in nestlings no increase in parasite prevalence from May to August was to note.

2.4.2.2 *Comparison of adults and nestlings*

Nestlings were infected with *Isospora* spp. at similar frequency (60%) than adults (53%) but at higher intensity. Przygodda and Scholtyseck (1961) found nestlings of various bird species to be infected with coccidians at lower frequencies than adult birds. Since coccidians take about six to seven days to develop, oocysts can de detected in feces at the earliest on the 6^{th} or 7^{th} day of life. Hatchwell et al. (1990) found no haematozoan parasites in the blood films of nestlings and also argued that infections had probably occurred but had not progressed far enough through their life cycle to appear in peripheral blood. Considering this developmental aspect, the infestation rate in blackbird nestlings was relatively high, especially under the assumption that prevalence was even underestimated due to the single-sample-assays in nestlings. The immune system of young birds is still not mature at the nestlings age and, thus, more vulnerable to the challenges of parasitic infection than in adults (Rose 1967; Roitt et al. 1996; Ros et al. 1997). This higher vulnerability may affect susceptibility and further establishment of infections.

2.4.2.3 *Isospora infections within nests*

In contrast to ectoparasitic infections which are easily transmitted among nestlings of the same nest (by close body contact or by parasites living inside the nest and visiting nestlings only for blood meals), isosporan infections were distributed randomly among nestlings. Nestling's feces are surrounded by a fecal sac, which parents regularily remove from the nest. Thus, transmission probability through infective fecal material is highly reduced within nests. Sometimes, however, nests are used for more than one breeding event. In these cases, infections may be aquired by oocyst-contaminated old layers of nesting material. Nevertheless, the main source of infection at this age is probably the ingestion of infected food and, thus, a random event in a first instant. This fact makes isosporan parasites suitable to study effects of parasites on individual nestling fitness, because randomly infected and not

infected nest mates share the same environmental conditions and the same parents' phenotypic and genotypic (if they are no extra pair young) qualities.

2.4.3 *Multiple infections*

Multiple infections were as frequent as infections with only one parasite type. Binder (1971) also reports that multiple infections were common in his population. Associations of parasites, both within and among taxonomic levels are a common phenomenon reported for various parasites in birds (Moore and Simberloff 1990; Forbes et al. 1994; Fedynich et al. 1996; Janovy 1997). The different sections of the alimentary tract each present a habitat which may be used by one or several parasite species (Janovy 1997). In dual infections, *Isospora* spp. and *Capillaria* spp., the most prevalent parasites in this study, co-occurred with all other parasites. Cestodes and acanthocephalans did not co-occur. Forbes et al. (1994) in a study on blue grouse showed that haemoparasitic patterns of interspecific association varied. They suggested that the presence or absence of specific infections accounted for some variation in the likelihood of co-occurrence. Thus, the study of parasite assemblages is important with respect to understanding the impact of infections on aspects of the host's physiology, immunology, behaviour, ecology and population dynamics. The frequent occurrence of multiple infections, furthermore, elucidates the need for multi-parasite models of parasite-host interactions (Dobson 1990).

2.4.4 *Males and females*

Overall infection prevalence did not differ in males and females. Adult females, however, were infected with *Capillaria* spp. significantly more frequently and more intensively than males and they also were more frequently affected by multiple infections. Studies investigating a general sexual bias of parasitism have yielded controversial results (Poulin 1996; McCurdy 1998). This

is not further surprising, considering the three most frequent cited causes for a sexual bias: 1) hormonal differences which can lead to differential suppression or activation of certain parts of the immune system (Grossman 1989; Bentley et al. 1998; Zuk & Johnsen 1998), 2) differences in parasite exposure (Zuk 1990; Zuk & McKean 1996) and, 3) differences in physiological stress due to different energetically demanding activities (Zuk 1990; Zuk & McKean 1996). The testing of all these aspects requires carefully designed experiments, since many variables, such as the parasites used, their life cycle, their pathogenicity, the sampling technique, the biology of the host, its breeding status, the local climatic and environmental conditions and temporal variables may all lead to confounding results if not properly treated.

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Parental expenditure and intestinal parasites in Eurasian Blackbirds (*Turdus merula*)

Abstract - In birds, parental feeding is assumed to be the most costly part of reproduction. If immunological response to parasitic infection also involves costs to an individual, a negative relationship between parental feeding expenditure and the ability to fight parasitic infections is predicted. In a population of wild blackbirds, *Turdus merula,* the correlation between feeding expenditure and the infection with three intestinal parasites was analyzed. The presence or absence of parasites was determined by microscopic examination of parasitic-egg-concentrated feces from adult birds. Parental feeding expenditure was determined as the number of days hatchlings have been fed. Infection frequencies between successful breeders feeding chicks and unsuccessful breeders did not differ, probably because infection prevalence prior to the breeding season was not randomly distributed between (future) successful and unsuccessful breeders. In successfully breeding males, likelihood of infection increased with paternal feeding expenditure. Because of an overall high infection prevalence in females, no such relationship was found in females. Our findings suggest, that female blackbirds are generally more susceptible to infections. Furthermore, we show that, at least male blackbirds, seem to incur high feeding costs, which are reflected in decreased ability to resist infections.

3.1 Introduction

It is a fundamental assumption of life-history theory that an organism has only a limited amount of energy and resources at its disposal (Stearns 1992). Consequently, not all life-history traits (e.g. reproductive life span, growth rate, number and size of offspring, age and size at maturity) can be maximized at the same time (Williams 1966). Every investment can only occur at the expense of another investment. At a physiological level, this means that individuals are faced with energy allocation trade-offs. If two or more processes compete for

the same limited resources within a single individual, a "decision" has to be made regarding resource allocation between the competing processes (Levins 1968; Sibly and Calow 1986). Every prediction of a physiological trade-off makes the basic assumption that the processes concerned result in costs to the individual.

Consider a reproducing individual which is exposed to parasitic infection, a condition which is probably very common in natural populations. Assuming that both reproduction and defense against parasites are costly, the individual is faced with a trade-off between the amount of resources or energy it can invest in present reproduction, and that required to combat infection. Several studies have addressed the question of reproductive costs, and most show strong evidence that individuals indeed incur costs through reproduction, measured in 1) reduced survival (Nilsson and Svensson 1996; Stjernman et al. 2004), 2) reduced future fecundity (Roskraft 1985; Gustafsson and Sutherland 1988; Gustafsson et al. 1994), 3) reduced future sexual attractiveness (Gustafsson et al. 1995), 4) reduced future condition (Festa-Bianchet and Jorgenson 1997) and acceleration of senescence (Gustafsson and Pärt 1990). In these studies it is assumed that the measured fitness costs stem from the most costly components of reproduction, among which the feeding of young holds a central place (Clutton-Brock 1991). The energy expenditure of birds feeding nestlings has been shown to increase to 3.0 to 4.0 times the basic metabolic rate (Drent and Daan 1980; Walsberg 1983; Masman et al. 1989). Less attention has been paid to measurements of energetic costs arising from immune responses. Protozoans and helminth parasites can induce a variety of immune reactions in their hosts, ranging from phagocytic takeup, intracellular defences, antibody-dependent cellular cytotoxicity and intestinal T-cell-mediated inflammatory responses (Wakelin 1994; Wakelin and Apanius 1997). Although it seems reasonable to assume that immune reactions are costly (Sheldon and Verhulst 1996) direct estimates of energetic costs of immune responses are difficult to obtain (Råberg et al. 1998; Owens and Wilson 1999). A growing number of studies, however, provides indirect evidence of these costs (for reviews see Schmid-Hempel 2002; Zuk and Stoehr 2002), as e.g. reduced weight gain after immunization (Klasing et al. 1991; Deerenberg et al. 1997), or weight loss after

immune challenge (Bonneaud 2003). Although there is still some controversy about where costs of immunity actually arise, a concurring opinion seems to establish in the literature that, in a broad sense, there is a “cost of immunity” may the costs arise from the maintenance of an immune system per se, from the maintenance of specific kinds of resistance, from the potential of mounting a successful immune response or from actually mounting an immune response (Svensson et al. 1998; Williams et al. 1999; Lochmiller and Deerenberg 2000; Ots et al. 2001; Råberg et al. 2002; Schmid-Hempel 2002; Bonneaud et al. 2003, Verhulst et al. 2005).

If a trade-off exists between parental expenditure and immunological defense, then a higher investment in costly parental activities should be associated with a higher probability of parasitic infection, as a result of a lower immunocompetence. Although there is still a big gap in our knowledge of how parental effort, immunocompetence and parasite resistance are linked to each other on a physiological level (Norris and Evans 2000), two studies provide indirect evidence that parental effort, immunocompetence and parasite resistance are directly linked to each other (Apanius et al. 1994; Nordling et al. 1998). Furthermore, several studies support the idea of a trade-off between parental effort and immunocompetence (Deerenberg et al. 1997; Nordling et al. 1998; Ilmonen et al. 2000; Råberg et al. 2000; Moreno et al. 2001; Ardia et al. 2003; Verhulst et al. 2005), and between parental effort and parasite resistance (Norris et al. 1994; Richner et al. 1995; Oppliger et al. 1996; Ots and Hõrak 1996; Allander 1997; Oppliger et al. 1997; Nordling et al. 1998; Wiehn and Korpimäki 1998; Fargallo and Merino 1999; Merino et al. 2000). All of the latter studies considered infections with haemoparasites, but only a few studies were conducted in birds considering intestinal parasites. Intestinal parasites occur in a wide array of wild birds (Janovy 1997) and have been shown to influence fecundity and breeding success of red grouse (Hudson 1986; Hudson and Dobson 1991), migratory performance in Bar tailed Godwits (Piersma et al. 2001) and carotenoid-based plumage pigmentation in male house finches (Brawner et al. 2000). Thus, they may play as an important role in shaping life history traits as has been suggested for haemo- and ectoparasites. In the present correlational study on a free ranging population of blackbirds the focus

is on intestinal helminths and coccidians. I investigate the relationship between natural parental feeding expenditure and the probability of intestinal parasitic infections.

3.2 Methods

3.2.1 *Study site and study population*

This study was conducted from 1996 to 1998 as part of an ongoing survey of a free ranging population of blackbirds in the Botanical Garden of Bonn, Germany. The study area comprised 8.2 ha. of evergreen and deciduous vegetation and open grass areas. The number of breeding pairs varied between 34 to 36 pairs per year. All adult birds were captured in mist nets prior to the breeding season and marked with aluminium rings and a combination of colored rings for easy field identification. From March to August all breeding attempts (nests with at least one egg) were recorded, and all nests inspected on a regular basis to record egg production and loss, onset of incubation, day of hatching, nestling number and day of fledging.

3.2.2 *Blackbird parasites*

Prevalence of intestinal parasites was determined by evaluation of presence or absence of parasitic eggs and oocysts in the feces. Only three of the most prevalent intestinal parasites, coccidians of the genus *Isospora*, nematodes of the genus *Capillaria* and cestodes of the orders Caryophyllidea and Cyclophyllidea were considered, evaluated by screening feces of 103 blackbirds.

Isospora spp. are sporozoan parasites usually parasitizing cells of the intestinal tract. Ingested oocysts release sporozoites which penetrate into intestinal epithelial cells where they undergo asexual reproduction by schizogony. Schizogony is followed by sexual reproduction which ends with the production of oocysts. Oocysts are excreted with the feces about six to seven days after infection. They sporulate to the infective stage under oxygenated,

humid conditions. Oocysts found in blackbird feces are of 18 – 25 µm in length, and probably belong to different species. For blackbirds, *Isospora lacazei* and *Isospora turdi* (Boughton 1937; Pellerdy 1974) have been reported.

Capillaria spp. are nematodes parasitizing various sections of the gut. In many species, earthworms, a major component of blackbirds' diet, serve as intermediate hosts. After ingestion of infected intermediate hosts, in which development to infective larvae occurred, larvae develop to sexually mature worms within about three weeks. Eggs excreted with blackbirds' feces are typically asymmetric, lemonshaped and 60 – 70 µm in length. *Capillaria longicollis* (Binder 1971), *C. similis, C. exilis* and *C. ovopunctata* have all been reported for blackbirds (Wakelin 1966).

Cestodes also parasitize various parts of the intestinal tract. Their life cycles are diverse, but all require at least one intermediate host for development (snails, earthworms, arthropods). Cestode eggs from our blackbird samples varied in length from 41 µm to 66 µm, containing hooked larvae. We confined our diagnosis to order level. In the literature the occurrence of representants from the families Davaineidae, Dilepididae and Hymenolepididae have been reported (Index catalogue 1968; Binder 1971, Lübcke and Furrer 1985).

3.2.3 *Collection of fecal samples*

In order to minimize disturbance to birds and to reduce parasitic egg output biases due to “shocksamples“ (samples with high percentage of water), which can occur when birds are captured, an observational method to obtain fecal samples was chosen. Birds were observed in activities which preferentially take place on the ground (feeding, collecting nesting material and collecting food for chicks) and followed until they defecated. Feces were collected only, if the position and the identity of the sample could be determined unambiguously. With this observational method it was possible to gather more than one sample for most birds without disturbing breeding activities. Only samples collected within a 40 day period (May 1st to June 9th) were considered, to reduce biases due to possible seasonal parasitic fluctuations, although overall parasite prevalence in this blackbird population has been shown to remain constant from

May to August (Misof in prep.). The considered 40 day period coincides with the mid-point of the breeding season (mid March – August), and was selected to both enable the inclusion of as many breeding birds as possible and to be as short as possible. Samples were collected only in the morning hours, to avoid sampling biases due to daily fluctuations of egg/oocyst output (Brawner and Hill 1999; Misof 2004). Analysis was restricted to the breeding seasons of 1996 and 1998. Sample series from 24 males and 18 females were obtained for analysis.

3.2.4 *Analysis of fecal samples*

Fecal samples were stored at 4°C in 2% potassium bichromate. A modified flotation method with a saturated $NaCl/ZnCl_2$ solution (Bürger and Stoye 1983) was used to concentrate parasite eggs. After removal of large particles (seeds, grass, pebbles) the sample was centrifuged for 10 minutes at 300 g. The supernatant was then removed and 6 ml of flotation medium added and carefully mixed. Four compartments of two McMaster counting chambers were then filled and a total volume of 0.6 ml screened for parasite eggs.

3.2.5 *Infection status*

Parasitic infection was considered only as present or absent, because the relationship between fecal egg- or oocyst-counts and infection intensity is notoriously difficult to interpret, even in well-studied systems. (Doster and Goater 1997; Thienpont et al. 1979; Keymer and Hiorns 1986). Although infection intensity is probably the more powerful tool in analyzing parasite-dependent processes, we abstained from using it because the factors potentially leading to distorted intensity estimates are numerous: in helminths, male individuals and larval stages cannot be detected; the number of eggs laid is species specific and usually decreases with worm age; density dependent effects on egg-laying occur and egg-laying in most worms is not a continuous process (Thienpont et al. 1979); in protozoans, high temporal variability of parasite counts occurs, even in a 24 hour period under controlled laboratory conditions (Doster and Goater 1997; Kruszewicz 1995).

The terms “infected“ and “not infected“ used in this study refer to the success or failure to detect parasite eggs in fecal samples. The term “overall

infection" refers to indicate infections with at least one of the three parasite types. In most cases, more than one fecal sample for each bird was obtained. If parasite eggs were found in a single fecal sample the bird was scored as "infected". Only if no eggs were found in all the samples was a bird scored as "uninfected". Birds for which only a single sample was available, and in which no eggs could be detected, were excluded from the study.

3.2.6 *Breeding status and feeding expenditure*

All birds had reached egg-laying status by the time the first sample was taken. Hence, they were all subject to the endocrine changes that accompany reproduction, and which are thought to be potential mediators of parasite susceptibility (Grossman 1984). Birds were termed successful breeders (SBs), if they had at least one hatched chick prior to the day on which their last fecal sample was taken. Birds which did not successfully hatch chicks up to the day on which their last fecal sample was collected, were termed unsuccessful breeders (UBs). Blackbirds start breeding in March when clutch and brood loss are usually high but replacement clutches are readily laid. Thus, the assignment of SBs and UBs was not bound on calendar date. Feeding expenditure was scored as the sum of days on which nestlings had been fed, prior to the date on which the respective fecal sample was taken. This sum included feeding days of preceding breeding attempts of the same bird in the same season. The counting procedure of feeding days was repeated for all considered samples of a bird and, finally, a mean number of feeding days was determined. This very general measurement of parental feeding expenditure was intentionally used, in order to weight out any bias, which may arise in dependence on the considered factors. So is the "real" feeding expenditure dependent on: 1) the number of chicks, but as well on the age of chicks: blackbird nestlings get fed during their first two days of life only twice to three times a day (Hune, unpubl. data), thus, the feeding effort of parents may not be comparable between two cases on which, i. e. one nestlings has been fed for five days or on which five nestlings have been fed for one day; 2) the feeding experience of the parent: it probably takes less effort for experienced parents to gather a certain amount of food than it takes for less experienced parents; 4) the weather conditions: it is much harder to find

earthworms on dry days compared to wet days, but from a thermostatic point of view it may be less energy consuming to forage on warm, dry days compared to cold, wet days; 5) the travelling distance to good feeding grounds: even if nests are visited similarily frequent, parents with bad territories may have to invest more effort into the same number of feeding visits.

3.2.7 *Data analysis*

Simple G-tests were performed to analyze the distribution of parasitic infections in the population. All p-values represent two tailed tests. The confidence limit was set at 95%. Logistic regression was used to test for effects of feeding expenditure and of calendar date on infection probability of successful breeders (SBs). Infection status (infected/ not infected) was treated as dependent binary variable. Number of feeding days, calendar day and sex were included as independent variables. The final model was obtained by stepwise inclusion of variables based on the likelihood ratio statistics. To test for sex specific effects, two additional models were generated for males and females separately. All statistical analyses were run using the software package SPSS for Windows, version 10.0.

3.3 Results

3.3.1 *Prevalence and distribution of infections*

Overall Infection prevalence and infection prevalences with the three parasite types did not differ significantly between 1996 and 1998 (all $P>0.4$). Thus, data for the two years were pooled in all following analyses. Infection prevalence was high: from 42 adult blackbirds examined, 34 (81%) were infected with at least one of the three parasite types. The most prevalent parasites were nematodes of the genus *Capillaria*, present in 28 of 42 birds (67%). Cestode infections could be detected in 14 birds (33%), and infections with protozoans of the genus *Isospora* were present in 11 birds (26%). The comparison between overall infection prevalence between males and females

yield a significant difference, with females being infected more frequently than males (Table 1). Infection prevalence in females was extremely high, with all but one female being infected with either one of the three parasite types (Table 1). However, if the parasites were considered separately, no differences in infection frequencies between males and females were found (Table 1).

Table1. Comparison of infection prevalences between males and females (G-tests, df = 1) for infections with at least one of the three parasites and for infections with each of the three parasites separately. The number and the percentage (in brackets) of infected individuals are given.

	males (n = 24)	females (n = 18)	G	P
infected	17 (71)	17 (95)	4.20	0.04
Isospora spp.	6 (25)	5 (28)	0.04	0.84
Capillaria spp.	14 (58)	14 (78)	1.80	0.18
Cestodes	7 (29)	7 (39)	0.44	0.51

3.3.2 *Breeding status and infection prevalence*

29 birds were successful in hatching chicks (SBs) up to the time the last sample was obtained from these birds. 13 birds still had no chicks hatched at the time the samples were obtained (UBs). There was no significant difference in infection prevalence between SBs (n=29) and UBs (n=13) (P > 0.6). The separate consideration of the three parasites did also not result in significant differences between SBs and UBs (Table 2), although oocysts of the protozoans *Isospora* spp. were, with one exception, found amongst SBs only (P=0.05). Contrary to our prediction, eggs of *Capillaria* spp. were found in 11 (85 %) UBs compared to 17 (59%) SBs. This result, however, was not significant (P=0.09).

In contrast to the higher infection prevalence of females with respect to the whole study population (Table 1) there was no difference in overall infection

prevalence between male and female SBs. Consideration of the three parasites separately did also not result in a difference between male and female infection prevalences (Table 2).

Table 2. Comparison of infection prevalences between successful breeders (SBs) and unsuccessful breeders (Ubs) and between male and female successful breeders (SBs) (G-tests, df = 1). Infections with at least one of the three parasites and with each of the three parasites separately are presented. The number and the percentage (in brackets) of infected individuals are given.

	SBs (n = 29)	UBs (n = 13)	G	P	male SBs (n = 17)	female SBs (n = 12)	G	P
infected	23 (73)	11 (85)	0.17	0.68	12 (71)	11 (92)	2.09	0.15
Isospora spp.	10 (35)	1 (8)	3.89	0.05	5 (29)	5 (42)	0.47	0.77
Capillaria spp.	17 (59)	11 (85)	3.00	0.09	9 (53)	8 (67)	0.55	0.46
Cestodes	10 (35)	4 (31)	0.06	0.81	6 (35)	4 (33)	0.01	0.91

3.3.3 *Feeding expenditure and infection status*

Logistic regression analysis yielded a significant deviation from the null model for the independent variable feeding days, i.e. with increasing number of feeding days the probability of infection for SBs increased (Table 3). There was no effect of season on the likelihood of infection. Sex also had no significant effect on the infection probability of SBs (Table 3). The separate consideration of a males' and a females' feeding expenditure and of the respective calendar date on the infection probability yielded a significant effect for the variable feeding days, but in males only (Table 3). The probability of parasitic infection increased significantly with the number of days a male had fed nestlings, irrespective of calendar date (Fig. 1). No such effect was found in females (Table 3).

Table 3. Logistic regression models for the relationship between the variables feeding days, calendar day (season) and sex on the infection status (dependent variable) of successful breeders (SBs) (model 1) and of male and female SBs respectively (model 2 and 3). The first model was significant with $\chi^2 = 17.102$, df = 1, $p < 0.001$. The second model was significant with $\chi^2 = 10.921$, df = 1, $p = 0.001$.

Model description	LR χ^2	Δ LR χ^2	coefficient	S.E.	df	*P*
SBs (*n* = 29)						
Null Model	29.569				3	
feeding days	17.102	12.467	0.630	0.266	1	0.018*
calendar day	-	-	-	-	1	ns
Sex	-	-	-	-	1	ns
Male SBs (*n* = 17)						
Null Model	20.597				2	
feeding days	10.921	9.676	0.508	0.243	1	0.037*
calendar day	-	-	-	-	1	ns
Female SBs (*n* = 12)						
Null Model	6.884				2	
feeding days	-	-	-	-	1	ns
calendar day	-	-	-	-	1	ns

3. 4 Discussion

Life-history theory predicts that high physical activity (e.g. feeding nestlings) should be associated with low immunological resistance to parasitic infections. The hypothesis of a trade-off between physical energy expenditure and expenditure in immune defense makes two predictions: 1) that birds feeding nestlings (SBs) should be more frequently parasitized than non-feeding

birds (UBs), and 2) that in SBs, the likelihood of infection should be higher in birds with higher parental feeding expenditure.

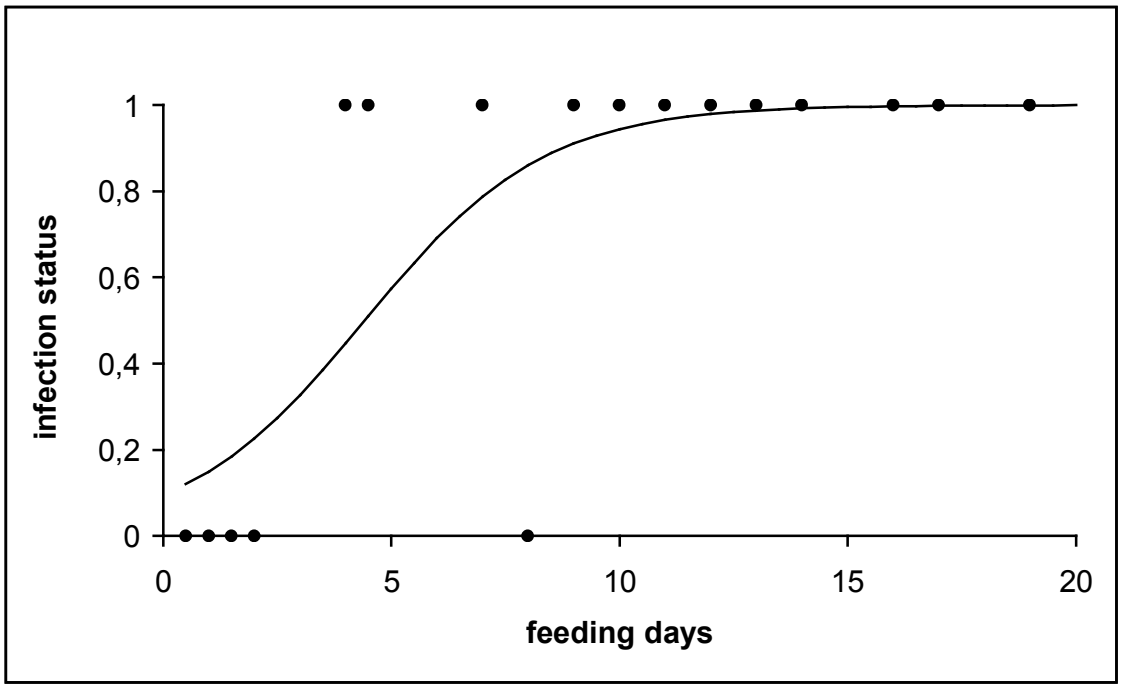

Fig. 1. The relationship between infection status and number of days a male successful breeder fed his chicks. The solid line depicts the fitted values from the regression model. Points represent the observed values of individuals.

In our study, the prediction that SBs should be more frequently parasitized than UBs, did not hold. Overall Infection prevalence between the two groups did not differ (Table 2). We can think of two plausible explanations. 1) Investments in other costly activities, prior to the nestling feeding stage (e.g. egg-laying, territory defense, mate guarding, incubating) have already influenced the infection status of birds. The generally higher infection prevalence in females (Table 1) demonstrates, that female blackbirds are, more than males, vulnerable to parasitic infections at this stage in the breeding cycle. This is perhaps due to the costly activity of egg laying in which all females of this study already invested. The mean number of eggs laid was 5,0 in UBs and 4.0 in SBs. Egg laying in a similar sized passerine bird, the Starling (*Sturnus vulgaris*) has been shown to constitute 281 % BMR (one day's basal metabolic

rate) (Walsberg 1983). Furthermore, in blackbirds, females are exclusively responsible for nest building and incubating (Glutz von Blotzheim 1988).

2) Prior to the breeding season, parasitic infections were probably not randomly distributed between (future) successful and (future) unsuccessful breeders. Thus, the result is an outcome of two intermingling processes: the cause and the consequence of parasitism. Let us consider that birds infected with parasites prior to the breeding season fail to breed successfully for the time being, whereas birds not infected and, thus, in better condition breed successfully right away. Due to their additional expenses by feeding nestlings, however, even these breeders in good condition become more susceptible to infection and, as a consequence, become infected with parasites. In order to disentangle both processes, i.e. the cause and the consequence of parasitism, one would have to check on the infection status of all birds, both prior and during the breeding season. Merilä and Andersson (1999), Dawson and Bortolotti (2001) and Sanz et al. (2002) have already stressed the importance of checking on the health status of study birds prior to experimental manipulation, because the initial health status of birds might obscure the relationship between reproductive effort and parasitism.

The second prediction, that for SBs the likelihood of infection should be higher in birds that had invested more in feeding their young, held. The infection probability was higher in birds which had fed nestlings for more days (Table 3). We can discount the possibility that the positive correlation was merely the result of a seasonal increase in infection prevalence, because calendar date had no effect on the likelihood of infection. We are able to distinguish temporal components of time from both variables (feeding days and calendar date), because, if breeding is highly asynchronous, as is the case in blackbirds, calendar date is not related to a fixed stage in the breeding cycle.

The separate consideration of males and females, yielded a significant relationship in males only (Table 3), but it is obvious, that the lack of a relationship in females results from the generally high infection prevalence in female SBs (92%; only a single female not infected). Thus, we do not preclude the existence of a trade-off between feeding expenditure and ability to mitigate

parasitic infections in females. Instead, we suggest that not prevalence of infection, but intensity may have risen with increasing feeding expenditure.

Apart from various experimental studies which all support the idea of a physiological trade-off between immunological functions and energetically demanding physical activities (Apanius et al. 1994; Norris et al. 1994; Richner et al. 1995; Oppliger et al. 1996; Ots and Hõrak 1996; Allander 1997, Deerenberg et al. 1997; Hakkarainen 1998; Nordling et al. 1998; Wiehn and Korpimäki 1998; Fargallo and Merino 1999; Brawner et al. 2000; Ilmonen et al. 2000; Merino et al. 2000; Råberg et al. 2000; Piersma et al. 2001; Ardia et al. 2003; Verhulst et al. 2005), three correlational studies also support the predictions of a physiological allocation trade-off: 1) the influencial study of Festa-Bianchet (1989) who found higher fecal counts of lungworm larvae (*Protostrongylus sp*.) in lactating compared to non-lactating Bighorn ewes (*Ovis canadensis),* 2) a study of Oppliger et al. (1997) who showed that Great-Tit females which were found to be infected after hatching of the nestlings were those which had laid larger clutches, and 3) a study of Ots and Hõrak (1996), who found a positive correlation between a measure of reproductive effort (prefledging brood weight) and an indicator of health state (heterophile : lymphocyte ratio in the peripheral blood stream) in Great Tits.

The results of the present study support at least one prediction of a trade-off between likelihood of infection with gastro-intestinal parasites and the energetic demands of chick feeding. In correlational studies factors such as individual and environmental quality can be expected to override associations between two traits (Stearns 1992). However, if the predicted correlation between two traits can be found despite the confounding effects of individual and environmental quality, as we have done for males in a population of wild blackbirds, a conflict in optimizing both traits, feeding chicks and being immunocompetent against parasitic infections, seems to constitute a real feature of the species‘ life-history.

3.5 References

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4

Variables of growth are positively associated with coccidian infection in nestlings of Eurasian Blackbirds (*Turdus merula*)

Abstract - Following life history theory, negative relationships between parasitic infection and somatic growth can be predicted. Several studies, which almost exclusively considered blood sucking ectoparasites, investigated the relationship between parasitic infection and growth in bird nestlings, but their findings have been very controversial. In this study the relationship between infections with the sporozoan endoparasites *Isospora spp*. and aspects of somatic growth in nestlings of Eurasian Blackbirds (*Turdus merula*) was examined. Tarsus length, wing length and the weight of 188 nestlings, originating from 82 nests were measured when nestlings were 8 to 10 days old. At the same occasion fecal droppings were collected and subsequently analysed for the presence or absence of isosporan oocysts. I found that tarsus length and wing length were significantly influenced by infection status, with tarsi and wings being longer in chicks, infected with *Isopora* spp. Weight also was higher in infected chicks, although this relationship was not significant. Furthermore, tarsus length was dependent on the year of birth, whereas wing length was influenced by nest origin.

I conclude that infections with *Isospora* spp. do not impose the predicted trade-off on blackbird nestlings, probably because infections detectable at this age, are not (yet) seriously interfering with nestling health. Rather, infections with *Isopora* spp. seem to be a function of feeding rate. Chicks which get more food are also those which are bigger, but they are also those which are at a higher risk of getting infected. This point of view opens exciting aspects for future research.

4.1 Introduction

Parasites, per definition, are assumed to negatively affect fitness components of their hosts by substracting energy or other materials from their

bodies. Under the assumption that energy and resources are limited, life history theory predicts that an animal faced with parasitic infection has to trade-off its available energy or resources between all aspects of life which are relevant for its fitness (Stearns 1992). The immune system of birds is still not mature at the nestling age and is more vulnerable to parasitic infection than in adults (Rose 1967; Roitt et al. 1996; Ros et al. 1997). Thus, a parasitic infection very probably entails some form of cost for a young bird. A crucial fitness relevant aspect for a nestling is growth, in which it invests most of its energy and materials (Weathers 1992). Nestling size and condition at fledging have been shown to be a good predictor of survival in Eurasian blackbirds (Magrath 1991) and other bird species (Nur 1984; Tinbergen and Boerlijst 1990; Ringsby et al. 1998; Takagi 2001; Legge 2002). Thus, an infected nestling will have to 'decide' how to allocate its resources to maintenance, growth, immune defence and to repair of parasite induced damage. Several studies have focused on negative effects of parasites on growth and condition of nestling birds. The results, however, were inconsistent: the hypothesized negative associations between infections and somatic growth were found in many studies (Richner et al. 1993; Møller et al. 1994; Kruszewicz 1995; Merino and Potti 1995; Christe et al. 1996; Dufva and Allander 1996; Santos Alves 1997; Bosch and Figuerola 1999; Nilsson 2003), but just as many studies failed to find negative effects of parasites on their nestling hosts (Rogers et al. 1991; Roby et al. 1992; Young 1993; Tompkins et al. 1996; Bauchau 1997; Merino and Potti 1998; Szabo et al. 2002). This was even the case in a very small species, the house wren *Troglodytes aedon*, in which parasites consumed an estimated 10-30 g of blood per brood before nestlings fledged (Johnson and Albrecht 1993). But not only did many studies fail to find the predicted negative association, some even found a positive association between infection and somatic growth (Moore and Bell 1983; Mazgajski and Kedra 1998; Saino et al. 1998; Szép and Møller 1999). These controversial results suggest, that several factors shape the complex relationship between parasitic infection and growth in bird nestlings and that effects depend on the parasie-host system studied.

Blood sucking ectoparasites (flies, fleas, ticks and mites) have been the most commonly used parasites in all these studies, whereas endoparasites

have largely been neglected. As with regard to the optimization of life history decisions in nestlings, endoparasites differ in one main aspect from most blood sucking (nest) ectoparasites: they do not leave their hosts at fledging. Therefore, the optimal life-history decision of nestlings infected with endoparasites must take into account a longer infection period than just the nestling period. Thus, they may end up with other strategies than nestlings infected with blood sucking ectoparasites.

Coccidians from the genus *Isospora* are sporozoan endoparasites belonging to the family Eimeriidae. They mainly infect cells of the intestinal mucosa, although also other tissues have been found to get infected (Box 1981, Bush et al. 2001). Eimeriidae are well known from economically important poultry where they can cause haemorrhage, anemia, reduced efficiency in secreting enzymes and absorbing nutrients and sometimes even extensive destruction of the intestinal tissue (Olsen 1974; Long 1982). It is known that coccidians from the genera *Eimeria* and *Isospora* occur in many wild bird species (Scholtyseck 1956, Frank 1980, Hubalek 1994, McQuistion 2000), but not much is known about their pathogenicity in wild birds. Similar to captive species, health impediment in wild birds is probably associated with a number of factors, among which are 1) the number of ingested infective oocysts, 2) the rate of asexual multiplication of the parasite in host cells, 3) the turnover rate of host epithelial cells in relation to parasite generations and 4) the general vulnerability of the host to disease. *Isospora* spp. has, so far, been assumed to have a direct life cycle similar to that of *Eimeria*, but recent studies suggest that the asexual and sexual phase probably occur in different hosts (Bush et al. 2001). In species with direct life cycle, infection occurs by ingestion of infective oocysts. Once ingested, sporozoites are released into the intestinal lumen where they penetrate into epithelial cells. There they undergo one or more cycles of asexual reproduction (schizogony) before sexual development occurs. Oocysts, the products of the sexual phase are excreted with the feces about six to seven days after infection. They sporulate to the infective stage under oxygenated, humid conditions. In urban environments in which optimal feeding areas, like open grass patches, are extensively used by many birds, a reservoir of infective oocysts may build up. Thus, infection prevalence and

intensity in these urban populations are probably higher than in rural areas. For this reason, the urban population of Eurasian Blackbirds breeding in the Botanical Garden of Bonn, situated in the centre of the city seemed suitable for investigating effects of endoparasitic infection on aspects of somatic growth in nestlings.

4.2 Methods

4.2.1 *Study area and study species*

This study was conducted throughout the breeding seasons of 1996, 1997 and 1998 on a population of individually marked Eurasian Blackbirds (*Turdus merula* Linne´ 1758) in the Botanical Garden of Bonn (Germany). The study area comprised 8.5 ha of evergreen and deciduous vegetation as well as patches of open grass areas. About 35 pairs breed in the garden each year. Eurasian Blackbirds are socially monogamous thrushes which hold long-term territories maintained by both sexes throughout the year (Snow 1956). All adult birds were captured in mist nets prior to the breeding season and were individually marked with colored rings.

From March to August nests were located by systematic searches and were then inspected on a regular basis. 255 nestlings were ringed, weighed and measured at age 8d to 10d. Blackbird nestlings fledge 13 to 14 days after hatching.

4.2.2 *Nestling measurements*

Nestlings were weighted to the nearest 0.5 g with a spring balance. At this age mass is a good indicator of future growth and survival (Magrath 1991). Left and right tarsus length were measured with a digital caliper to the nearest 0.1 mm and the mean value of right and left leg calculated. Furthermore, the length of the left wing chord was measured with a ruler to the nearest of 0.5 mm.

Finally, a size independent condition index was calculated by using the

residuals from the regression of body mass (ln transformed) on tarsus length (ln transformed) (Jakob et al. 1996).

4.2.3 *Collection and analysis of fecal samples*

During ringing fecal droppings from 188 nestlings, originating from 82 nests, were collected in order to analyze feces for the presence or absence of parasitic oocysts of *Isopora* spp.. Fecal samples were collected only if unambiguous individual assignment was possible, and mutual contamination of droppings was excluded. Oocysts found in blackbird feces are of 18 – 25 µm in size, and probably belong to different species. For blackbirds, *Isospora lacazei* and *Isospora turdi* (Scholtyseck 1956; Pellerdy 1974) have been reported.

Fecal samples were stored in 2% potassium bichromate. In the laboratory the presence of oocysts was evaluated by a modified flotation method. A saturated $NaCl/ZnCl_2$ solution was used to concentrate oocysts (Bürger and Stoye 1983). After removal of large particles (seeds, grass, pebbles) the sample was centrifuged for 10 minutes at 300 g. The supernatant was then removed and 6 ml of flotation medium added and carefully mixed. Four compartments of two McMaster counting chambers were then filled and a total volume of 0.6 ml screened for oocysts.

4.2.4 *Determination of sex*

On the same occasion as ringing took place, a small blood sample was withdrawn from the brachial vein with a capillary. Blood was transferred to an EDTA coated tube and then frozen until analysis. Nestling sex was determined by genetic analysis of the blood (Rütten, unpublished thesis), following Griffiths & Tiwari's (1995) method using the restriction enzyme Hae III.

4.2.5 *Data analysis*

Univariate ANOVAs were computed to check whether the measured variables of growth (weight, tarsus length, wing length, condition) were influenced by any of the following factors: sex, year of birth, infection status with *Isospora* spp. and origin (nest and family). Origin of nest refers to the nest of birth, whereas origin of family refers to the parents, since many parents

raised nestlings from more than one nest. The three growth variables (weight, tarsus length and wing length) were controlled for age by using the residuals from the regressions of age on the untransformed values of weight, tarsus length and wing length, respectively. Thus, normality of the independent variables was best met, with tarsus length and wing length being normally distributed (Kolmogorov-Smirnov test: tarsus: $z=1.306$, $p=0.066$, wing: $z=0.970$, $p= 0.303$), and weight slightly deviating from normality ($z=1.440$, $p=0.032$). Anova results, however, tend to be robust to such small deviations at large sample sizes (Sokal and Rohlf 1995). Three of the dependent variables are highly correlated (Pearson's correlation: tarsus*weight $r=0.732$, tarsus*wing $r=0.771$, weight*wing $r=0.671$; all $p<0.01$). Thus, separate ANOVAs were computed. Year of birth, sex and infection status were incorporated in each analysis as independent fixed factors. Nest and family were incorporated as independent random factors in order to control for within nest or genetic dependencies. Sample sizes in tests vary because of various missing data.

In contrast to ectoparasitic infections which usually affect all chicks in a nest, infections with *Isospora* spp. are not transmitted by body contact but by ingestion of infective parasitic stages. Thus, in 31 out of 82 nests fecal samples from infected as well as not infected siblings from the same nest could be collected. The mean values of the measured growth parameters (weight, tarsus length, wing length and condition) of infected and not infected chicks from those 31 nests were compared in paired t-tests. Since all chicks of a nest had been exposed to the same conditions during growth (e.g. to the same properties of the nest, the same weather conditions, the same food availability, the same parents) this set-up assured the most accurate comparison possible between infected and not infected nestlings.

In a further step I tried to find out whether infected and not infected chicks differ in their development from day 8 to 10. Two nests for which the exact age of nestlings was not known were excluded from the analyses. Linear regressions of age on 1) weight, 2) tarsus length and 3) wing length were computed for infected and not infected chicks from the remaining 29 nests and regression slopes and elevations were tested for significant differences by the t-testing procedure according to Zar (1999).

All other analyses were performed using the statistical package SPSS 12.1. The rejection level was defined at $\alpha = 0.05$. All p-values are two-tailed.

4.3 Results

4.3.1 *Distribution of infections and sex ratio*

The analysis of fecal samples showed that, at the age of 8 to 10 days, at least 60% of nestlings (112 infected, 76 not infected) had already been infected with *Isospora* spp. Furthermore, infection prevalences in the nestling population did not significantly differ between years ($G=5.498$; $df=2$; $p=0.06$; $n=188$) and within breeding seasons (April to August) ($G=7.206$; $df= 4$; $p= 0.13$; $n=188$).

The genetic analysis of sex revealed a very balanced sex-ratio of 1:1 (85 males, 84 females) within our nestling population. Female and male chicks did not differ in infection frequency ($G=0.064$; $df =1$; $p=0.80$; $n=169$).

4.3.2 *Which factors are related to the size, weight and condition of nestlings?*

The results of the ANOVAs from n = 158 (159) chicks are summarised in table 1 and illustrated in figure 1. The size of a chick (tarsus length and wing length) was dependent on the infection status of the chick. Interestingly, infected chicks had significantly longer tarsi and longer wings than not infected chicks. Tarsus length, however, was also dependent on the year of birth, and wing length on the origin of nest. The variable weight did not significantly depend on any of the tested factors. However, the trend seemed to be the same as for tarsus length (Fig.1c). Infected chicks were heavier than not infected ones, but only in 1996 and 1998. A chick's condition was only dependent on the year of birth. Probably, the origin of nest also had some influence on the condition of a nestling, but this effect was not significant. None of the interaction terms had any significant effect on the weight, the size and the condition of nestlings. This means that , irrespective of infection status sex and year of birth had no influence on the condition, the size and the weight of a nestling.

Table.1 Results of univariate ANOVAs with (a) weight, (b) tarsus length, (c) wing length and (d) condition as dependent variables, sex, year and infection as fixed factors and family and nest as random factors. MS denotes mean square.

	d.f. effect	MS effect	d.f. error	MS error	*F*	*p*
(a) tarsus length (n=158)						
sex	1	0.017	82	0.696	0.024	0.877
year	2	3.388	18.933	0.816	4.151	0.032
infection	1	7.423	82	0.696	10.665	0.002
family	51	1.463	14.556	0.836	1.749	0.121
nest	17	0.824	82	0.696	1.184	0.296
infection * sex	1	0.082	82	0.696	0.117	0.733
infection * year	2	0.144	82	0.696	0.207	0.813
(b) wing length (n=159)						
Sex	1	0.192	82	0.646	0.298	0.587
Year	2	0.767	21.652	1.358	0.565	0.577
infection	1	5.274	82	0.646	8.159	0.005
family	51	0.932	16.722	1.603	0.581	0.930
Nest	18	1.522	82	0.646	2.355	0.005
infection * sex	1	1.278	82	0.646	1.978	0.163
infection * year	2	0.701	82	0.646	1.084	0.343
(c) weight (n=159)						
Sex	1	0.680	82	0.772	0.880	0.351
Year	2	2.891	24.089	1.049	2.756	0.084
Infection	1	2.390	82	0.772	3.093	0.082
Family	51	1.256	15.930	1.144	1.097	0.439
Nest	18	1.113	82	0.772	1.440	0.135
infection * sex	1	0.000	82	0.772	0.000	0.986
infection * year	2	0.690	82	0.772	0.893	0.414
(d) condition (n=158)						
sex	1	0.533	82	0.305	1.748	0.190
year	2	2.194	18.413	0.480	4.571	0.024
infection	1	0.190	82	0.305	0.623	0.432
family	51	0.509	15.188	0.509	0.999	0.531
nest	17	0.429	82	0.305	1.611	0.080
infection * sex	1	0.037	82	0.305	0.122	0.728
infection * year	2	0.308	82	0.305	1.009	0.369

4.3.3 *Comparison of growth parameters between infected and not infected chicks from the same nest*

In agreement with the results of the ANOVAs, I found infected chicks of a nest (n = 31) to have significantly longer tarsi and wings than their not infected nest mates (n = 31) (tarsus length: $t = -3.070$; $p = 0.005$; wing length: $t = -2.883$; $p = 0.007$). As far as the weight and condition are concerned, infected chicks were as heavy and in as good a condition as not infected chicks (weight: $t = -1.519$; $p = 0.139$; condition: $t < 0.001$; $p > 0.9$).

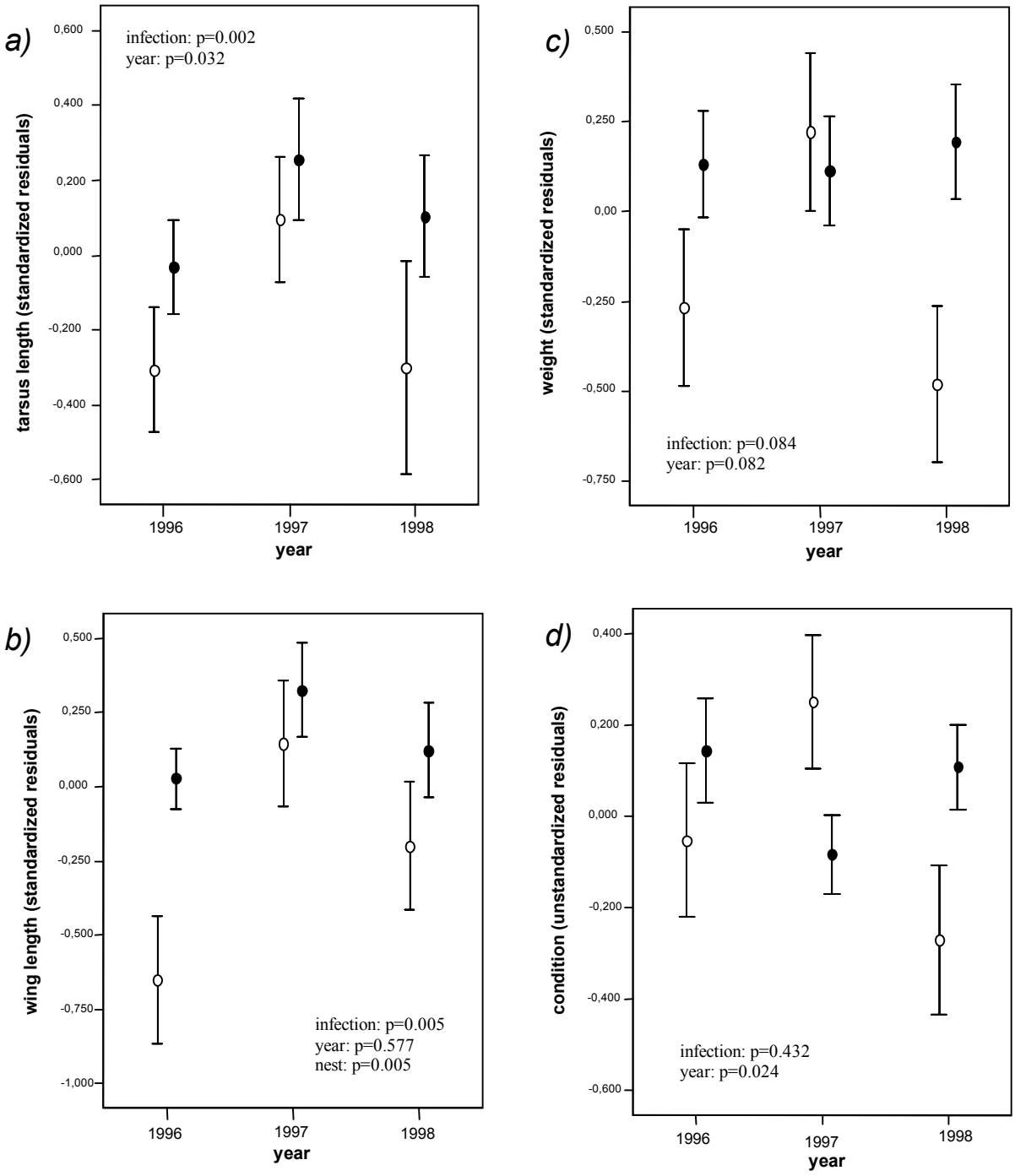

Fig. 1 Influence of year and infection status (filled circles denote infected, open circles not infected chicks).on (a) tarsus length (n=158), (b) wing length (n=159), (c) weight (n=159) and (d) condition (n=158) of blackbird nestlings.

In order to test at which point in time during their development these mean body differences between infected and not infected chicks emerge I first regressed weight, tarsus length, wing length and condition on the age of infected and not infected chicks, respectively. Slopes and elevations of these regressions from infected and not infected siblings were then compared. The results of the regressions are summarised in table 2 and illustrated in figure 2. The relationships between age and the growth variables (weight, tarsus length and wing length) were significant for not infected nestlings which indicate that these chicks significantly grew from age 8d to 10d. The same was true for the infected chicks from these nests with the exception of weight gain which was near to significance. The results of the regressions on the condition were not significant, both for infected and not infected chicks which indicates that the condition of all chicks remained constant over day 8 to 10.

Table 2. Results of regressions of the growth variables on the age of nestlings. n = 29.

	dependent variable	R^2	*b*	SE	t	*p*
(a) Chicks not infected (n = 29)	tarsus length	1.41	10.303	4.896	2.105	0.045
	wing length	0.282	5.206	1.599	3.256	0.003
	weight	0.162	4.145	1.813	2.286	0.030
	condition	0.029	0.019	0.021	0.903	0.375
(b) Chicks infected (n = 29)	tarsus length	0.198	6.630	2.565	2.585	0.015
	wing length	0.491	5.196	1.018	5.103	>0.001
	weight	0.127	2.375	1.199	1.981	0.058
	condition	0.002	0.004	0.016	0.253	0.802

More important than the regressions themselves are the results from the statistical comparison of the regression parameters between infected and not infected chicks. The regression slopes were not significantly different between infected and not infected chicks for any of the four variables (all $p > 0.5$; weight: $t = 0.375$, $df = 54$; tarsus length: $t = 0.665$, $df = 54$, wing length: $t = 0.005$, $df = 54$; condition: $t = 0.569$, $df = 54$). These results indicate that all chicks, infected and not infected, grew at a similar rate from day 8 to 10. The comparison of the elevations between the regression functions, however, resulted in significant differences between infected and not infected chicks for weight ($t = 2.743$; $df =$

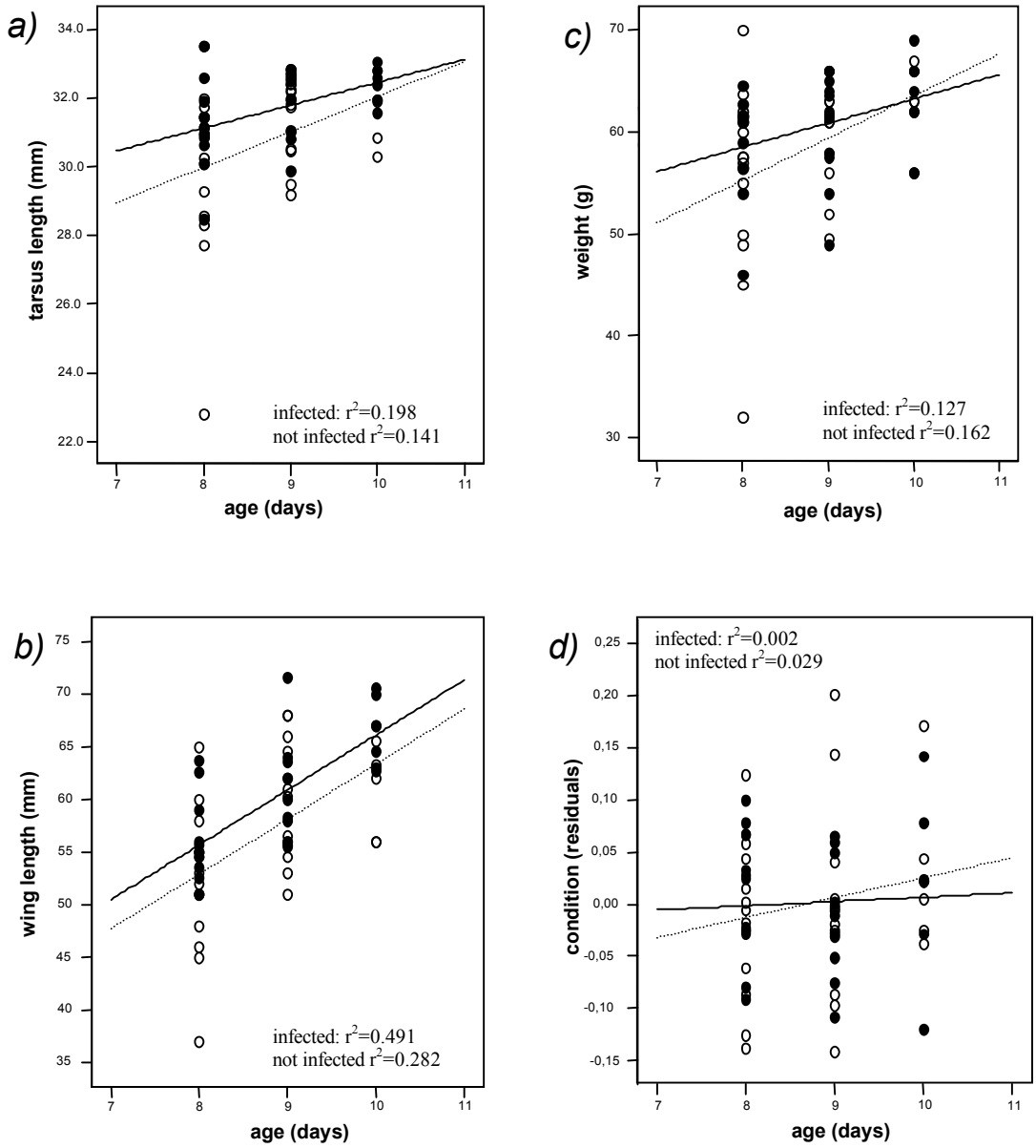

Fig. 2 Linear regression curves for (a) tarsus growth, (b) wing growth, (c) weight gain and (c) condition of infected chicks (black line) and not infected chicks (dotted line) originating from 29 nests. Mean nest values for infected chicks are presented by filled circles, mean nest values for not infected chicks from the same nest as open circles.

55; $p < 0,01$) and tarsus length ($t = 2,061$; $df = 55$; $p < 0,05$). No differences were found for wing length and for condition between infected and not

infected chicks (wing length: t = 1,944; df = 55; p >0,05, condition; t = 0,2551; df = 55; p > 0,5). These results indicate that chicks found infected at an age of 8 to 10 days were already heavier and bigger in their first days of life.

4.4 Discussion

A negative relationship between the presence of an infection and aspects of growth, as has been expected by theory, could not be found. Infections with the protozoan endoparasites *Isospora* spp. did not negatively influence any of the measured growth parameters of blackbird nestlings. Quite on the contrary, at an age of 8 to 10 days infected nestlings had significantly longer wings and longer tarsi than uninfected nestlings. Their weight did not significantly differ from that of not infected chicks, although they were, on average, heavier than their not infected nest mates (Fig.1).

Although these results were surprising from a theoretical point of view, previous studies had shown that the association between parasitic infection and growth presented itself as very diverse, with nestlings being negatively affected by parasitic infection or not being affected at all, or even being positively affected. Only a few studies have so far reported a positive relationship between parasitic infection and aspects of somatic growth in bird nestlings (Moore and Bell 1983; Mazgajski and Kedra 1998; Szép and Møller 1999; Saino et al. 1998). In two of the cited studies blood sucking ectoparasites were the parasites of concern. Saino et al. (1998) studied the effects of a dipteran ectoparasite on *Hirundo rustica* nestling growth and immune defense. They experimentally increased ectoparasite intensity in some nests and found that nestlings exposed to increased infestations had larger rate of feather growth but were in worse condition, since they had lower plasma protein content and larger blood cell sedimentation rate. They did not find significant effects of parasites on tarsus growth and body mass. They interpreted the positive correlation between parasite count in the nest and retrix length as the

result of nestlings investing heavily in feather growth, in order to reduce their stay in the infested nest. Similar to those results, Szép and Møller (1999) found that in sand martin nestlings (*Riparia riparia*) wing length was positively correlated and tarsus length negatively correlated to tick load. They also interpreted this finding as sand martin nestlings from heavily parasitized nests investing more in the growth of feathers (wing length) to the detriment of skeleton growth (tarsus length), in order to facilitate early fledging.

For infected blackbird nestlings, however, it does not make sense to fledge early, since this is no means to get rid of their endoparasites. Thus, other mechanisms must play a role in the enhancement of feather and skeletal growth of nestlings in this parasite-host system. The study best comparable to the present study is from Mazgajski and Kędra (1998), who also studied the effect of the microparasite *Isospora* spp. on the growth of starling nestlings (*Sturnus vulgaris*). As in the present study, they found a positive effect of parasitic infection on two of the measured three growth parameters (weight, tarsus length, wing length) of starling nestlings. They compared nests with no infections and high and and low infections, respectively. Chicks from most heavily infected nests were on average heavier and had longer wings than nestlings from less or not infected nests. No difference was found in tarsus length between nests of different infection levels. Mazgajski and Kędra (1998) conclude from their study that 1) *Isospora* spp. are not harmful to starling nestlings and 2) that those which are fed more have also greater chance of getting infected with isosporan oocysts. I do not conclude from my results that *Isospora* spp. infections are generally harmless to blackbird nestlings, because there are several reasons why negative effects may not show up where or when they are expected to. 1) Infections may be very light and show no measurable negative effects on chicks. 2) Parents may compensate for the negative effects of parasites by increased feeding, as has been shown in a study on great tits (Christe et al. 1996), on blue tits (Tripet and Richner 1997) and on marsh tits (Wesolowski 2001). 3) Nestlings may compensate for negative effects by developmental plasticity during growth. Bize et al. (2003) showed that parasitized Alpine swift nestlings had lower growth rates than deparasitized nestlings before the peak of parasite infestation, but greater

growth rates after the peak. They grew for 3 additional days and finally fledged at the same weight and size as deparasitized nestlings. 4) Parasites may affect other aspects than growth. Szabo et al. (2002) found no effects of haematophagous mites on body mass or fledging success in nestling house sparrows, but an effect on haematological parameters. Parasites generated a non-specific immune response with inflammatory processes and anaemia. 5) Negative effects may not show up during the nestling period but later in life. Haematophagous ectoparasites reduced the clutch sizes of the nestlings' first and subsequent clutches in Great tits, although they did not significantly affect the probability of nestling recruitment as local breeders (Fitze et al. 2004). Considering the above mentioned aspects I conclude that, under the environmental conditions met during my study, naturally occuring infections with *Isopora spp*. did not severily impede the health of blackbird chicks during their nestling stage and, thus, did not lead to a trade-off between fighting negative effects of infection and investing in growth.

The answer to the question, however, whether the parasite is harmful or not, still does not account for a positive relationship between growth and infection. I completely agree with Mazgajskis and Kedras (1998) conclusion, that nestlings which are better fed (and thus are heavier and bigger) also have a greater chance of getting infected. Infections with *Isospora* spp. are, in a first instant, random processes. Infection occurs by ingestion of a contaminated food item. Comparable to European Blackbirds, starlings are medium-sized omnivorous birds which preferentially feed on the ground. Since the study on starlings was also conducted in a park located within a big city (Warszawa), oral transmission by infected food items brought to nestlings is expected to be comparable in these two study populations. In fact, infection prevalence of nests was 67% in starlings (Mazgajski and Kedra 1998) and 70% in our blackbird nestling population. Dependent on the degree of environmental infestation and on the pathogenicity of parasites, getting fed, while being essential for growing, represents a risk for the nestling. The comparison of the regression elevations suggested that nestlings which were found to be infected at an age of 8 to 10 days were heavier and had longer tarsi in their first days of life, compared to nestlings which were found to be free of infection at an age of

8 to 10 days. Thus, chicks being heavier and bigger early in life probably get fed more and, as a consequence, get infected at a higher chance. Thus, getting fed more is not a consequence of infection, as if for compensating negative effects of parasites, but infection is a consequence of getting fed more. Moore and Bell (1983) in their study on an acanthocephalan endoparasite (*Plagiorhynchus cylindraceus*) in the starling also found infected nestlings to be heavier than their not infected siblings and concluded, that consuming a greater number of prey also means being more likely to acquire the parasite. If infection probability is a function of feeding rate, interesting theoretical questions about nestling-parent relationship and optimal decision-making arise. Is it adaptive for chicks growing up in areas of high parasitic infestation to beg less in order to get fed less and, as a consequence to remain small but healthy? Should parents adapt their feeding rates and habits to the degree of parasitic infestation in the area? Should they feed less in areas of high infestation and produce smaller, but healthier chicks? Or should they change their feeding habits and look for food preferentially on places with lower incidence of infective oocysts, e.g. on trees and bushes? This view opens new, exciting aspects of the *Isopora*-blackbird parasite-host system. Comparable to the system of European Oystercatchers feeding on parasitized mussels (Norris 1999) or of Steller's eiders feeding on parasitized amphipods (Bustnes and Galaktionov 2004) an alternative trade-off, namely between energy intake and risk of infection can be predicted.

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5

Diurnal cycle of *Isospora* spp. oocyst shedding in Eurasian Blackbirds (*Turdus merula*)

Abstract - Diurnal fluctuations in the appearance of parasites have been recognized for more than 60 years, but have largely been ignored in studies examining the role of parasites in connection with evolutionary aspects of behaviour, ecology and population dynamics. The disregard of diurnal fluctuations, however, can influence the reliability and interpretation of data. I examined the shedding of *Isospora* spp. oocysts in faeces of naturally infected, free living Eurasian Blackbirds (*Turdus merula*). Adult birds and nestlings shed coccidian oocysts (*Isospora* spp.) predominantly in the afternoon. The results are in agreement with earlier studies on coccidian oocyst shedding in other bird species. They are discussed with regard to these studies and to practical implications for future investigators on this field.

5.1 Introduction

With their influential paper, Hamilton and Zuk (1982) have brought parasites into the centre of discussion about sexual selection. They proposed that animals should choose mates for heritable disease resistance by scrutiny of characters whose full expression is dependent on health and vigour. Infections with blood parasites were used as health parameter with the consequence that protozoan blood parasites became a favourite group for testing the Hamilton-Zuk hypothesis. This was done "largely in ignorance of the natural history of the parasites involved" as Weatherhead and Bennett (1991) already pointed out. As a consequence, tests of the predictions were based on bold assumptions, resulting in critical discussions (Endler and Lyles 1989; Moller 1990; Clayton

1991; Weatherhead and Bennett 1991; 1992). Specifically the assumption, that a bird's parasite burden could be reliably determined by the examination of a single blood smear was repeatedly criticized (Cox 1989; Pruett-Jones et al. 1991; Weatherhead and Bennett 1991;1992; Cooper and Anwar 2001). These criticisms addressed the disregard of annual (Weatherhead and Bennett 1991; 1992; Sanz et al. 2002), seasonal (Weatherhead and Bennett 1991; 1992; Allander and Sundberg 1997; Hatchwell et al. 2000) and geographical (Merilä et al. 1995) sources of variation in parasite prevalence and intensity. Less attention, however, has been paid to shorter periodic phenomena, as e.g. daily or diurnal periodicities. One of such neglected phenomena is the diurnal shedding of coccidian oocysts in the faeces of host birds. The most commonly used indirect method for the diagnosis of gastrointestinal parasitic infections is the examination of faecal material for parasitic stages (oocysts, eggs, larvae). If the shedding of parasitic stages, however, underlies diurnal fluctuations, which has been demonstrated for oocysts of coccidians in sparrows (Boughton 1933; Kruszewicz 1995), in house finches (Brawner and Hill 1999), in pigeons (Boughton 1937) and in dark-eyed juncos (Hudman et al. 2000), then the probability to detect an infection, or to determine the abundance of an infection, will vary with collection time and may, thus, result in distorted estimates on infection frequencies and intensities. As a consequence, diurnal fluctuations may play a role in all studies, in which behavioural, ecological and evolutionary aspects of parasites and their hosts are examined, namely on the level of individuals, populations and species. For this reason it is surprising, that the importance to consider diurnal fluctuations has only recently received attention (Brawner and Hill 1999; Hudman et al. 2000; Cooper and Anwar 2001).

In this study I examine the shedding of *Isospora* spp. oocysts for diurnal fluctuations in the faeces of naturally infected, free living adults and nestlings of European blackbirds *(Turdus merula*). I show that a diurnal periodicity of oocyst shedding can be recorded from undisturbed, free ranging adult birds, which has so far been shown only for dark eyed juncos (Hudman et al. 2000). Furthermore, I show that the same diurnal periodicity in oocyst shedding can be recorded from nestlings, which were trapped on nests. With this study I want to confirm the results of other studies and to provide further evidence that the

phenomenon of a diurnal oocyst shedding actually exists in nature and not only in the lab. Additionally, I want to remind to the risks of hasty interpretations of parasitic assays.

5.2 Methods

This study was part of an ongoing survey (1995-2000) on a free ranging, color-banded population of European blackbirds (*Turdus merula*) in the Botanical Garden of Bonn (Germany). Fecal samples from adult birds were collected in 1996 (May-June), 1998 (May-August) and 2000 (June-July). They were collected by an observational method, which did not require capturing of birds. Birds were observed in activities which preferentially take place on the ground (feeding, collecting nesting material, collecting food for nestlings) and were followed until they defaecated. Faeces were collected only if the identity of the samples could be determined unambiguously. For 19 adult individuals, both between 7:00 and 12:00 (morning samples) and between 12:00 and 18:00 (afternoon samples) one or more samples were collected. If in both, morning and afternoon samples, no oocysts could be detected the respective bird was declared as not infected.

In the frame of the survey, most nestlings were weighed, measured (tarsus length, bill length, wing length) and ringed at the age of 8 to10 days. From April to July of the years 1996, 1997 and 1998 one faecal sample of each nestling was collected when possible. Morning samples were obtained from 38 nestlings and afternoon samples from 150 nestlings. Because the sample sizes of morning and afternoon samples were that unbalanced, an additional, smaller data set was created: for each of the 38 morning samples one afternoon sample was selected which best matched a morning sample with respect to collection date. Of course, the selection occurred in total ignorance about the infection status of the nestlings. Thus, a reduced data set with 76 independent samples from 76 nestlings was created, 38 samples collected in the morning and 38 collected in the afternoon.

Faecal samples were stored at 4°C in 2% potassium bichromate. They were examined using a flotation method with saturated NaCl/$ZnCl_2$ solution (Bürger and Stoye 1983). After removal of large particles (seeds, grass, pebbles), samples were centrifuged for 10 minutes at 300 g. The supernatant was removed and pellets weighted to the nearest of 0.01 mg. 6 ml of flotation medium was then added and carefully mixed to avoid production of air bubbles. Four compartments of McMaster counting chambers, each with a volume of 0.15 ml, were then filled. The floating oocysts of each chamber were counted microscopically and a mean oocyst count per sample was calculated. This mean oocyst count was divided by the mass of the faecal sample to obtain standardized counts.

The parasites of interest, *Isospora* spp. belong to the family Eimeriidae. They undergo an alternating cycle of sexual and asexual reproduction. In most species the asexual phase occurs in the intestinal epithelium where one or more asexual cycles are completed before sexual development occurs (Olsen 1974). Oocysts, the products of the sexual phase, are released into the intestinal tract and passed in faeces. Under the proper humidity, temperature and oxygen conditions oocysts sporulate and, upon ingestion, are infective to a host.

Parasite counts, especially microparasite counts, show a highly variable distribution in the host population (Goater and Holmes 1997). Therefore, non-parametric testing was used throughout. All testing was performed two tailed and α set at 0.05.

5.3 Results and Discussion

90% of adult birds (17 of 19) were infected with *Isopora* spp. In 8 birds oocysts were found in both, morning and afternoon samples, in 8 birds oocysts were found only in afternoon samples and in a single bird oocysts were found only in the morning sample. Thus, it was significantly more likely to detect oocysts in morning samples than in afternoon samples (McNemar-test: df=1,

p=0.039, n=19). In nestlings, a similar distribution of apparently infected and not infected birds could be found. In both data sets, oocysts were more likely to be found in samples collected in the afternoon. If the complete data set was considered, oocysts were detected in 65% of afternoon samples (98 of 150) but only in 37% of morning samples (14 of 38). In the reduced (balanced) data set 63% of afternoon samples contained isosporan oocysts (24 of 38), but only 37% of morning samples (14 of 38). In both cases, the difference is significant (G tests, df=1: complete data set: n=188; G=10.063; *p*=0.002; reduced data set: n=76; G=5.326; p=0.021).

The comparison of oocyst counts from morning and afternoon samples yielded significant differences for both, adult birds and nestlings. In both cases, significant lower mean oocyst counts were found in morning samples than in afternoon samples (Tab 1; Fig.1).

Table 1. Comparisons of oocyst counts from faeces of European Blackbirds collected in the morning and in the afternoon.

		Morning samples	Afternoon samples	Z	*p*
Adult birds	N	19	19		
	Median	1.1	55.7		
	25 percentile	0.0	30.6		
	75 percentile	21.3	142.1		
	Wilcoxon-test			-2.533	0.011
Nestlings (full data set)	N	38	144		
	Median	0.0	20.2		
	25 percentile	0.0	0.0		
	75 percentile	6.1	189.4		
	Mann Whitney U-test			-4.005	>0.001
Nestlings (reduced data set)	N	38	38		
	Median	0.0	24.2		
	25 percentile	0.0	0.0		
	75 percentile	6.1	163.4		
	Mann Whitney U-test			-3.098	0.002

The results of a higher likelihood to find isosporan oocysts in afternoon samples (presence/absence data) and to find higher oocyst counts in afternoon samples (abundance data) are in accordance with earlier studies on the diurnal oocyst excretion of *Isospora* spp., carried out in dark-eyed juncos (*Junco hiemalis*) (Hudman et al. 2000), in house finches *(Carpodacus mexicanus*)

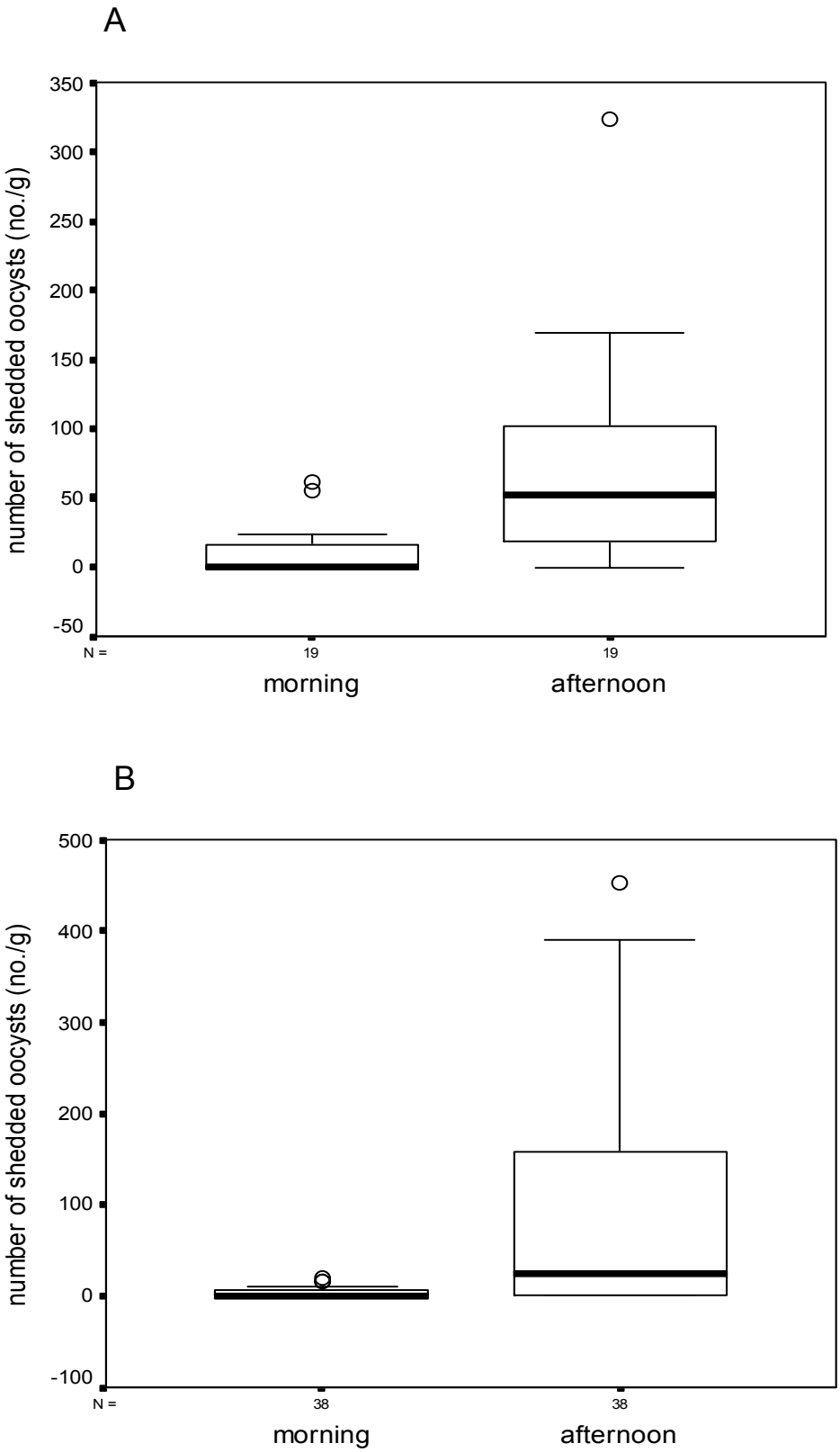

Fig. 1. Mean number of shedded oocysts per gram from faecal samples of adult birds (A) and of nestlings (B) collected during mornings (6:00-12:00) and afternoons (12:01-18:00). Box-plots indicate the median and the 25th and 75th percentiles. Error bars (whiskers) outline the largest and smallest value that is not an outlier. Open circles indicate outliers. Extremes are excluded from graphic presentation.

(Brawner and Hill 1999), in house sparrows (*Passer domesticus)* (Boughton 1933; Kruszewicz 1995) and in tree sparrows (*Passer montanus*) (Kruszewicz 1995). In his seminal study, Boughton (1933) reports that, in house sparrows, the largest quantity of oocysts was found in faecal droppings from 14:00 to 20:00. Sporadically, he also examined various bird species from Milwaukee Zoological Garden (Boughton 1933) and found that, in most bird groups, a higher percentage of *Isopora* spp. was shed in the afternoon. Similarly, Brawner and Hill (1999) observed that house finches shed more oocysts at 16:00 and 20:00 than at 08:00 and 12:00. Kruszewicz (1995) found isosporan oocysts in faeces of tree sparrows between 10:00 and 18:00 only. The oocyst output from house sparrows started about 14:00 in his experiment and finished at midnight. Hudman et al. (2000) collected faeces from dark-eyed juncos both, between 15:00 and 5:00 and between 5:00 and 12:00. Birds were kept in cages only for the collection of faeces otherwise being free ranging. In agreement with the findings from lab settings, they were more likely to detect isosporan oocysts in faeces produced at night than during the day. Our results on adult free ranging European blackbirds, thus, confirm the evidence that this pattern of a diurnal periodicity, with oocysts being shed predominantly in the afternoon, exists in nature and not just in the lab. My results on European blackbird nestlings, which shed oocysts more frequently and at higher abundance in the afternoon, corroborates a study of Kruszewics (1995) who artificially infected 9 house sparrow nestlings and 8 tree sparrow nestlings with isosporan oocysts. In his study almost no oocysts were excreted in the morning hours and the highest oocyst production took place between 18:00 and 22:00. Because nestlings of my study were naturally infected and trapped on nests, the results further indicate that the same pattern of a diurnal shedding can actually be recorded in nature.

The importance to consider large-scale temporal phenomena, as e.g. seasonal and annual fluctuations in infection prevalence and abundance has repeatedly been emphasized and critically discussed (Sanz et al. 2002; Weatherhead and Bennett 1991; 1992; Allander and Sundberg 1997; Hatchwell et al. 2000; Allander and Bennett 1994). The importance of diurnal phenomena, however, has only recently been addressed. Cooper and Anwar (2001) mention

the potential of failing to detect infections with blood parasites in a blood smear by reason of periodicities. Hudman et al. (2000) in their study on dark-eyed juncos take account of the diurnal periodicity in the shedding of isosporan oocysts and recommend that future studies of coccidia focus on samples collected at night. Brawner and Hill (1999) devoted a whole paper to this subject. I emphatically agree with them in highlighting the importance of diurnal periodicities in studies on behavioural, ecological and evolutionary aspects of parasite-host associations. The results of theirs and my study show, that the simple aspect of the time of day at which samples are collected, can have a deep impact on the reliability and validity of data. Similar to their findings in house finches, my results in European blackbirds indicate, that it is unsuitable to collect faecal samples in the morning hours if *Isospora* is the organism of interest. This, however, must not be a general pattern. Boughton (1937) found oocysts of a, not further specified, species of *Eimeria* to be shed predominantly in the morning hours. Thus, as Brawner and Hill (1999) already pointed out, it should be a major step prior to any study involving parasites, to find the most *consistent* method in assessing parasite prevalence or abundance. First, a technically reliable method to detect infections or to measure their abundance in a given sample has to be developed. Then, (at least in a preliminary study) repeated sampling from the same individuals within short time periods should be conducted. If this repeated sampling does not lead to repeatable results, one can proceed on the assumption that the shedding of oocysts underlies a periodicity. In contrast to annual or seasonal periodicities, which probably represent “real” fluctuations in parasite prevalence or abundance, e.g. seasonal phenomena known as "spring rise" or "autumn rise" (Greve 1985; Aktinson and Van Riper 1991), diurnal periodic phenomena, as the one described here, probably represent fluctuations in the *detectability* of parasites, rather than fluctuations in parasite prevalence or abundance. Cooper and Anwar (2001), relating to blood parasites, already pointed out, that the failure to find parasites does not necessarily indicate that they are absent. Unfortunately almost nothing is known about the underlying physiological, anatomical or developmental mechanisms leading to a diurnal periodicity of oocyst shedding and, thus, oocyst detectability. Experiments carried out by Boughton (1933; 1988)

suggest, that the periodicity is dependent on the host's hours of activity and rest. The physiological changes of the host which trigger a periodic oocyst shedding, and its advantages for the parasite or the host, however, are still unclear. Furthermore, the anatomy of the host's digestive tract may play a role in the shedding periodicity. In birds, caeca, if present, are equipped with a sphincter and evacuated separately from the rectum. The caecal evacuation rhythm ranges from 1:7 to 1:12 (ratio of caecal to rectal evacuations; Duke 1986). European blackbirds have paired caeca and it is known that isosporans also parasitize caecal epithelial cells (Boughton 1930). Coccidians colonizing caeca would, thus, be dependent on the caecal evacuation rhythm to shed oocysts with faeces.

The diurnal shedding of oocysts is not the only periodic phenomenon in coccidians which can result in variable time-dependent *probabilities* to detect infections or to measure their abundance. In *Eimeria,* oocysts from a single infection are shed over several days. The numer of oocysts shed is starting low, increasing to a maximum and decreasing until the infection has run its course (Fuller et al. 1995). Furthermore, several infections can occur simultaneously. Thus, an observed oocyst shedding pattern may be the product of different simultaneously occurring periodic phenomena. In view of this potential complexity, my recommendation for future investigators is 1) to focus analyses on presence/absence data because they are less likely to lead to distorted results 2) to collect several samples from each individual within short time periods and 3) to collect samples at an appropriate time, when oocyst shedding is expected to be at its peak. I believe that these considerations have validity also for other parasite-host systems, since a high variability of *apparent* parasite presence or abundance in repeated measurements is not an exclusive phenomenon of protozoan parasites (Doster and Goater 1997).

5.4.References

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6

Parasitic eggs and oocysts in the feces of Eurasian Blackbirds (*Turdus merula*):

a model to estimate the quality of a parasitic assay

Abstract – Indirect assays of endoparasitic infections commonly involve the examination of body excretions and/or of blood for parasitic stages. Several biological aspects of the host, of the parasites and of their association, however, account for the fact that parasites will not be detected with the same probability at all times. Whereas the success to find parasitic stages clearly denotes that the respective individual is infected, the failure to detect them does not necessarily means that the individual is not infected. We developed an estimation parameter p_{inf} which is designed to estimate the probability of incorrectly assigning the infection status "not infected" to an infected individual from which one or more samples were collected but no parasitic stages were found. We present data from a wild population of Eurasian blackbirds and their most common gastrointestinal parasites: protozoans of the genus *Isospora*, nematodes of the genus *Capillaria*, cestodes and acanthocephalans. The evaluation of the estimation parameter showed that at least three samples had to be evaluated from each bird to reliably (with a probability of 95%) denote an individual as not infected with *Capillaria spp.*, or as not infected with either one of the four parasite types. For a reliable judgement of the infection status with the three other parasite types (*Isospora* spp., cestodes and acanthocephalans) the evaluation of four, five and seven samples, respectively, would have been necessary. The implications from the model and the results from our data are discussed with regard to practical aspects of methodological planning.

6.1 Introduction

Two of the major questions currently being asked about host-parasite evolution are the role of parasites in sexual selection and in life-history evolution

(Clayton and Moore 1997). Studies devoted to test hypotheses about parasite-host systems all require a reliable method to assess a host's infection status (the presence or absence of an infection) and/or intensity of an infection. If the host of concern is required to stay alive, which is usually the case in ecological, behavioural and evolutionary studies, only indirect methods can be applied for the assay of endoparasitic infections, i.e. infections of the blood, digestive tract and inner organs. The most commonly applied methods include the examination of blood smears (blood parasites) and the examination of body excretions (parasites of the intestinal tract and of other inner organs). From the latter, feces are the most important, as eggs and larvae of all gastro-intestinal parasites and many others leave the hosts' body by this vehicle (Soulsby 1968). However, several biological aspects of the host (gastro-intestinal anatomy, consistency of feces), of the parasites (age, sex, size, developmental and reproductive cycle, susceptibility to crowding effect) and of their association (physiological and immunological interactions) account for the fact, that infections, although present, will not be detected with the same probability and the same intensity at all times (Boughton 1933; Thienpont et al. 1979; Levine 1985; Shaw and Moss 1989; Moore and Simberloff 1990; Moss et al. 1993; Fuller et al. 1995; Arneberg et al. 1998; Brawner and Hill 1999; Tompkins and Hudson 1999; Hudman et al. 2000; Brown and Grenfell 2001; Misof 2004). Here we will focus on the evaluation of presence or absence of infections and will not consider infection intensities, which are even more difficult to interpret (Weatherhead and Bennett 1991; Doster and Goater 1997). As other authors have already pointed out (Cox 1989; Gregory and Blackburn 1991; Weatherhead and Bennett 1992) the success to find parasites or parasitic stages in a single blood smear or a single fecal sample unequivocally indicates the presence of an infection at a given time, whereas the failure to find parasites or parasitic stages may indicate two things: 1) that the individual is actually free of infection or 2) that the individual is infected but we are unable to detect the infection. The probability to detect an actually present infection, and, thus, the accuracy of an assay, can be increased by taking multiple samples from each individual, but the problem will nevertheless persist whether repeated negative findings indicate the real absence of an infection or a failure to detect an

actually present infection. While there is no reason to reject positive results, negative results present a problem and have to be interpreted with caution.

In this paper we develop an estimation parameter p_{inf} which is designed to estimate the probability of failing to detect an actually present infection in an individual. The analysis is based on data from a free living population of Eurasian Blackbirds (*Turdus merula*), for which fecal samples were scored for the presence (1-score) or absence (0-score) of oocysts and eggs of four gastrointestinal parasitic taxa, protozoans of the genus *Isospora*, nematodes of the genus *Capillaria*, cestodes and acanthocephalans.

6.2 Methods

6.2.1 *Sample collection and analysis*

During the breeding seasons of 1996-1998 fecal sample-series (n = 37) of three samples each were collected from adult, free living, color-banded blackbirds in the Botanical Garden of Bonn, Germany. The three samples of each bird were collected within the range of twelve days at some time between April and August. They were stored in a 2% potassium bichromate solution at 4°C up to further analysis. Fecal samples were examined using a flotation method with saturated $NaCl/ZnCl_2$ solution (Bürger and Stoye 1983). After removal of large particles (seeds, grass, pebbles), samples were centrifuged for 10 minutes at 300 g. The supernatant was removed and pellets weighted to the nearest of 0.01 mg. 6 ml of flotation medium was then added and carefully mixed to avoid production of air bubbles. Four compartments of McMaster counting chambers, each with a volume of 0.15 ml, were filled and screened for the presence or absence of oocysts from the protozoans *Isospora* spp., for eggs from the nematodes *Capillaria* spp., from cestodes and from acanthocephalans.

If oocysts or eggs were found in a sample it was assigned a "1"; if no oocysts or eggs were found it was assigned a "0". This way, five matrices with each 37 x 3 scores (= a total of 111 scores) were obtained for 1) overall infection (infection with at least one of the four parasite taxa) 2) infections with

Isospora spp., 3) infections with *Capillaria* spp., 4) infections with cestodes and 5) infections with acanthocaphalans.

6.2.2 *Assumptions of the model*

6.2.2.1 *Time interval and infection status*

The infection status of a bird is expected to change over time due to 1) seasonal and environmental aspects, 2) behavioural, physiological and immunological processes and 3) the finite life span of parasites (Mehlhorn et al. 1986; Crompton 1991; Wakelin and Apanius 1997; Bush et al. 2001) A once aquired and established infection, however, is not expected to disappear overnight because physiological and immunological processes of the host (phagocytosis, cytotoxicity, intracellular defense mechanisms, antigen recognition, antibody production), responsible for the successful interruption or termination of the parasites' development (e.g. inflammation of the attachment site, expulsion of worms) have to take place (Wakelin 1994; Wakelin and Apanius 1997). The establishment of an infection, such that it can be diagnosed by an indirect method is also not a sudden event, because migration to specific niches in the host organism has to occur, attachment to the site (most helminths) or intrusion into epithelial cells (most protozoans). The prepatent period of most parasites takes at least six days (Mehlhorn et al. 1986), which means that an infection is already present (and, in most cases, detrimental) before it can be measured by an indirect method. We assumed that the infection status of a bird was less probable to switch from "infected" to "not infected", and vice versa, the shorter the time period was we considered. For practical reasons we chose a period of 12 days and made the assumption that the infection status of birds did not change within this period.

6.2.2.2 *Denotation of 0-scores and 1-scores*

As a consequence from the above made assumption, and from the fact that there is no reason to reject a positive finding of infection (Cox 1989; Gregory and Blackburn 1991; Weatherhead and Bennett 1992) we defined that

even a single 1-score in a three-sample-series denotes that the respective bird is infected. Thus, the remaining 0-scores of the respective mixed sample-series were assumed to be false, i.e. in these cases we assumed to have failed to detect an actually present infection. For the evaluation of the estimation parameter p_{inf} we focus on infected birds, i.e. on birds with at least one 1-score in the sample-series. Birds with 0-scores exclusively are not (yet) considered, because their true infection status can not be determined a priori, as will be discussed in the following sections.

6.2.3 *The model*

For infected birds two cases can be distinguished: a series of 1-scores (the ideal case) and a series of mixed results of 1-scores and 0-scores. The probability to be wrong when measuring a 0-score for an actually infected bird can be evaluated (taking each single measurement into account) as

$$p_{inf} = \frac{\textit{number of all false measurements for infected birds}}{\textit{number of all measurements for infected birds}} \qquad (1)$$

Equation (1) gives a rough estimation of the probability p_{inf} to measure a false “zero”. From equation (1) follows that the the quality of the parasitic assay is high (which means that there is a low probability to assign a “zero” to an infected animal), if in the data set exclusive 1-score-series are frequent and mixed series are rare. Correspondingly, the quality of the parasitic assay is low, if exclusive 0-score-series occur rarely and mixed series are frequent. Thus, the probability to be wrong depends on both, the quality of the testing method and on the number of measurements. The additional consideration of n measurements for the same individual reduces the probability for a false series of zeroscores to p_{inf}^{n}.

To give investigators a tool to estimate whether a series of 0-scores reliably denotes that the respective individual is not infected (at a significance

level of α=0.05), p_{inf} for the considered test method has to be calculated following equation (1). The number of required measurements for the respective assay can then easily be evaluated from equation (2)

$$p_{inf}^{n} < 0.05. \qquad (2)$$

6.3 Results

6.3.1 *Allocation of 0-scores and 1-scores*

The results of the analysis of the 37 three-sample-series (111 samples) are summarized in table 1. All possible 8 states of "0-score and 1-score sequences occurred (000, 111; 110; 011; 100; 001; 101; 010). For infected birds the ideal case of three 1-scores was obtained at the most in 30 % of cases (*Isospora* spp.:14 %, *Capillaria* spp.:28 %, cestodes: 0 % acanthocephalans: 22%, infection with any of the four parasites: 30 %). Thus, for at least 70 % of detected infections mixed sample series were obtained.

Table 1 Frequency of occurrence of the 8 possible states of the 3-measurement series. For each parasite 37 sample-series were considered.

Sample 1	Sample 2	Sample 3	Overall infection	*Isospora* spp.	*Capillaria* spp.	Cestodes	Acanthocephalans
0	**0**	**0**	7	23	19	31	28
1	**1**	**1**	9	2	5	0	2
1	**1**	**0**	4	2	2	0	0
0	**1**	**1**	5	3	5	0	1
1	**0**	**0**	0	1	1	0	0
0	**0**	**1**	8	5	3	4	4
1	**0**	**1**	2	0	1	1	0
0	**1**	**0**	2	1	1	1	2

6.3.2 *Estimates of p_{inf}*

The probability to falsely assign the infection status "not infected" to an actually infected bird was evaluated for each parasite type. These estimates of

p_{inf} are summarized in table 2. In neither case (infection with the four parasite types) was it possible to reliably (at a significance level of α = 0.05) denote an animal as “not infected” after the evaluation of one and of two measurements. Only after the evaluation of three samples was it possible to reliably denote an animal as “not infected” with *Capillaria* spp. (p=0.04) or as not infected with any of the four parasites (p=0.04). For the three other parasites it was not possible to reliably denote an individual as “not infected” if three 0-scores were obtained. For infections with *Isospora* spp. four measurements would have been necessary to correctly denote an individual as “not infected” with a probability of 95% (because p_{inf} = 0.45 and p_{inf}^4< 0.05). For infections with acanthocephalans five measurements would have been necessary to pass a reliable judgement on the infection status (because p_{inf} =0.48 and p_{inf}^5< 0.05) and for infections with cestodes seven measurements would have been necessary (because p_{inf} = 0.61 and p_{inf}^7< 0.05).

Table 2 The probability to measure a series of wrong zero-score(s) for an infected bird after 1, 2 or 3 measurements, respectively.

	p_{inf}	p_{inf}^2	p_{inf}^3
overall infection	0.34	0.12	0.04
Isospora spp.	0.45	0.20	0.09
Capillaria spp.	0.33	0.11	0.04
cestodes	0.61	0.37	0.23
acanthocephalans	0.48	0.23	0.11

6.4 Discussion

The evaluation of the estimation parameter p_{inf} showed that for each parasite type a different number of samples was necessary to reach a reliable judgement (at a 95 % significance level) of the infection status of a bird. At least three samples had to be evaluated from each bird to reliably denote an

individual with three 0-scores as not infected with *Capillaria* spp. , or as not infected with any of the four parasite types. For a reliable judgement of the infection status with the three other parasite types (*Isospora* spp., acanthocephalans and cestodes) the evaluation of four, five and seven samples, respectively, would have been necessary.

The results of this study emphasize the importance of a careful methodological planning in parasite-related studies. A preliminary test on the effectiveness and reliability of the method, as the one presented here, may be of great value in the compilation of the most suitable and effective method for the respective study. Only a few studies critically examined the reliability of commonly applied methods (Gregory and Blackburn 1991; Weatherhead and Bennett 1991, 1992; Allander and Sundberg 1997; Ots et al. 1998), on which most investigators in good faith rely. A concurring opinion seems to exist that infection prevalence will usually be underestimated rather than overestimated, since only the interpretation of negative findings is problematical (Cox 1989, Gregory and Blackburn 1991; Weatherhead and Bennett 1992; Cooper and Anwar 2001).

The model illustrates that in the case of infections with a rare parasite (low infection prevalence in the population) a higher sample size of individuals is required for the evaluation of p_{inf} , since individuals with exclusively 0-scores have to be excluded from the analysis. Thus, the low estimation parameter found for cestode infections in this study does not necessarily denote that the applied method is inaccurate: if cestode prevalence in the population is actually low, a better estimate of p_{inf} may have been achieved by an increase in (bird) sample size and, thus, by a relative increase in 1-scores.

Since the estimation parameter p_{inf} is dependent on the portion of 1-scores in the data set, the value of p_{inf} can be improved by 1) a more effective or sensible method in detecting parasitic stages and 2) by increasing the number of repeated measurements.

In terms of fact, the improvement of the method may require a thorough study of the parasite's biology and its interaction with the host. It is known that biological aspects of the host (gastro-intestinal anatomy, consistency of feces), of the parasites (age, sex, size, developmental and reproductive cycle,

susceptibility to crowding effect) and of their association (physiological and immunological interactions) account for the fact, that infections will not be detected with the same probability and the same intensity at all times (Boughton 1933; Thienpont et al. 1979; Levine 1985; Shaw and Moss 1989a, b; Moore and Simberloff 1990; Moss et al. 1993; Fuller et al. 1995; Arneberg et al. 1998; Brawner and Hill 1999, Tompkins and Hudson 1999; Hudman et al. 2000; Brown and Grenfell 2001; Misof 2004). Most frequently, temporal phenomena are responsible for a variation in parasite detectability. The egg output of helminth parasites in feces has been shown to underly seosonal patterns, due to young worms maturing and beginning to lay eggs and mature worms becoming older and less fecund (Moss et al. 1993). The developmental cycle of most haemoparasites includes intracellular and extracellular stages. Thus, they cannot be found in circulating blood as long as they undergo schizogonic cycles in tissues (Aktinson and van Riper 1991). A diurnal periodicity of parasite detectability has been described for trypanosomes (Levine 1985) and for isosporan parasites (Boughton 1933; Kruszewicz 1995; Brawner and Hill 1999; Hudman et al. 2000; Misof 2004). Since temporal phenomena play a major role in influencing parasite detectability, the most reliable and effective method will have to take into account at which point in time parasitic stages will regularily be detected in samples. In order to improve the accuracy of an indirect parasitic assay it may, thus, be of higher relevance to know about the temporal patterns of the respective parasites than to invest in costly alternative methods, as e.g. in serological procedures (Mehlhorn et al. 1986). It is certainly a worthful undertaking to pay attention to the special features of the parasite-host system of concern.

In view of the still scarce knowledge about the biology of gastrointestinal parasites and their wild bird hosts, however, it may not be practicable to follow this good meant advise. In these cases the accuracy of the assay and, thus, the probability to detect actually present infections, may be improved by increasing the sample size per bird. Following equation (2), the estimation parameter p_{inf} exponentially improves by every additional sample taken per bird. However, also the collection of additional samples per bird, although worthful, may not be practicable in certain cases, e.g. because animals would have to be repeatedly

caught or disturbed, or because several parasites with different sampling optima have to be considered and sampling would become too time consuming.

A low reliability of correctly assigning the status “not infected” to individuals may then have to be accepted. In these cases, investigators must bear in mind that their assay is based on a wobbly foundation and have to take great care in the interpretation of these results.

Parasites play an increasingly important role in understanding evolutionary, ecological, demographical, physiological, immunological and behavioural processes (Toft et al. 1991; Bush et al. 2001). Thus, it is, more than ever, important to encourage interdisciplinary studies, which not only lead to a new understanding of the complex relationships between parasites and hosts, but which also yield the prerequisites for the development of reliable indirect methods in parasitic assays. It may be about time to review methods of (indirect) parasitic assays on live animals, to introduce standards and, finally, to develop more sensible techniques for the detection of parasitic stages.

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7

General discussion

The aim of the present study was 1) to investigate the gastrointestinal parasitic fauna of blackbirds and their potential role in mediating fitness aspects in adult and nestling blackbirds and 2) to examine special properties of a parasite-host system for practical implications on indirect parasitic assays.

7.1 *The gastrointestinal parasitic fauna of blackbirds*

The examination of fecal samples from 272 birds showed that four parasite taxa commonly occurred in blackbirds: the protozoans *Isospora* spp., the nematodes *Capillaria* spp., cestodes and acanthocephalans. Overall infection prevalence was high (82%). *Isospora* spp. and *Capillaria* spp. were the most prevalent parasites, present in 53% and 56% of birds, respectively. This result was not in congruence with findings from a study on the intestinal helminth fauna on blackbirds, conducted in a comparable habitat 35 years ago (Binder 1971), in which the cestode *Dilepis undula* and the nematode *Porrocaecum ensicaudatum* were the most prevalent parasites. Overall infection prevalence between the sexes did not differ significantly, but females were more often and more intensively infected with *Capillaria* spp.. Furthermore, they were more frequently affected by multiple infections.

The composition of the parasitic fauna of a population is heavily dependent on the abiotic and biotic factors of the habitat and of the population, such as climatic conditions, vegetation structure, feeding habits and population density. These factors influence the availability of intermediate hosts or infective stages and/or the probability of ingesting them. Since the four parasitic taxa considered in this study have more or less different requirements for successful

development and transmission (Frank 1976), they were likely to be found at different frequencies in the study population. The differences in the ocurrence and prevalence of parasites found between my study and the study on the intestinal helminth fauna on blackbirds, conducted in a comparable habitat 35 years ago (Binder 1971) can also be explained by local differences in the host 's exposure to infective stages, arising from variable local abiotic and biotic conditions. Alternatively, variations in the host's susceptibility and (or) immune responsiveness may cause variation in the harboured parasite communities (Goater et al. 1995). As we have shown in the study on parental feeding expenditure and intestinal parasite infection (Chapter 3), blackbirds suffer higher susceptibility to parasites during certain stages of the reproductive cycle, probably due to a compromise in immune function during periods of high physical demand. The differences in infection prevalence found between males and females in the present thesis (Chapter 2 and Chapter 3) may as well have arisen from compromises in immune function operating more on one sex (females) at the time of sampling. On the other hand, high local parasitic pressure may induce immunological responses in offspring or a transfer of maternal immunity to their offspring, as a few studies have shown (Smith et al. 1994; Gasparini et al. 2001; Buechler et al. 2002; Walker et al. 2003). Thus, genetic resistances to parasites may arise which represent adaptations to the local parasite pressure.

The findings from this study clearly demonstrate, that the parasite communities and prevalences assessed from single populations can not be taken as representative for the whole species, a fact which should be considered in most parasite related studies.

7.2 *Gastrointestinal parasites as mediators of fitness in blackbirds ?*

Parasites, per definition, are assumed to negatively affect fitness components of their hosts by substracting energy or other materials from their bodies. Under the assumption that energy and resources are limited, life history theory predicts that an animal faced with parasitic infection has to trade-off its

available energy or resources between the combat of infection and other fitness relevant aspects of its' life (Stearns 1992). I examined two energy consuming fitness relevant activities in the life of a bird (the feeding of nestlings and somatic growth) and their respective association to parasitic infection. Under the assumption that the combat against parasites involves energetic costs for the individual, I predicted that birds investing more in parental feeding should be parasitized more frequently than birds investing less and, second, that nestlings infected with parasites should invest less in growth than nestlings not infected with parasites.

Although direct estimates of energetic costs of immune responses are difficult to obtain (Råberg et al. 1998; Owens and Wilson 1999), a growing number of studies provides indirect evidence of these costs (for reviews see Schmid-Hempel 2002; Zuk and Stoehr 2002) and a concurring opinion seems to establish in the literature that, in a broad sense, there is a "cost of immunity" (Svensson et al. 1998; Williams et al. 1999; Ots et al. 2001; Råberg et al. 2002; Schmid-Hempel 2002; Bonneaud et al. 2003, Verhulst et al. 2005). For the two other functions, the feeding of nestlings and somatic growth, energetic costs arising from high metabolic activity have also been documented (Drent and Daan 1980; Walsberg 1983; Masman et al. 1989; Weathers 1992).

7.2.1. *Feeding expenditure and parasitic infection*

I predicted that successful breeders feeding nestlings (SBs) should be more frequently parasitized than unsuccessful breeders not engaged in feeding nestlings (UBs). Furthermore, I predicted that among SBs, the likelihood of infection should be higher for birds which had invested more in parental feeding. I did not find SBs and UBs to differ in their infection prevalence, but the probability to get infected rose for SB with the number of days they engaged in feeding their offspring. However, this was true for males only, since all but one successful breeding females were already infected at the nestling feeding stage.

Probably, investments in other costly activities, prior to the nestling feeding stage (e.g. egg-laying, territory defense, mate guarding, incubating) had already influenced the infection status of birds in my study, such that all birds,

future successful breeders and unsuccessful breeders, had already suffered from a compromise in immune function. The generally higher infection prevalence in females supports this explanation, because in blackbirds, females are exclusively responsible for nest building and incubating (in addition to egg laying) (Glutz von Blotzheim 1988). On the other hand, the similar infection frequencies of UBs and SBs may be the outcome of two intermingling processes, the consequence of infection (not being successful because of being infected) and the cause of infection (becoming infected because of working hard). Since I did not check on the infection status of birds prior to the breeding season I cannot discriminate between both processes. Nevertheless, infections seemed to be the consequence of a stressful activity (the feeding of nestlings), because male successful breeders were more likely to suffer from parasitic infection the more they had invested in feeding their young. Thus, the results of the present study, at least partly, support the prediction of a trade-off between likelihood of infection with gastro-intestinal parasites and the energetic demands of chick feeding. In correlational studies factors such as individual and environmental quality can be expected to override associations between two traits (Stearns 1992). However, if the predicted correlation between two traits can be found despite the confounding effects of individual and environmental quality, as we have done for males in a population of wild blackbirds, a conflict in optimizing both traits, feeding chicks and being immunocompetent against parasitic infections, seems to constitute a real feature of the species‘ life-history.

7.2.2 *Somatic growth and coccidian infection in nestlings*

In blackbird nestlings a negative relationship between the presence of infections with *Isospora* spp. and aspects of somatic growth (weight, tarsus length, wing length and condition) was predicted. However, the predicted relationship was not found. Infections with the protozoan endoparasites *Isospora* spp. did not negatively influence any of the measured growth parameters of blackbird nestlings. Quite on the contrary, at an age of 8 to 10 days infected nestlings had significantly longer wings and longer tarsi than uninfected nestlings. Their weight did not significantly differ from that of not infected chicks, although they were, on average, heavier than their not infected

nest mates.

Although these results were surprising from a theoretical point of view, previous studies in other bird species, investigating the relationship between parasitic infection and aspects of somatic growth have yielded very controversial results (Rogers et al. 1991; Richner et al. 1993; Young 1993; Dufva and Allander 1996; Merino and Potti 1998; Nilsson 2003) with a few studies even reporting on a positive relationship between ectoparasitic infection and aspects of nestling growth (Saino et al. 1998; Szép and Møller 1999) and endoparasitic infection and nestling growth (Moore and Bell 1983; Mazgajski and Kedra 1998). Infection with *Isospora* spp. and with many other gastrointestinal parasites is, in a first instant, a random process because infection occurs by ingestion of a contaminated food item or an infected intermediate host. Thus, dependent on the degree of environmental infestation, getting fed, while being essential for growing, also involves an infection risk for the nestling. Moore and Bell (1983) and Mazgajski and Kedra (1998) who also considered gastrointestinal parasites in their studies (the acanthocephalan *Plagiorhynchus cylindraceus* and protozoans of the genus *Isospora*) also concluded from their findings that the infection risk was associated with the amount of ingested food.

If the probability of infection is really a function of feeding rate, interesting theoretical questions about nestling-parent relationships and optimal decision-making arise. The trade-off between investment in growth and in immune function may only be of secondary importance for these chicks, and instead, comparable to the system of European Oystercatchers feeding on parasitized mussels (Norris 1999) or of Steller's eiders feeding on parasitized amphipods (Bustnes and Galaktionov 2004) an alternative trade-off, namely between energy intake and risk of infection becomes of primary importance.

7.3 A critical view on methodological aspects of indirect parasitic assays

7.3.1. *Diurnal fluctuation of oocyst shedding in feces*

The commonly practised method of assessing a birds haematozoan infection status by the examination of a single blood smear has been repeatedly criticized in the past (Cox 1989; Pruett-Jones et al. 1991; Weatherhead and Bennett 1991;1992; Ots et al. 1998; Cooper and Anwar 2001). Various biological aspects of the host (age, sex, reproductive status, gastro-intestinal anatomy, consistency of feces, resistance to parasites), of the parasites (age, sex, size, developmental and reproductive cycle, susceptibility to crowding effect) and of their association (physiological and immunological responses) can distort results about the "true" infection status of the host. Among these aspects periodic phenomena play a central role, because they cause parasites not to be detected with the same ease and probability at all times. I had a closer look on a short periodic phenomenon, the daily fluctuation of oocyst shedding in the feces of adult and nestling blackbirds and found that oocysts of the protozoans *Isospora* spp. were detected more frequently and at higher intensities in samples collected in the afternoon as compared to samples collected in the mornings. My results corroborate findings from earlier studies on coccidian oocyst shedding in other bird species (Brawner and Hill 1999; Hudman et al. 2000) and show that the time of day at which samples are collected can have a significant effect on the reliability and validity of data. Comparable to the results in house finches (Brawner and Hill 1999) my results in blackbirds suggest that it is unsuitable to collect feces in the morning hours if *Isospora* spp. are the parasites of interest. Since the physiological and immunological processes underlying such a periodicity are still not understood no general pattern can be predicted for other parasite-host systems. Thus, it should be a major step prior to any study involving parasites to determine the most consistent method for assessing parasite prevalence or abundance. It may be necessary to focus on presence/absence data, because intensity measurements are even more prone to misinterpretations (Doster and Goater 1997; Hobbs et al. 1999). Furthermore,

the examination of several samples from each individual is recommended to increase the probability to detect an actually present infection.

7.3.2. *A model to estimate the reliability of zero-scores*

One conclusion which was drawn from the above finding is that the consideration of several samples may reduce the probability of failing to detect an actually present infection. However, even if several samples from each individual are considered, the problem may persist whether the failure in detecting parasitic stages in the sample actually denotes that the individual is free of infection or whether an infection is just not detected. The model which was developed was based on the absence/presence data of the four most prevalent parasites in the feces of blackbirds (*Isospora* spp, *Capillaria* spp., cestodes and acanthocephalans). The estimation parameter p_{inf} showed that, at an assumed significance level of $\alpha = 95\%$ it was not possible in the present blackbird population to reliably denote an individual as “not infected” after the evaluation of a single sample and not after the evaluation of two samples. Only after the evaluation of three samples was it possible in two cases (infections with any of the four parasite types and infections with *Capillaria* spp.) to reliably denote an individual, for which a sample series of three 0-scores was obtained, as “not infected”. For a reliable judgement of the infection status with the three other parasite types (*Isospora* spp., acanthocephalans and cestodes) the evaluation of four, five and seven samples, respectively, would have been necessary. Since the estimation parameter p_{inf} is dependent on the portion of (reliable) 1-scores in the data set, the value of p_{inf} can be improved by 1) a more effective or sensible method in detecting parasitic stages and 2) by increasing the number of repeated measurements. Both aspects may sometimes be hard to realize in practice and investigators may have to accept a low reliability of correctly assigning the status “not infected” to individuals. They must, however, bear in mind that this aspect of their assay is based on a wobbly foundation and have to take great care in the interpretation of these results.

Since parasites play an increasingly important role in understanding evolutionary, ecological, demographical, physiological, immunological and behavioural processes, it may be about time to review methods of (indirect)

parasitic assays on live animals, to introduce standards and, finally, to develop more sensible techniques for the detection of parasitic stages.

7.4 References

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Summary

Parasites are increasingly being recognized as potent agents of selection. Gastrointestinal parasites, however, have largely been ignored in testing the role of parasites in mediating sexual selection and life history decisions. One aim of the present thesis was to contribute to the still scarce knowledge about gastrointestinal parasites in ecological, evolutionary and behavioural studies.

Fecal samples were collected from an urban, individually marked population of Eurasian Blackbirds (*Turdus merula* L.,1758). A flotation method with saturated NaCl/$ZnCl_2$ solution was used to concentrate parasitic stages from the samples. Absence or presence of parasitic stages was used as the main measure of infection. For the sake of completedness oocysts and eggs were also counted to obtain a measure of infection intensity, which' interpretation, however, is notoriously difficult.

Four parasitic taxa commonly occurred in the population: protozoans of the genus *Isospora*, nematodes of the genus *Capillaria*, cestodes and acanthocephalans. *Isospora* spp. and *Capillaria* spp. were the most frequent parasites, infecting 53 % and 56 % of birds, respectively. These results markedly differed from a similar study conducted 35 years ago (Binder 1971) and explicitly demonstrate, that the parasite communities and prevalences assessed from single populations can not be taken as representative for the whole species, as has repeatedly been done in the past.

I investigated whether four of the most prevalent parasites found in the feces of blackbirds impose fitness costs on their hosts. It is a central assumption of life-history theory that the availability of energy or resources is limited. Thus, the investment in one costly trait can only occur at the expense of another costly trait (Stearns 1992). In adult male blackbirds I found a cost of parasitism. Males that had invested more heavily in the feeding of nestlings, measured as the number of days nestlings had been fed, were also more likely to be infected with gastrointestinal parasites. Female infection prevalence was very high (95%) and significantly higher than in males. In blackbirds, females

are alone responsible for nest building, egg laying and incubating. Due to these high investments prior to the nestling feeding stage they are, probably, more than males vulnerable to parasitic infection. During the nestling feeding stage, however, males also trade their health for reproduction.

In nestlings no direct cost of parasitism on aspects of somatic growth could be found. Quite on the contrary, nestling size, measured as tarsus length and wing length were positively associated with prevalence of coccidian infection. I conclude that infections with *Isospora* spp. do not impose the predicted trade-off on blackbird nestlings. Rather, infections with *Isopora* spp. seem to be a function of feeding rate. Thus, an alternative trade-off, namely between energy intake and risk of infection seemes to become of primary importance for blackbird nestlings.

The second aim of this thesis was to critically elucidate methodological aspects of indirect parasitic assays. It is a still commonly practized method to assess a bird's infection status by the examination of a single sample, although it is known that several properties of the host and of the parasites influence the probability to detect parasitic stages with the same frequency and intensity at all times.

I examined a periodic phenomenon, the diurnal shedding of isosporan oocysts in the feces of adult and nestling blackbirds. I found oocysts to be shedded more frequently and at higher intensities in the afternoon. This finding is in congruence with results on *Isospora* oocyst shedding in other bird species and suggests that it should be a major step prior to any study involving parasites to determine the most consistent method for assessing parasite prevalence or abundance.

In order to give future investigators a tool to estimate the quality of their indirect method, I developed a model, based on repeated measurement data of presence or absence of the four most prevalent parasites in fecal samples from adult blackbirds. The parameter p_{inf} determines the probabilty to falsely denote an infected individual as "not infected" if no parasites could be found in its' sample(s). The model showed that in the blackbird population at least three samples had to be evaluated from each bird to reliably (with a probability of 95%) denote an individual with three 0-scores as not infected with *Capillaria*

spp. , or as not infected with any of the four parasite types. For a reliable judgement of the infection status with the three other parasite types (*Isospora* spp., cestodes and acanthocephalans) the evaluation of four and more samples would have been necessary. These results show that great consciousness and caution have to be used in the methodological planning of an indirect parasitic assay and in the interpretation of its' results.