Blood parasitaemia in a high latitude flexible breeder, the white-winged crossbill, *Loxia leucoptera*: contribution of seasonal relapse versus new inoculations

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SUMMARY

We measured seasonal changes in the prevalence of haematozoa (*Leucocytozoon fringillinarum, Haemoproteus fringillae*, and *Trypanosoma avium*) in free-ranging White-winged Crossbills, *Loxia leucoptera*, over 1·5 year in Fairbanks, Alaska, USA. This prevalence was low during early winter. *L. fringillinarum* prevalence increased in late winter/early spring, in the absence of vectors, suggesting relapse of latent infection. By contrast, the prevalence of *T. avium* and *H. fringillae* did not increase until mid-spring, coincident with the emergence of putative vectors and suggestive of new inoculations. The winter breeding period was not associated with lower body condition or elevated blood heterophil/lymphocyte ratios than the summer post-breeding period. Thus, birds unlikely perceived their breeding effort as particularly stressful. Adult males in May and June had low plasma testosterone and their blood prevalence of *L. fringillinarum*, but not other haemoparasites, was higher than in adult females. This difference may have resulted from sex differences in behaviour and/or plumage colouration – bright red in males, dull green/yellow in females. Species in which reproduction and vector abundance are seasonally dissociated may constitute important models for investigating the respective contribution of reproductive hormones, breeding effort, and vector abundance to patent and latent hemoparasitic infections and to new inoculations.

Key words: *Leucocytozoon*, *Trypanosoma*, *Haemoproteus*, testosterone, reproduction, sex difference, sexual dimorphism.

INTRODUCTION

Most avian species inhabiting temperate regions breed in spring and summer. In these species, the prevalence of blood parasites is generally higher during than outside the reproductive period (Weatherhead and Bennett, 1991; Rintamaki *et al*. 1999; Deviche *et al*. 2001; Garvin and Greiner, 2003; Murata *et al*. 2007; Cosgrove *et al*. 2008; Hartup *et al*. 2008), but the factors that account for this difference and their relative contribution to haematozoan infections remain debated.

One such factor is the abundance of appropriate haematozoan vectors (Klei and DeGiusti, 1975; Bennett *et al*. 1982; Godfrey *et al*. 1990; Earle *et al*. 1991). For example, geographical differences in infection with *Haemoproteus chloris* between populations of Greenfinches, *Carduelis chloris*, parallel putative differences in the abundance of the vectors for this parasite (Merilä *et al*. 1995). Similarly, differences in haematozoan (*Leucocytozoon* spp., *Haemoproteus* spp., *Plasmodium* spp. and *Trypanosoma* spp.) infection between mainland and island populations of passerines are associated with the presence or absence, respectively, of appropriate vectors (Super and van Riper, 1995). If vector abundance plays a primary role in determining haematozoan prevalence through new infections, a seasonal increase in vector abundance is predicted to be temporally associated with an increase in patent haematozoan infections.

A second factor is the breeding effort. This effort is associated with an elevated blood heterophil/lymphocyte (H/L) ratio (Ots and Hõrak, 1996), which increases in response to chronic stress or administration of the stress hormone corticosterone (Gross and Siegel, 1983; Gross, 1988; Ilmonen *et al*. 2003; Ots and Hõrak, 1996). These observations suggest that the breeding effort constitutes a major stressful event in the annual cycle with the potential to decrease resistance to new parasitic infections and/or precipitate a relapse of latent infections. Consistent with this contention, avian studies found a trade-off between reproductive investment and resistance to haemoparasitic infections (*Leucocytozoon sp.*: Norris *et al*. 1994; *Haemoproteus sp.*: Ots and Hõrak, 1996; *Haemoproteus majoris*: Allander, 1997; Gustafsson *et al*. 1994), and avian as well as mammalian studies indicate that the activity of the

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reproductive system can influence these infections (Bensch *et al*. 2007; Wood *et al*. 2007; Arriero *et al*. 2008; Fokidis *et al*. 2008). If the seasonal breeding effort is, indeed, physiologically stressful, it is predicted in previously infected birds that it will be associated with increased haematozoan prevalence resulting from relapse and/or parasite reproduction, independent of vector abundance.

The gonadal hormone testosterone (T) is also thought to influence haematozoan infections. Plasma T in many species is seasonally higher in males than females (Deviche and Gulledge, 2000; Ketterson *et al*. 2005; Spinney *et al*. 2006) and this difference parallels a sex difference in blood parasite prevalence (Kiyota *et al*. 1984; Zuk and McKean, 1996, but see Hartup *et al*. 2008 and Pawelczyk *et al*. 2003). In addition, adult male Dark-eyed Juncos, *Junco hyemalis*, that were undergoing a first reproductive cycle had lower plasma T and also lower haematozoan prevalence than older males (Deviche *et al*. 2001), and T administration to free-ranging males of this species increased their blood parasite prevalence (Deviche *et al*. 2006). In the Large Vesper Mouse, *Callomys callosus*, inoculation with *Trypanosoma cruzi* resulted in lower blood prevalence of this parasite in castrated than control animals and T treatment to castrates increased their parasite prevalence to control levels (Do Prado *et al*. 1999). Given to House Mice, *Mus musculus*, T treatment increased the survival of the intestinal nematode, *Heterakis spumosa* (Harder *et al*. 1992) and it enhanced susceptibility to new parasitic infections with the intestinal roundworm, *Strongyloides ratti* (Kiyota *et al*. 1984). If elevated plasma T stimulates re-emergence of tissue-bound parasites in the blood, for example secondary to immunosuppression, periods of elevated plasma T in males are predicted to be associated with an increase in patent infections, even in absence of appropriate vectors.

In previous studies using free-ranging birds, the relative contribution of the above factors to blood parasite prevalence was difficult to assess because most species reproduce and males have seasonally elevated plasma T at the same time of the year that vectors are most abundant (Garvin and Greiner, 2003; Rintamaki *et al*. 1999). To address this issue, we investigated seasonal changes in blood parasite prevalence and diversity in a population of White-winged Crossbills, *Loxia leucoptera*. Crossbills have a specialized diet consisting primarily of conifer seeds, which are available irregularly both geographically and temporally (Bent, 1968; Newton, 1972; Benkman, 1992). To accommodate this irregularity, crossbills have evolved flexible reproductive cycles that can extend from January to November (Benkman, 1992; Deviche and Sharp, 2001). Crossbills in the present study began to breed in winter and plasma T in males was seasonally elevated in late winter and early spring, when ambient temperatures were well below freezing and haematozoan vectors absent (Deviche and Sharp, 2001; Werner, 1992). By summer, when putative vectors became abundant, birds had completed their breeding cycle and T in males was circulating at baseline concentrations (Deviche and Sharp, 2001; see Results section). We reasoned that if the winter breeding effort or a seasonal increase in plasma T (in males) is associated with increased blood parasite prevalence, as predicted by other studies, this increase must result from an increase in reproduction (*Trypanosoma* sp.) or a relapse (*Haemoproteus* sp. and *Leucocytozoon* sp.; see Results section) of parasites rather than new inoculations. By contrast, if a seasonal increase in haematozoan vector abundance is primarily responsible for controlling seasonal changes in blood parasitaemia through new infections, prevalence was predicted not to increase until the spring emergence of vectors.

To further investigate the control of haematozoan parasite infections, we compared seasonal changes in parasite prevalence in adult males and females and determined whether juvenile birds can be infected with these parasites. To determine whether the late winter breeding period was associated with chronic stress, we determined the body condition of birds sampled during this season, measured the blood H/L ratios of these birds, and compared the results with those from birds sampled in summer, after completion of breeding. We hypothesized that if birds in winter-early spring are exposed to chronic stress resulting from environmental factors or breeding effort, these birds would be in lesser body condition and have higher H/L ratios than summer birds.

Few studies have analysed changes in haematozoan prevalence throughout the year in free-ranging passerines (Blue Tit, *Cyanistes caeruleus*: Cosgrove *et al*. 2008; Rufous-winged Sparrow, *Aimophila carpalis*: Deviche *et al*. 2005; House Finch, *Carpodacus mexicanus*: Hartup *et al*. 2008) and other birds (Rock Pigeon, *Columba livia*: Klei and DeGiusti, 1975; Japanese Rock Ptarmigan, *Lagopus mutus japonicus*: Murata *et al*. 2007; Helmeted Guineafowl, *Numida meleagris*: Earle *et al*. 1991; Red-bellied Woodpecker, *Melanerpes carolinus*: Schrader *et al*. 2003). A better understanding of the factors that control avian haematozoan infections requires additional seasonal studies using species that inhabit a diversity of habitats and are, therefore, exposed to a variety of environmental conditions during and outside their breeding season. To our knowledge, the present work is the first to describe changes in blood parasitaemia throughout the year in a subarctic passerine.

MATERIALS AND METHODS

Bird capture and sampling

We caught 131 adult male and 102 adult female White-winged Crossbills at the same locality in

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Fairbanks (Alaska, USA; 65°52′N/147°54′W) between 3 May 1998 and 6 August 1999, using Japanese mist nets and a live decoy bird placed next to the net. This sample consisted of 226 first captures (126 males, 100 females) and 7 recaptures (5 males, 2 females). Data from recaptured birds were considered independent from first capture data and were retained in the analyses as long as birds were recaptured at least 1 month after the first capture. Starting in 1999, adults (=after-hatch-year, AHY) were separated into 2 age classes (second-year, SY: $n=104$; after-second-year, ASY: $n=69$; unknown: $n=4$) based on plumage characteristics (Deviche, 2000; Pyle, 1997). Birds were sexed based on plumage colour and previously described morphological characteristics (developed cloacal protuberance in males; incubation patch in females: Pyle, 1997).

To determine whether hatch-year (HY) birds can be infected during their first summer, we analysed blood parasites in samples collected from birds of this age class caught in July and August 1998 ($n=11$) and 1999 ($n=19$). When sampled, HY crossbills were no more than a few months old as determined by incomplete skull pneumatization (Pyle, 1997).

Birds were caught between 6:20 a.m. and 4:10 p.m. (Alaska Standard Time). All activities were pre-approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee and were conducted with appropriate scientific collecting permits from the Alaska Department of Fish and Game, US Fish and Wildlife Service, and the US Geological Survey Bird Banding Laboratory.

Within 5–10 min of capture, we removed birds from the mist net and collected a blood sample (max. 700 μl) from a wing vein as described by Deviche and Sharp (2001). Five to 10 μl of blood were used to immediately prepare 1 or 2 thin blood smears on 25×75 mm glass microscope slides (Bennett, 1970). Smears covered, on average, a surface equal to 500–800 mm^2. The remainder of the blood was used to measure circulating concentrations of reproductive hormones, including T, in males (Deviche and Sharp, 2001). Slides were dried at room temperature, dehydrated, stained using the Giemsa method, and cover-slipped as described by Deviche *et al*. (2006). Representative slides with smears containing the 3 parasite species under study (see Results section) were deposited at the US National Parasite Collection (Beltsville, MD; Accession numbers: USNPC 102020-102023). After blood collection, we measured the wing chord of each bird to the nearest mm, weighed birds to the nearest 0·1 g, corrected these weights for the volume of blood taken, marked birds with an individually numbered aluminum tarsal band (US Geological Survey, Washington, DC), and released them at the capture site.

Blood smear analysis

Representative smears were studied using light microscopy at 1000× magnification under oil immersion to identify parasite species. Parasites were identified to species level using morphological criteria established by Peirce and Baker (1976) and Valkiunas (2005). For quantification, each smear was examined first at low (200×; 10 min) and then at high (400×; 5 min) magnification and birds were classified as negative or positive for each haematozoan species (see Results section; Deviche *et al*. 2001). We defined observed prevalence (hereafter prevalence) as the proportion (%) of sampled birds in which we detected blood infection with at least 1 individual of a particular parasite species (Bush *et al*. 1997). We also measured the abundance of *Leucocytozoon fringillinarum* (hereafter *L*. *fringillinarum*), defined as the number of these parasites per 100 visual fields of a smear studied at 400× magnification (Bush *et al*. 1997; Deviche *et al*. 2001). Given the area of individual circular visual fields at this magnification (diameter: 50 μm; area: 2×10^{-3} mm^2) and the smear area (500–800 mm^2; see above), abundance data could be obtained from non-overlapping fields. Parasites other than *L*. *fringillinarum* were generally present in low numbers and their abundance was not measured (Levine, 1985). Smears were analysed without knowledge of sex, age, or sampling date.

To determine the reliability and consistency of parasite detection, 25 randomly chosen smears, each from a different bird, were studied as described above by 2 observers (P.D.; H.B.F.) who independently measured the abundance of *L*. *fringillinarum* and the prevalence of *Trypanosoma avium* and *Haemoproteus fringillae* (hereafter *T*. *avium* and *H*. *fringillae*). In addition, each observer measured these parameters in pairs of smears prepared from a same blood sample of 25 randomly chosen birds.

We compared prevalence in summer-caught HY crossbills ($n=30$; see above) and AHY birds caught during the same period (1998: $n=11$ males and 7 females; 1999: $n=8$ males and 1 female). The proportion of HY birds during the considered period was higher in 1999 (HY/AHY: 2.1) than 1998 (id., 0·6; Fisher exact probability test: $P=0{\cdot}034$). In addition, HY crossbills in July and August had not begun to moult into first pre-basic plumage and their sex could not be determined. The sex ratio of the sampled adult population ($n=231$ birds, see above) was 43% females. For blood parasite data in HY and AHY birds to be as comparable as possible, it was necessary that (a) the proportion of HY to AHY birds be similar in 1998 and 1999 and (b) the assumed sex ratio of the HY sample be similar to that of the AHY sample (43%). This was achieved by excluding smears from 10 *randomly* chosen adult males and 2 *randomly* chosen adult females caught in 1998 from the data set before statistical analysis. Excluding

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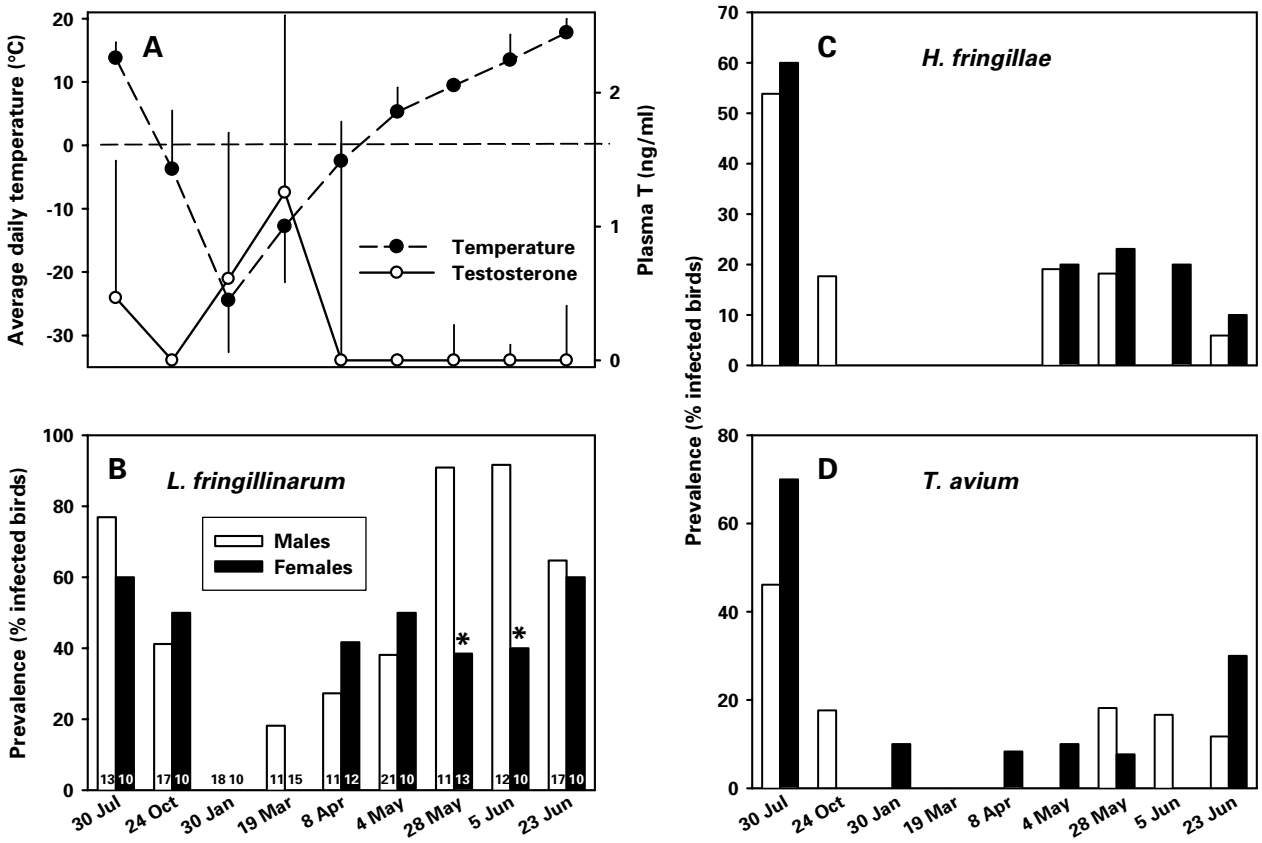

Fig. 1. (A) Seasonal changes in average daily temperatures (means ± S.E.M.) and male crossbill plasma testosterone (T; medians + 0·5 interquartile intervals) at the same sampling location in Fairbanks, Alaska. (B–C) Seasonal changes in prevalence of (b) *Leucocytozoon fringillinarum*, (c) *Haemoproteus fringillae*, and (d) *Trypanosoma avium* measured in thin blood smears from adult male and female White-winged Crossbills, *Loxia leucoptera*. Numbers at the bottom of panel B columns indicate sample sizes. Sample sizes are the same for the other panels. * Indicates an intersexual difference ($P < 0{\cdot}05$ and non-overlapping 95% confidence intervals) in prevalence.

these birds resulted in an AHY sample consisting of 1 male and 5 females (1998) and 8 males and 1 female (1999), for a combined total of 15 AHY birds. Using this approach, HY/AHY ratios were similar in 1998 (11/6) and 1999 (19/9), and so were the assumed HY and effective adult sex ratios (6/15 = 40% females).

Blood leucocyte profiles

We compared the blood abundance of 2 types of leucocytes (heterophils and lymphocytes) in adults sampled in summer 1998 (10 males, 10 females; average sampling date: 30 July), when birds had the highest seasonal average prevalence of blood parasites (see Fig. 1) with that in winter 1999 (11 males, 15 females; average sampling date: 19 March), when males had seasonally elevated plasma testosterone and birds of both sexes had low prevalence of blood parasites (Fig. 1). For this, we counted the number of heterophils (H) and lymphocytes (L) seen in non-overlapping microscope visual fields of smears studied at 400× magnification and for a constant duration (40 min per smear). Abundance data were used to calculate H/L ratios. Ratios were used in statistical analyses only when the corresponding total number of immune cells (H + L) was at least 30 ($n = 32$ birds).

Body condition

We used the same adults as used for leucocyte profile analysis to determine if body condition is sex dependent and differs between winter and summer. Body condition, a measure of stored energy reserves, was calculated using the residuals of a linear regression between wing chord and body mass (Costantini *et al.* 2007; Ochs and Dawson, 2008). It was assumed that birds with a larger body mass relative to their body size (estimated by wing chord) were in better body condition.

Ambient temperatures

Daily minimum and maximum temperatures during the study period were obtained from the Alaska Science Center (http://alaska.usgs.gov/science/biology/index.php) and used to calculate average daily temperatures.

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Testosterone assay

Plasma T concentrations in samples collected from adult males were measured using a commercial, validated radioimmunoassay (Deviche and Sharp, 2001). Hormone concentrations were not measured in samples obtained from females or HY birds.

Statistical analyses

We used χ^2 tests to measure associations between parasite species in smears obtained from adult birds. Analysis of these associations required 6 independent χ^2 tests (see Results section). A Bonferroni correction was accordingly applied and differences were considered statistically significant when corresponding probabilities were equal to or less than 0·008 (=0·05/6).

We used Pearson product moment correlations, McNemar tests for the significance of change, and Wilcoxon matched-pair signed-rank tests (Siegel, 1956) to determine the consistency and repeatability of parasite detection between observers and between pairs of smears collected from the same bird. Statistical associations between various independent variables (sex, age, plasma T, and average daily temperature on capture day) and prevalence of each parasite species were analysed by multiple logistic regressions.

Binary comparisons of prevalence between sexes at different times of the year (see below) and between age groups (SY *vs* ASY; HY *vs* AHY) were done using Fisher exact probability tests. When a difference in prevalence was statistically significant ($P \leqslant 0{\cdot}05$), we used a confidence interval for proportion calculator (http://www.dimensionresearch.com/resources/calculators/conf_prop.html) to calculate the 95% confidence interval for each group of data considered. Differences were considered biologically meaningful and are discussed as such only if a Fisher exact probability test was statistically significant *and* confidence intervals calculated for the two corresponding groups of data did not overlap.

To examine seasonal changes and sex differences in adult blood parasite prevalence and diversity, data (131 males and 100 females; see above) were sorted by capture date and then divided into consecutive, non-overlapping blocks of data, each consisting of at least 10 males and 10 females. Prevalence in males and females within each data block was compared statistically as described above.

Seasonal (winter *vs* summer) and sex differences in immune function (H and L abundance; H/L ratios), wing chord, body mass, and body condition were analysed by two-way analysis of variance with season and sex as independent variables. Body mass data were not normally distributed and were, therefore, ranked before analysis (Conover and Iman, 1981).

RESULTS

Parasite identification and overall prevalence

Blood smears were found to contain 3 protozoan parasite species: *L. fringillinarum*, *T. avium*, and *H. fringillae*. Forty-eight percent of adult birds sampled were found to contain at least 1 parasite species. The most prevalent parasite species was *L. fringillinarum* (42% of the samples). *T. avium* and *H. fringillae* had prevalences of 13%. The percentages of adult birds sampled in which we observed 1, 2, or 3 parasite species were 34%, 10%, and 5%, respectively.

Inter- and intra-observer consistencies

Data from 2 observers independently measuring the abundance of *L. fringillinarum* in the same smears were correlated ($r^2=0{\cdot}995$, $P<0{\cdot}0001$) and did not differ (Wilcoxon test: $P=0{\cdot}206$). These observers detected *T. avium* and *H. fringillae* with similar effectiveness (McNemar tests: Ps $>0{\cdot}05$). *L. fringillinarum* abundance measured in paired samples obtained from the same birds was consistent ($r^2=0{\cdot}962$, $P<0{\cdot}0001$) and there was no within-pair difference in *T. avium* or *H. fringillae* detectability (McNemar tests: $P>0{\cdot}05$). Thus, the method used to detect blood parasites in smears and to quantify prevalence was reliable and consistent.

Associations between parasite species

In adults, no association was detected between infections with *H. fringillae* and *L. fringillinarum*. Indeed, the proportions of blood samples containing *L. fringillinarum* did not differ whether these samples contained or not *H. fringillae* ($P=0{\cdot}037$; critical statistical significance level=0·008: see Materials and Methods section and Table 1). Likewise, the likelihood of blood infection with *H. fringillae* did not differ whether birds were or were not infected with *L. fringillinarum* ($P=0{\cdot}037$).

By contrast, blood infection with *H. fringillae* was associated with increased probability of infection with *T. avium*. Furthermore, infection with *T. avium* was associated with increased probability of infection with *H. fringillae* or *L. fringillinarum*, and infection with *L. fringillinarum* was associated with increased probability of infection with *T. avium* (Table 1).

Seasonal changes in ambient temperature and adult haematozoan prevalence

Average daily temperatures varied between $-24{\cdot}4 \pm 8{\cdot}2$ °C (January 1999) and $17{\cdot}8 \pm 2{\cdot}2$ °C (June 1999; Fig. 1A). These temperatures stayed below freezing from October 1998 to 1 April 1999 (except 25 March 1999: +1 °C). The *minimum* daily temperature in 1999 did not reach 0 °C until 19 April. *Maximum*

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Table 1. Observed frequency of blood infection (% birds infected) with one parasite species in the absence (Neg.) or presence (Pres.) of another parasite species in smears obtained from 231 adult White-winged Crossbills, *Loxia leucoptera*

	H. fringillae	*T. avium*	*L. fringillinarum*
H. fringillae			
Neg. ($n=200$)	—	8%	40%
Pos. ($n=31$)		42%*	61% n.s.
T. avium			
Neg. ($n=202$)	9%	—	36%
Pos. ($n=29$)	45%*		90%*
L. fringillinarum			
Neg. ($n=133$)	9%	2%	—
Pos. ($n=98$)	19% n.s.	27%*	

* $P<0.001$ (χ^2 test) for comparison of Neg. with corresponding Pos. data.
n.s., Not significant ($P>0.008$; χ^2 test).

daily temperatures never exceeded 0 °C between 26 October 1998 and 14 March 1999.

The prevalence of *L. fringillinarum* (male and female data combined, $n=231$) changed seasonally and was positively associated with average daily temperatures (multiple logistic regression: Ps $<0{\cdot}001$; Fig. 1A, B). This association was observed also when male ($n=131$) and female ($n=100$) data were analysed separately (P's $<0{\cdot}001$). Only January samples were found not to contain *L. fringillinarum*. By March the prevalence of this parasite in males had increased to 18% and by early April it was 27% in males and 42% in females. The late winter and early spring increase in prevalence took place as ambient temperatures were below freezing (Fig. 1A). The highest prevalence of *L. fringillinarum* occurred during the summer, when ambient temperatures were highest and putative vectors most abundant. Inclusion of sex as factor in the logistic regression models revealed that males had, on average, higher *L. fringillinarum* prevalence than females ($P=0{\cdot}029$). Pairwise comparisons (Fisher exact probability tests followed with confidence interval calculation) revealed higher prevalence of this parasite in males than females only in May and early June 1999 (Fig. 1B).

The prevalence of *T. avium* and *H. fringillae* was also positively associated to average daily temperatures (multiple logistic regressions, $n=231$, Ps $<0{\cdot}001$; Fig. 1C, D). These parasites either were not found in blood (*H. fringillae*) or were found at low ($\leqslant 10\%$) frequency (*T. avium*) until May (Fig. 1C, D). In contrast to the situation for *L. fringillinarum*, the prevalence of *T. avium* and *H. fringillae* was not sexually dimorphic (Ps $>0{\cdot}60$).

Plasma testosterone

Plasma T in adult males changed seasonally (Kruskal-Wallis analysis of variance on ranks: $H=23{\cdot}6$, $P=0{\cdot}003$; Fig. 1A). Plasma T was low in October 1998, increased between that month and March 1999, when peak levels were measured and average ambient temperatures were well below freezing. Plasma T then decreased and remained low (average: 0 ng/ml) for the rest of the sampling period. Slightly elevated levels in July 1998 did not differ significantly from levels measured in April–June 1999.

Association between adult male plasma T and haematozoan prevalence

We used data obtained from adult males in which plasma T concentrations were measured (1998: $n=33$; 1999: $n=85$) to determine whether plasma T was related to parasite prevalence. Multiple logistic regression analyses with ambient temperature and plasma T as independent variables revealed no statistical relation between plasma T and prevalence of any parasite species (Ps $>0{\cdot}60$; Fig. 1).

Age differences in adult haematozoan prevalence

Data collected in 1999 were used to compare prevalence in SY and ASY birds. For each parasite species, we performed a multiple logistic regression with age, sex, and ambient temperature as independent variables. Besides confirming the above-described positive associations between prevalence of each parasite species and temperature, these analyses revealed that overall, SY birds had lower *H. fringillae* blood prevalence than ASY birds ($P=0{\cdot}002$; Fig. 2). Separate analyses of data obtained for male and female belonging to each age class revealed a lower prevalence of this parasite in SY than ASY females ($P=0{\cdot}006$; Fig. 2). The prevalence of *H. fringillae* in SY and ASY males also differed statistically ($P=0{\cdot}042$; Fig. 2), but this difference was not considered biologically meaningful because confidence intervals overlapped. Multiple logistic regressions found no age-associated difference in adult prevalence of *L. fringillinarum* or *T. avium*.

Adult blood immune cells and haematozoan prevalence

The abundance of blood heterophils and lymphocytes was higher in summer than in winter (heterophils: $F_{1,42}=37{\cdot}1$, $P<0{\cdot}001$; lymphocytes: $F_{1,42}=22{\cdot}9$, $P<0{\cdot}001$), but it was not sexually dimorphic and there was no season × sex interaction (Ps $>0{\cdot}1$; Fig. 3). The abundance of heterophils was positively related to that of lymphocytes in winter (Pearson product moment correlation coefficients: $r=0{\cdot}495$, $P=0{\cdot}01$) as well as summer (id.: $r=0{\cdot}835$, $P<0{\cdot}001$). As a result of this relation, H/L ratios did not differ between seasons or sexes and there was no season × sex interaction for this parameter (Ps $>0{\cdot}2$).

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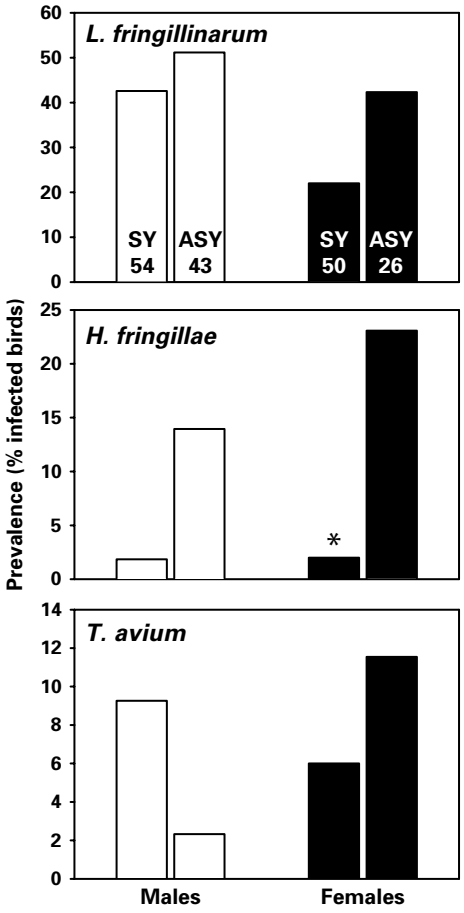

Fig. 2. Prevalence of (a) *Leucocytozoon fringillinarum*, (b) *Haemoproteus fringillae*, and (c) *Trypanosoma avium* in blood smears of second year (SY) and after second year (ASY) male and female White-winged Crossbills, *Loxia leucoptera*, sampled in 1999 at a single location in Fairbanks, Alaska. Numbers at the bottom of the upper panel columns indicate sample sizes. * Indicates a significant age difference in prevalence.

The low prevalence of blood parasites during winter (Fig. 1) did not warrant an analysis of relations between blood parasites and immune cell abundance during that season. In summer, the abundance of *L. fringillinarum* was positively correlated to that of heterophils ($n=20$; Pearson product moment correlation coefficient $=0{\cdot}53$, $P=0{\cdot}016$) but not lymphocytes (id., $P>0{\cdot}4$). By contrast, there was no relation between the prevalence of *T. avium* or *H. fringillae* and the abundance of immune cells. This abundance did not differ whether smears were positive or negative for either parasite species (Student's *t*-tests: $P>0{\cdot}4$).

Adult body size and condition

Males had a longer wing chord than females (males ($n=24$): $87\pm0{\cdot}3$ (S.E.) mm; females ($n=25$): $82\pm0{\cdot}4$ mm; $F_{1,45}=74{\cdot}5$, $P<0{\cdot}001$), but wing chord did not differ between winter and summer and there was no sex × season interaction for this variable (Ps $>0{\cdot}5$).

Males were on average heavier than females ($F_{1,45}=12$, $P=0{\cdot}001$) and birds were heavier in winter than summer ($F_{1,45}=6{\cdot}7$, $P=0{\cdot}013$; summer males: $27\pm0{\cdot}5$ (S.E.) g; winter males: $28\pm0{\cdot}3$ g; summer females: $25\pm0{\cdot}3$ g; winter females: $27\pm0{\cdot}7$ g). Crossbills were in better body condition in winter than summer (two-way analysis of variance of residuals of linear regression of wing chord over body mass: $F_{1,45}=7{\cdot}18$, $P=0{\cdot}01$), but body condition did not differ between sexes and there was no season × sex interaction for this variable (Ps $>0{\cdot}5$).

Haematozoan prevalence in hatch-year birds

Prevalence of *L. fringillinarum* and *H. fringillae* was lower in hatch-year birds than in adults (=AHY; Fisher exact probability tests: Ps $<0{\cdot}001$; no confidence interval overlap; Fig. 4). Prevalence of *T. avium* did not differ between the two age groups (Fisher exact probability test: $P=0{\cdot}09$).

DISCUSSION

The goals of the present study were to identify haematozoa in White-winged Crossbills, investigate seasonal and sex-and age-related changes in prevalence of these parasites, and identify factors that potentially account for these changes. Crossbills in the study population bred and males had high plasma T during winter and early spring (Deviche and Sharp, 2001). We hypothesized that if haematozoan prevalence increased at these times, this increase presumably resulted from re-emergence of parasites (i.e. increase in reproduction (*T. avium*) or relapse (*H. fringillae* and *L. fringillinarum*)) in the blood and not from new inoculations. Based on previous studies, we also hypothesized that this re-emergence may be related to the breeding effort and/or (in males) elevated plasma T. In late spring and summer, as ambient temperatures increased and putative vectors emerged, birds completed their breeding cycle and plasma T secretion in males had decreased to seasonally low to minimum levels (Deviche and Sharp, 2001; see Results section). At this time, an increase in haematozoan prevalence would presumably result from increased exposure to vectors causing new infections, as other avian studies suggest (see Introduction section). The findings are consistent with the conclusion that, depending on the time of year, seasonal changes in blood parasite prevalence in crossbills involve re-emergence of these parasites in the blood or new infections.

White-winged Crossbill blood samples contained 3 parasite species: *L. fringillinarum*, *T. avium*, and *H. fringillae*. This observation is consistent with a previous study identifying the same parasite genera in this species (Greiner *et al.* 1975). The ornithophilic vectors that transmit these parasite types include blackflies, *Simulium* spp. and biting midges,

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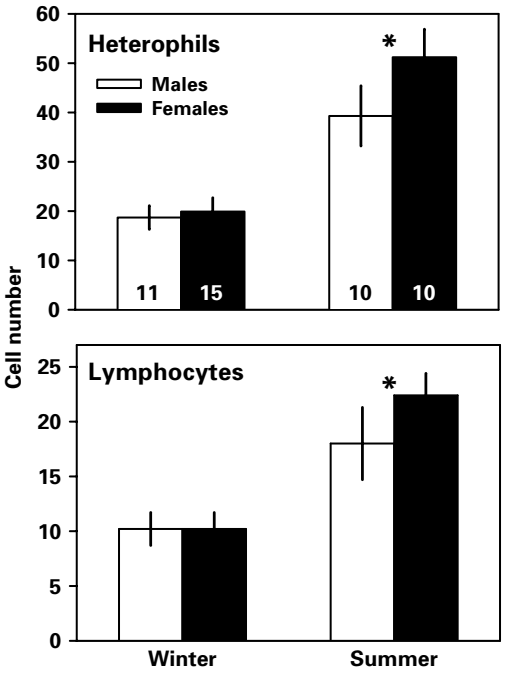

Fig. 3. Abundance (number of cells counted on non-overlapping microscope fields of blood smears studied at 400× magnification for 40 min; means ± S.E.) of heterophils and lymphocytes during winter and summer in adult male and female White-winged Crossbills, *Loxia leucoptera* sampled at a single location in Fairbanks, Alaska. Numbers at the bottom of the upper panel columns indicate sample sizes. * Denotes a seasonal difference ($P < 0{\cdot}05$, two-way analysis of variance).

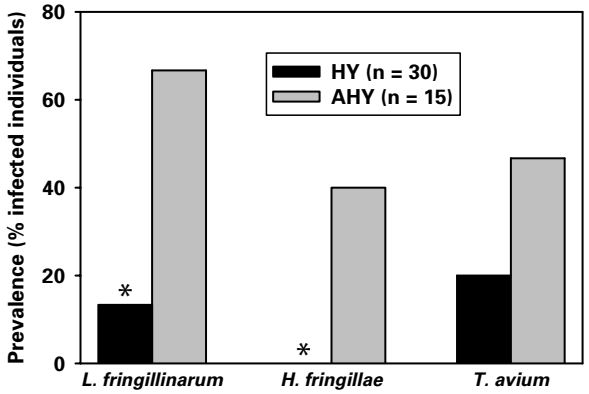

Fig. 4. Prevalence of *Leucocytozoon fringillinarum*, *Haemoproteus fringillae*, and *Trypanosoma avium* in blood smears of hatch-year (HY) and adult (AHY) White-winged Crossbills, *Loxia leucoptera*, sampled in 1998 and 1999 at a single location in Fairbanks, Alaska. * Denotes an age difference.

Culicoides spp. (Noblet *et al*. 1972; Yu *et al*. 2000; Steele and Noblet, 2001; Valkiunas and Iezhova, 2004; Votypka and Svobodova, 2004; Mullens *et al*. 2006; Hellgren *et al*. 2008), but no study has identified the specific vectors that transmit blood parasites to crossbills. Thus, it is not known whether associations between blood parasite species (*H. fringillae* and *T. avium*) or lack thereof (e.g. *H. fringillae* and *L. fringillinarum*) as observed in this study reflect aspects of the vector population dynamics or of the hosts' physiology or behaviour.

Some hatch-year crossbills were infected with *L. fringillinarum* and/or *T. avium*, demonstrating that these parasites can be transmitted to young (i.e. a few months old) birds. In adults, the prevalence of the 3 protozoan species changed seasonally in parallel with changes in ambient temperatures. Specifically, the prevalence of *L. fringillinarum* increased from 0% (January, average temperature below –20 °C) to 90% (males in May and June, average temperature approx. 10 °C). An increase in *L. fringillinarum* prevalence was noted as early as March (males: 18%) and April (females: 42%; Fig. 1B). The pre-patent period of *Leucocytozoon* sp. is thought to be 1–3 weeks (Kocan and Clark, 1966; Ahmed and Mohammed, 1978). If the same applies to crossbills and had the late winter increase in *L. fringillinarum* prevalence been caused by new inoculations, these inoculations would have taken place as ambient temperatures were consistently well below freezing. For example, the average temperature at the study site in mid-March was −12·8 °C. Such temperatures are generally considered to be incompatible with ornithophilic vector activity, suggesting that the late winter increase in *L. fringillinarum* prevalence resulted from relapse and not new inoculations. Consistent with this suggestion, birds can retain infections with *Leucocytozoon spp.* for at least 1 year after inoculation (Dick, 1978; Fallis *et al*. 1974). Previous research led to the proposal that a relapse may contribute to increased blood parasitaemia in other free-ranging birds (*Haemoproteus danilewsky* in Blue Jays, *Cyanocitta cristatamay*: Garvin and Schoech, 1996; *Leucocytozoon* spp. in Redstarts, *Phoenicurus phoenicurus*: Rintamaki *et al*. 1999), but the present work may provide the best evidence to date in support of this hypothesis.

As was the case for *L. fringillinarum*, the prevalence of *H. fringillae* and *T. avium* was nil to low during winter. Contrary to the situation for *L. fringillinarum*, however, the prevalence of these species did not increase until mid-May. Ambient temperatures at this time were above freezing and gradually increasing, and this was presumably associated with emergence of vectors (Werner, 1992). The degree of haemoparasitic infections in other avian species increases as a function of the vector abundance (Bennett *et al*. 1982; Godfrey *et al*. 1990; Merilä *et al*. 1995). These observations suggest that new inoculations contributed to the spring increase in prevalence of *H. fringillae* and *T. avium* seen in adult crossbills. Due to the absence of data on the identity of ornithophilic vectors in the study area and the phenology of vector emergence we can, however, not conclusively dissociate effects on *H. fringillae* and

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T. avium prevalence due to new inoculations from those potentially resulting from relapse (in the case of *H. fringillae*).

Control of late winter relapse of L. fringillinarum

What accounts for the putative late winter relapse of *L. fringillinarum* seen in crossbills? Based on previous work demonstrating correlations between blood parasitaemia and T and showing effects of this hormone on haematozoan infections (Introduction section), a first possibility is that T, which in male crossbills was seasonally elevated in late winter, stimulated a relapse of *L. fringillinarum*. Plasma T has to our knowledge not been measured in female crossbills but in other passerines, females during the breeding season generally have substantially lower plasma T than conspecific males (Introduction section). Considering all the adult samples, male crossbills had on average higher blood prevalence of *L. fringillinarum* than female crossbills. However, this was not always the case. In particular, *L. fringillinarum* prevalence was not sexually dimorphic in March and April, around the time that plasma T was elevated in males. Furthermore, the prevalence of *L. fringillinarum* was higher in males than females in late May and early June, when plasma T in males had decreased to baseline levels. Finally, there was no statistical correlation in males between plasma T and their prevalence of any parasite species. Collectively, these observations do not support the hypothesis that elevated plasma T caused a late winter relapse of *L. fringillinarum*. However, firm conclusions on this topic require additional studies examining whether reproductive hormones influence haematozoan infections in females and measuring blood *L. fringillinarum* prevalence as a function of time during which plasma T is elevated.

A second possibility is that breeding effort, independent of reproductive hormones, induced a relapse of *L. fringillinarum*. Hatch-year crossbills at the study site in 1999 were first caught in April, and by May these birds comprised 60% of all captures (Deviche and Sharp, 2001). Thus, crossbills bred successfully in the study area starting in winter and continuing in spring, at the same time that their blood *L. fringillinarum* prevalence increased. Previous passerine studies found a trade-off between reproductive investment and resistance to haemoparasitic infections, and the blood H/L ratio, which increases in response to an increase in breeding effort, is positively related to stress (Introduction section). This effort may, therefore, result in physiological stress that in turn causes a relapse of haematozoan infections during the breeding period. However, the present study provides no evidence to this effect: The blood abundance of heterophils and lymphocytes in adult crossbills increased between winter and summer, but H/L ratios did not change seasonally and, in addition, birds were heavier and in better body condition in winter than in summer. These observations do not support the hypothesis that crossbills experienced more stress during (winter) than at the end (summer) of their reproductive season and, therefore, that the putative late winter relapse of *L. fringillinarum* infection was stress- or breeding effort-related. Studies measuring additional stress-related parameters such as plasma corticosterone are warranted to test this conclusion. Furthermore, it is possible that only some winter-sampled crossbills in this study were actually breeding. If so, an effect of breeding effort on H/L ratios may have escaped detection due to the inclusion of non-breeding birds in the data sets.

Finally, exposure to increasing photoperiod, rather than breeding effort *per se*, may have stimulated a relapse of *L. fringillinarum*. Day length in the study area rapidly increases at the end of winter and during spring (Deviche and Sharp, 2001) and in the Blackcap, *Sylvia atricapilla*, transfer from a 12 h- to a 16 h-long day length increased the blood prevalence of *Haemoproteus belopolskyi* and *Trypanosoma* spp. even though birds were protected from vectors and did not breed (Valkiunas *et al.* 2004). The mechanism mediating this effect of day length is not identified. The same may occur in crossbills but as the blood of Blackcaps did not contain *Leucocytozoon* spp., it is unknown whether exposure to long days increases the patency also of this parasite.

Sex differences in blood parasite prevalence

Sex differences in avian haematozoan infections were reported in some previous studies (Red-winged Blackbird, *Agelaius phoeniceus*: Weatherhead and Bennett, 1991; Brown-headed Cowbird, *Molothrus ater*: Weatherhead and Bennett, 1992), but not in others (Norris *et al.* 1994; Rintamaki *et al.* 1999; Deviche *et al.* 2001; Hartup *et al.* 2008).

One factor that may contribute to differences between studies is that sex differences in haematozoan prevalence can be age- and parasite species-specific. Illustrating this observation, SY female crossbills had lower *H. fringillae* prevalence than older females. Furthermore, this difference was specific to *H. fringillae* and was not seen in males. A second factor is that sex differences in haematozoan infections can be seasonal. For example, adult male crossbills had higher *L. fringillinarum* prevalence than adult females (91% *vs* 39%) in late May and early June, but not at other times of the year.

What explains this season-dependent sex dimorphism in haematozoan infections? One hypothesis is that the sex difference in blood parasite prevalence results from reproductive hormone-mediated differences in physiological resistance to infections (Weatherhead and Bennett, 1991) or in behaviour. Testosterone generally circulates at higher

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concentrations in males than females and is immunosuppressive (Do Prado *et al*. 1998; de Souza *et al*. 2001; Deviche and Cortez, 2005). As a result, males during summer may be physiologically less resistant than females to the effects of new inoculations on their haematozoan prevalence. Consistent with this view, male rodents are often more susceptible to infectious agents than females (nematode, *Haemonchus contortus*, in sheep: Gauly *et al*. 2006; *Trypanosoma evansi* in rat: Al-Mohammed, 2006; *Plasmodium chabaudi* in House Mouse: Cernetich *et al*. 2006; *Trypanosoma cruzi* in House Mouse: Schuster and Schaub, 2001). Alternatively, T may influence haematozoan infections through behavioural changes. Testosterone promotes the propensity of birds to express male-typical behaviours, such as singing and territory defence (Van Duyse *et al*. 2002; Day *et al*. 2006; Strand *et al*. 2008), that presumably increase exposure to ornithophilic vectors and, therefore, the probability of new infections.

The present data do not exclude the possibility that male crossbills had higher prevalence of *L. fringillinarum* than females in summer due to a T-mediated sex difference in resistance to new infections or behaviour. However, the sex difference in *L. fringillinarum* prevalence was noted several months after plasma T in males had decreased to low levels. This finding does not support a short-term role for T in the control of the observed sex difference in *L. fringillinarum*, but males and females may have differed immunologically or behaviourally during summer due to factors other than their reproductive hormones.

Alternatively, sex differences in plumage brightness, rather than in immune function or behaviour, may have caused male crossbills to be more susceptible to attacks by *L. fringillinarum* vectors than females. Adult male crossbills generally have a bright red plumage whereas females have a drab green or yellowish plumage (Benkman, 1992; personal observation), and interspecific comparisons found a positive relation between plumage brightness and haemoparasitic infections (Hamilton and Zuk, 1982; Scheuerlein and Ricklefs, 2004). The factors that account for this relation, its generality, and whether it applies intraspecifically, remain debated. For example, Garvin and Remsen (1997) proposed that the reported association between plumage brightness and susceptibility to attacks by vectors reflects the fact that bird species living in canopies are, on average, more colourful than species living closer to the ground and some ornithophilic vectors are most common in tree canopies. Furthermore, a comparative study quantifying plumage colourfulness in *Aimophila* sparrows found no association between this parameter and blood parasite prevalence (Deviche *et al*. 2005) and intraspecifically, male plumage redness was independent of haemoparasite prevalence (Redpoll, *Carduelis flammea*: Seutin, 1994). Experimental studies examining the preference of vectors for specific plumage colours within avian species are warranted to test the hypothesis that these colours influence the vectors' choice of host.

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