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RESEARCH ARTICLE

Assessing the role of reproduction and stress in the spring emergence of haematozoan parasites in birds

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ABSTRACT

A spring emergence of avian haemosporidian infections is nearly universal among temperate zone birds and is often described as a cost of reproductive effort. We take advantage of the opportunistic (i.e. aseasonal) breeding schedule of the red crossbill (*Loxia curvirostra*) to determine the relative contributions of season versus host physiology to the timing and intensity of *Haemoproteus* infections in the temperate zone. Despite breeding activity in both the winter and summer, *Haemoproteus* infections were highly seasonal – occurring largely from May through September – and measures of host physiology (i.e. reproductive condition and stress parameters) did not explain parasite prevalence. However, within the spring–summer peak, infection intensity (i.e. parasite density) was positively correlated with plasma levels of testosterone and free corticosterone and negatively correlated with corticosterone binding globulin capacity. These data are discussed in terms of the behavioral ecology of host and vector, and suggest that both seasonal increases in vector activity and relapse of latent (i.e. dormant) infections contribute to the spring emergence in birds. Relapse of latent infections does not appear to be induced by reproductive activity or increased allostatic (i.e. energy) load, but rather by a season-specific change in host or parasite physiology (e.g. melatonin or endogenous rhythms).

KEY WORDS: *Haemoproteus*, Corticosterone, Testosterone, Corticosterone binding globulin, Relapse, Prevalence

INTRODUCTION

Parasites can be a potent selective force driving both the abundance and distribution of host species (Grenfell and Dobson, 1995). The fitness consequences of parasite infections are variable (Allander and Bennett, 1995; Dawson and Bortolotti, 2000; Grillo et al., 2012; Marzal et al., 2005; Siikamäki et al., 1997; Sol et al., 2003) and are probably dependent on the epizootiology of individual parasite species as well as the evolved relationship between each parasite–host species pair, individual host physiology and local environmental conditions (Atkinson and Van Riper, 1991). Avian haemosporidian infections peak during the spring and summer months in many temperate-zone hosts, when environmental conditions permit increased vector activity and hosts allocate large amounts of energy to reproduction. Proposed mechanisms for the seasonal peak in infections thus include changes in the behavior and physiology of the parasite, vector and host, but are difficult to tease apart given that many of these changes occur simultaneously in the temperate zone.

Arthropod vector life cycles are closely linked to temperature, thus seasonal fluctuations in temperate climates often preclude year-round transmission of haemosporidian parasites (Worms, 1972). In the interim, haemosporidian parasites enter a latent (dormant) phase, surviving in the tissues of avian hosts (Atkinson and Van Riper, 1991). Dormancy is thought to minimize clearance from the host and to seasonally reduce virulence, thereby enhancing overwinter survival of the parasite (Worms, 1972). Re-emergence of the parasite into the blood stream coincides with a peak in the probability of transmission (i.e. peak vector abundance) in many species (Atkinson and Van Riper, 1991). For example, most *Haemoproteus* spp. in the temperate zone show peak prevalence in the spring months when vectors (e.g. *Culicoides* spp.) become abundant. In contrast, infections of *Haemoproteus columbae* peak in the autumn, which mirrors a peak in the abundance of its vector species, *Pseudolynchia canriensis*, a hippoboscid fly (Klei and DeGiusti, 1975).

It remains unknown how haemosporidian parasites time seasonal emergence to coincide with seasonal peaks in vector abundance. It seems improbable that parasites detect and respond directly to environmental changes (temperature, photoperiod) given that they overwinter in the internal organs (e.g. liver, muscle) of endothermic hosts. It has been suggested that parasites may have an endogenous circannual clock to time re-emergence (Hawkins, 1975); however, relapse in birds held on constant photoperiod regimes often fails to show periodicity (Ahmed and Mohammed, 1978; Valkiŭnas et al., 2004). Alternatively, a seasonal change in host physiology could induce or facilitate re-emergence. The importance of host physiological changes remains an active source of debate and is the focus of this paper.

Avian physiology changes dramatically during the spring breeding season. Hormones mediate many of these processes, serving as broad-scale messengers that deliver signals generated in the brain to target cells throughout the body. Several hormone systems could function as cues for parasite emergence. Hormones involved in the hypothalamic-pituitary-gonadal (HPG) axis (e.g. gonadotropins, androgens, estrogens) mediate many of the cellular mechanisms involved in reproduction, such as gonadal growth, reproductive behaviors and allocation of resources to different processes (Wingfield and Farner, 1993). These hormones may, therefore, serve as cues for parasite emergence because reproduction in most birds is temporally coincident with high vector abundance in the spring. Testosterone has been shown to decrease immune function, thereby possibly enabling parasite re-emergence (Casto et al., 2001; Singh and Haldar, 2005) or increasing infection intensity (Deviche et al., 2006), though some studies have found no relationship between testosterone and timing of *Haemoproteus* emergence (Buttemer and Astheimer, 2000) or

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prevalence (Deviche et al., 2010). Glucocorticoids are also often elevated during early breeding (Romero, 2002) and may reflect high allostatic load or energy turnover (McEwen and Wingfield, 2003; Landys, 2006). Energy investment in migration and/or reproduction in the spring may also result in trade-offs that compromise immune function, thereby allowing for re-emergence of parasites (Atkinson and Van Riper, 1991; Norris et al., 1994; Allander, 1997). Indeed, seasonal fluctuations in immune function are evident and may be linked to the endocrine and nervous systems (Martin et al., 2008). In birds, corticosterone (CORT; the primary avian glucocorticoid) correlates with higher haemosporidian infection intensity (i.e. parasite density) in some species (Garvin and Schoech, 2006; Applegate and Beaudoin, 1970), and environmental stressors may induce relapse in others (Valkiŭnas et al., 2004). Finally, melatonin, an idoleamine hormone that is secreted primarily at night (Reiter, 1991), could offer a direct internal signal of daylength (i.e. short versus long pulses of production) and, thereby, a means for parasites to measure season. Changes in host physiology may therefore underlie seasonal variation in parasite prevalence and parasite density by triggering emergence of latent infections or altering host susceptibility. Further, a seasonal increase in vector abundance can contribute to seasonal peaks in parasite abundance and parasite density as uninfected juveniles and adults are exposed to and colonized by the parasite. Unfortunately, many of these factors occur in temporal coincidence during the spring breeding season, making causal relationships difficult to detect.

Crossbills (*Loxia* sp.) offer an interesting and unique model to disentangle the factors underlying the spring peak in avian haemosporidian infections because of their unusual breeding pattern. Crossbills breed opportunistically both during and outside (before and after) the typical spring relapse period (Newton, 1972; Benkman, 1990; Hahn, 1998; Hahn et al., 2008), offering a model to investigate (1) endocrine correlates of parasite infections independent of season and (2) temporal patterns of patent infections in adults relative to juveniles born in the winter (i.e. pre-vector emergence) and summer (i.e. post-vector emergence). Deviche et al. (Deviche et al., 2010) found support for a season-specific mechanism in white-winged crossbills (*Loxia leucoptera*), but the increasing daylength coincident with the early April breeding event in this study makes it difficult to separate effects of photoperiod from effects of reproductive activity. We build on these findings by sampling at multiple locations across multiple years, incorporating stress hormone and reproductive condition data and determining the temporal patterns of juvenile infections relative to adults in a close relative, the red crossbill (*Loxia curvirostra* Linnaeus 1758).

If blood parasites use cues generated by the host organism as it transitions into a breeding phase (e.g. responding to reproductive hormones or increased allostatic load associated with breeding), then we would expect to see a winter emergence of blood parasites in reproductively active crossbills and correlation with either reproductive or glucocorticoid hormones. If, however, parasites are using cues linked directly to photoperiod (e.g. melatonin) or if parasites are using an endogenous mechanism to time re-activation, then we would not expect a winter emergence.

RESULTS

Reproductive condition

The proportion of red crossbills in high reproductive condition varied seasonally, with high proportions of breeding red crossbills (i.e. >50% captures) occurring in the summer months of July, August and September and in January (contingency analysis; χ^2=167, d.f.=11, P<0.0001; Fig. 1A). As expected, testosterone levels predicted reproductive condition in males (high 1.8±0.18; low 1.2±0.16; $t_{1,96}$=2.46; P=0.01). Testosterone did not, however, correlate with any of the HPA-axis measures (i.e. baseline total CORT (P=0.16), baseline free CORT (P=0.42), induced CORT (P=0.81) or CORT binding globulin (CBG) capacity (P=0.19).

Infection status and parasite prevalence

Infection status was affected by region and month, which interacted significantly with age (Table 1, Fig. 1B). Infection status was not affected by sex, reproductive condition, fat deposit or body condition, or any of the interactions terms or hormone measures in our logistic model (Table 1).

Prevalence in adults increased significantly in May and remained at a higher level than winter months (except February) through October (contingency analysis; P<0.0001 followed by Mann–Whitney comparison of means with Holm–Bonferroni adjustments; Fig. 1B). In juveniles, prevalence did not rise from winter lows until June and remained elevated through November, with the exception of July and August, which did not differ from the winter months (contingency analysis; P=0.002 followed by Mann–Whitney comparison of means with Holm–Bonferroni adjustments; Fig. 1B). The first detection of

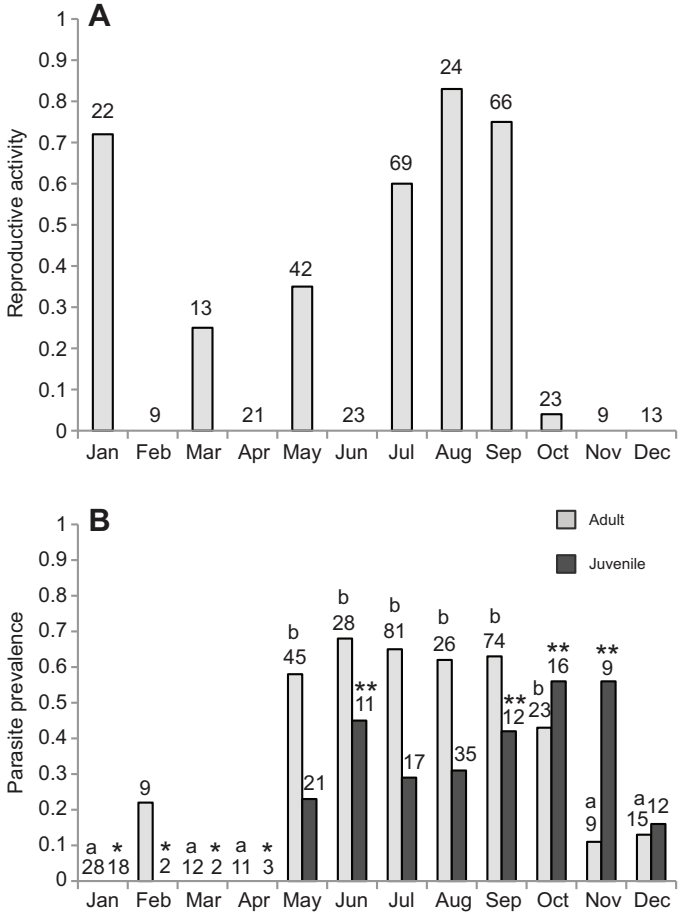

Fig. 1. Reproductive activity and prevalence of *Haemoproteus* infections in free-living red crossbills. Seasonal patterns of (A) reproductive activity (i.e. the proportion of adult red crossbills in high reproductive condition) and (B) parasite prevalence in free-living adult (light bars) and juvenile (dark bars) red crossbills. Sample sizes give number of individuals captured. Letters denote months that are significantly different in parasite prevalence in adults and asterisks denote months that are significantly different in parasite prevalence in juveniles (unlabeled months do not differ in parasite prevalence from any other month within the same age class).

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Table 1. Results of logistic regression model describing *Haemoproteus* infection status and general linear model describing *Haemoproteus* density in free-living red crossbills

	Infection status			Density		
	d.f.	*F* or *t*	*P*	d.f.	*F* or *t*	*P*
Full model	*N*=549			*N*=222		
Month	11	45.3	**<0.0001**	8	13.1	**<0.0001**
Region	4	23.0	**0.0001**	4	4.2	**0.003**
Age	1	0.0	0.99	1	0.17	0.68
Age × Month	10	24.0	**0.007**	–	–	–
Sex	1	0.1	0.73	1	14.2	**0.0002**
Sex × Month	10	10.3	0.41	–	–	–
Reproductive condition	1	0.0	0.99	1	2.52	0.11
Reproductive condition × Month	8	1.9	0.96	–	–	–
Reproductive condition × Sex	1	0.2	0.68	1	0.71	0.40
Fat	1	0.5	0.49	1	0.12	0.72
Body condition	1	0.5	0.47	1	3.2	0.08
CORT measures added	*N*=302			*N*=104		
Total baseline	1	0.4	0.51	1	0.32	0.74
Free baseline	1	0.7	0.69	1	2.55	**0.01**
Total induced	1	0.0	0.91	1	0.23	0.82
CBG	1	0.6	0.45	1	2.19	**0.03**
Testosterone added	*N*=132			*N*=52		
Testosterone	1	1.6	0.20	1	1.02	**0.02**

Bold indicates a statistically signficant contribution of the predictor variable to the model.

infection in the spring appeared earlier in adults (5 May, day-of-year 125) than in juveniles (23 May, day-of-year 143; Fig. 2), which probably contributed to the later increase in prevalence observed in juveniles (i.e. June in juveniles as compared with May in adults). Juveniles showed no infections in January through April, agreeing with the very low prevalence in adults during these months. Of 84 individuals captured between December and May, only two individuals had *Haemoproteus* present in the blood. These were both low-level infections [two and three *Haemoproteus* detected per 10,000 red blood cells (RBCs)]. Both infected birds were captured in February at different times of the same day (i.e. same capture location but probably different flocks) and were adult males in low reproductive condition and relatively poor body condition (i.e. both had negative body size versus mass residuals).

Parasite prevalence also varied among regions. The Olympic Peninsula region had a higher prevalence than other regions (Fig. 3).

Parasite density

Month, region and sex were related to parasite density (Table 1); however, age, reproductive condition, body condition, fat deposit and all interaction terms were not.

Among months containing active infections, parasite density was higher in July (4.1±0.2) and August (3.7±0.3) than in September (2.3±0.2) and October (1.9±0.3) (Tukey's test, $P<0.0001$). Males (3.3±0.1) had higher parasite density on average than did females (2.6±0.2) ($t_{1,218}=9.85$, $P=0.002$, $R^2=0.04$; Tukey's test, $P=0.008$). Parasite density varied by region ($F_{4,221}=9.5$, $P<0.0001$, $R^2=0.15$; Fig. 4): density in the Olympic Peninsula and Columbia River regions was higher than the Warner Mountain region (Tukey's test, $P<0.0001$) and the Teton Mountain region (Tukey's test, $P=0.006$ and 0.03, respectively). Captures in the Olympic Peninsula and Columbia River regions were almost exclusively Type 3 red crossbills (see Materials and methods for a description of red crossbill types), suggesting that type may contribute to regional differences in parasite density. The Teton Mountain region offers an opportunity to look at type differences in parasite density within the same capture region because 60% of all infected individuals captured there were Type 5, 30% were Type 4 and 10% were Type 3. There were no significant differences in parasite density between

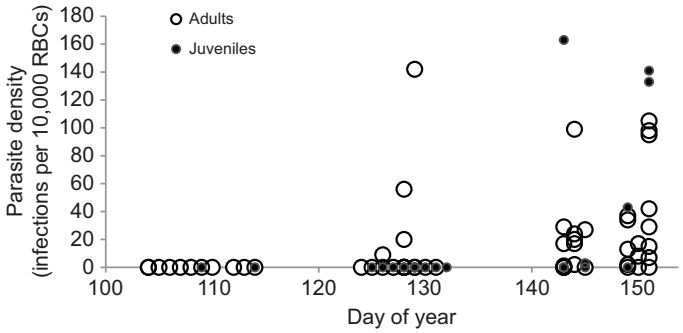

Fig. 2. Spring increase in parasite density in free-living red crossbills. Appearance of *Haemoproteus* parasites in red blood cells (RBCs) of adult red crossbills precedes first appearance in juveniles. Date is shown as day of year (represents 10 April–31 May).

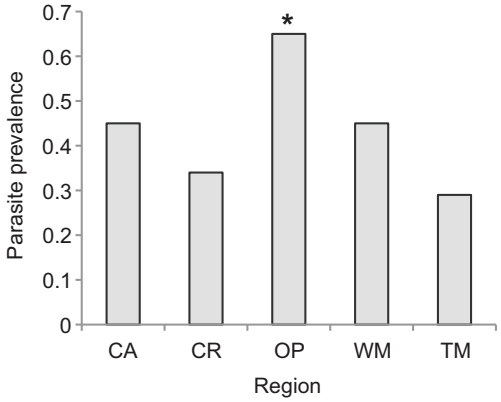

Fig. 3. Parasite prevalence in red crossbills by capture region. Prevalence is higher in the Olympic Peninsula (OP) region compared with the Northern California Coast (CA), Columbia River (CR), Teton Mountain (TM) or Warner Mountain (WM) regions.

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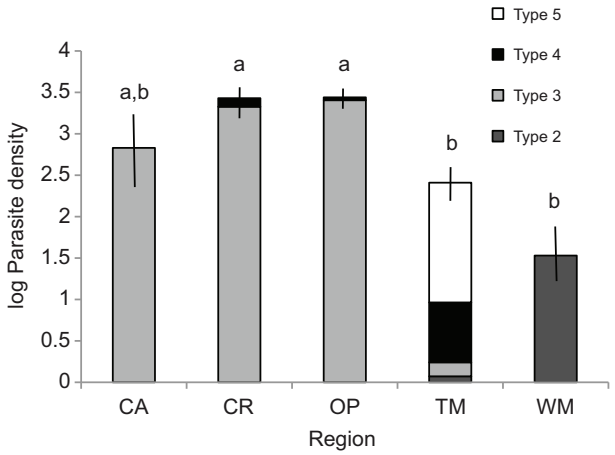

Fig. 4. Parasite density in red crossbills by capture region. Shading represents the proportion of eco-types caught at each location. Columns sharing letters are not significantly different. Error bars are ±s.e.m. Parasite load is significantly higher in the coastal regions [i.e. Columbia River (CR), Olympic Peninsula (OP)] than in the more interior Teton Mountain (TM) or Warner Mountain (WM) regions.

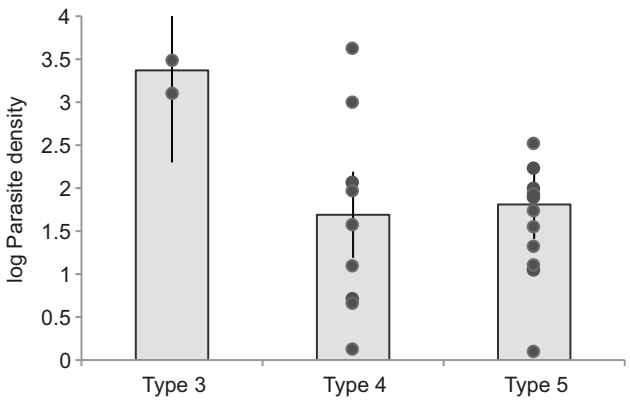

Fig. 5. Parasite density does not significantly vary by red crossbill eco-type within the Teton Mountain region. Circles represent individuals and columns represent group averages (±s.e.m.).

types (Fig. 5). However, sample size was small for Type 3 individuals (N=2), and these individuals had a parasite load typical of the Olympic Peninsula and Columbia River regions (i.e. similar to Type 3 individuals in other locations).

In our GLM including hormone data, CBG capacity, free baseline CORT and testosterone were associated with parasite density, whereas baseline total CORT and induced total CORT were not (Table 1). Parasite density covaried positively with testosterone ($F_{1,58}$=5.6, P=0.02, R^2=0.09; Fig. 6A) and CBG capacity ($F_{1,105}$=11.22, P=0.001, R^2=0.10; Fig. 6B) and negatively with free baseline CORT ($F_{1,103}$=12.9, P=0.0005, R^2=0.11). Parasite density was thus related to some hormone parameters whereas infection status was not.

DISCUSSION

We found no evidence for the hypothesis that reproductive activity or stress physiology drive the widespread spring emergence of haemosporidians observed in avian species, despite a relationship between some of these measures and the intensity of spring infection. Approximately 70% of the crossbills captured in January and 25% captured in March were in high reproductive condition, yet no acute phase infections were found during those months (Fig. 1). There was, therefore, no correlation between *Haemoproteus* prevalence and reproductive condition in red crossbills. Interestingly, the only two infections detected during winter months were both in males not in full breeding condition, again failing to lend support to the hypothesis that physiological changes associated with reproduction underlie relapses of chronic infections. Hormone levels also could not predict infection status: testosterone, total and free CORT and CBG all failed to correlate with infection status. This agrees with a study in white-winged crossbills where prevalence of one haemoprotozoan species (*Leucocytozoon fringillinarum*) was not linked to testosterone levels (Deviche et al., 2010). In total, these results suggest that reproductive and stress physiology are not responsible for triggering a relapse of infection in birds.

Instead, our data point towards a season-specific pattern underlying the haemosporidian spring emergence in birds. Prevalence in adult birds, whose infections could be the result of either new vector-borne infection or relapse, increased in May and peaked in June, when vectors are typically emerging in the temperate zone. Acute phase infections early in the spring may reflect relapse in adults that precedes vector emergence and new inoculation of winter-born juveniles (Fig. 2). In the Teton Mountains, average temperatures were still below 0°C in early May when prevalence in adults was already 24%. It is unlikely that these early spring infections were due to inoculation by *Haemoproteus* vectors (i.e. adult *Culicoides* spp.), given that *Culicoides* spp. are inactive at these temperatures (Fallis and Bennett, 1960; Purse et al., 2005; Tsutsui et al., 2011). Further, there were no infections detected in winter-born juveniles captured at this time (N=10), suggesting a lack of new inoculations in the population in early May. The spring peak in *Haemoproteus* spp. in wild populations is well documented (Dorney and Todd, 1960; Farmer, 1962; Khan and Fallis, 1969), and captive experiments excluding vector activity have revealed a seasonal relapse of latent infections that occurs between May and June (Allan and Mahrt, 1989). Interestingly, these cycles of recurrence showed periodicity in grouse (Allan and Mahrt, 1989) but not in pigeons (Ahmed and Mohammed, 1978), although the light cycle was not indicated in the latter study so it is unclear whether melatonin signals were different across these two studies. In the present study, uninfected adults captured in May were either not previously infected, were able to clear the infection prior to latency, or had latent infections that had not yet emerged. Our inability to distinguish among these different explanations may make it difficult to detect relationships between relapse and individual physiological parameters. This problem is absent, however, when considering parasite density because uninfected individuals were excluded from the analysis.

Infection intensity

While the spring emergence in infections was not generated by physiological changes associated with reproduction, physiological variation does appear to underlie individual variation in infection intensity (i.e. parasite density). Given the extensive literature on the immunosuppressive capacity of testosterone (Muehlenbein and Bribiescas, 2005; Roberts et al., 2004), it is not surprising that males with higher testosterone levels had higher parasite density (Fig. 6A). Interestingly, this effect may not relate directly to reproductive activity. Although males in high reproductive condition had higher testosterone levels on average than did males in low reproductive condition, there was no relationship between

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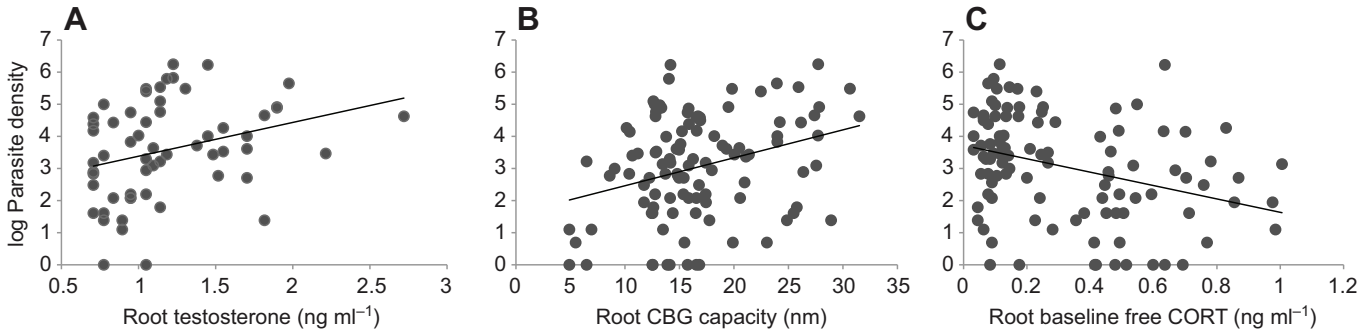

Fig. 6. Relationships between hormonal measures and infection intensity in red crossbills. Parasite density in red crossbills correlates positively with (A) testosterone (ng ml^{-1}) and (B) corticosterone binding globulin (CBG) capacity (nm) and negatively with (C) baseline free corticosterone (CORT) (ng ml^{-1}). Circles represent individuals. Testosterone data are from males only.

reproductive condition and parasite density. The inclusion of females in the metric for reproductive condition may mask an effect in males; however, there was also no reproductive condition × sex interaction effect on parasite density. This suggests that the effect may be generated directly through some mechanism of testosterone action rather than by an indirect effect of investment in reproduction. In support of this hypothesis, testosterone implants increased abundance of *Leucocytozoon fringillinarum* in dark-eyed juncos without influencing the secondary sexual characteristic of cloacal protuberance length (Deviche et al., 2006). Testosterone implants also reduced immune function in bush quail (Singh and Haldar, 2005) and dark-eyed juncos (Casto et al., 2001), but no correlation was found between plasma testosterone and *Haemoproteus* infections in blue jays (Garvin and Schoech, 2006) or white winged crossbills (Deviche et al., 2010). It is unclear whether these differences are due to differences in the behavior, ecology or physiology of the host, vector or parasite species. If testosterone directly generates higher infection intensities, then we would expect to see a sex bias in parasite density given the comparatively low testosterone levels in females. Indeed, adult males in this study and others (Deviche et al., 2010) had higher parasite density relative to females and younger males (Deviche et al., 2001), supporting a link between testosterone and infection intensity that may be relatively independent of reproductive investment.

It is possible that investment in reproduction does not affect immune function and, thereby, infection intensity unless an individual is struggling to meet immediate energy demands (i.e. high allostatic load relative to energy supply) (McEwen and Wingfield, 2003). Elevated baseline CORT levels are expected to reflect such a scenario, yet we detected no relationship between baseline total CORT and parasite density. This also suggests that high intensity infections are not activating an acute stress response, nor are they increasing the sensitivity of the HPA axis to capture and handling stress (i.e. there was no relationship between parasite density and induced CORT). Further, high intensity infections are not more common in individuals of low body condition. In combination, these data suggest that *Haemoproteus* infections are not increasing with allostatic load in red crossbills. Interestingly, however, CBG capacity did vary with parasite density: individuals with high parasite density had higher CBG capacity and, therefore, lower free CORT (Fig. 6B). CBG is thought to have evolved with immune function capacities (Wilckens, 1995), thus it is possible that a positive correlation with parasite density reflects the upregulation of CBG capacity in response to infection intensity. CBG is recruited to sites of inflammation where it is cleaved, releasing CORT to act locally to reduce inflammation and prevent over reaction of the immune response (Pemberton et al., 1988). Haemosporidian infections are known to induce strong inflammatory responses in birds (Atkinson and Van Riper, 1991; (Atkinson et al., 1986; Desser, 1967). It is therefore possible that higher CBG capacity in infected birds simultaneously decreases free CORT levels in the blood plasma but increases local levels at CBG recruitment sites (Pemberton et al., 1988). Alternatively, testosterone is known to increase CBG capacity in some birds (Deviche et al., 2001; Zysling et al., 2006), suggesting that testosterone may drive increases in both CBG capacity and parasite density. In this study, testosterone and CBG capacity did not correlate, although complex interactions can obscure such mechanisms.

Age-specific parasite prevalence

Temporal patterns of parasite prevalence in juvenile red crossbills differed from those found in adults, suggesting that the factors underlying the spring prevalence peak may vary between these two groups. Juveniles in passerine species typically show a peak in haemosporidian prevalence that is later than in adults (Deviche et al., 2001). This is not surprising given that juveniles are typically born after vectors have emerged and it takes some time before new infections are detectable in the blood. Prevalence in juvenile crossbills in the present study increased later than in adults (i.e. June rather than May) and decreased later as well (i.e. November rather than October), supporting the hypothesis that peak haemosporidian prevalence occurs later in juveniles. Crossbills are distinct, however, in that there are two major breeding seasons and, hence, two infusions of juveniles into the population: winter/spring and summer (Hahn et al., 2008). These seasons are separated by a nomadic migration in early summer on one end and the autumn molt on the other (Cornelius and Hahn, 2012; Cornelius et al., 2011). Bimodal patterns of juvenile recruitment predicts a bimodal distribution in parasite prevalence of juveniles assuming equal inoculation rates: prevalence would increase as winter-born juveniles are inoculated in the spring, decline briefly in the summer as new (and therefore uninfected) juveniles flood the population, and then peak a second time in the late summer/early autumn as vectors inoculate summer-born juveniles. Although sample sizes do not allow us to draw strong conclusions, our data are consistent with such a bimodal distribution given that prevalence in July and August is not significantly different from that in winter months. A combination of vector activity and fluctuation in population recruitment may, therefore, drive the age-specific patterns in parasite prevalence, and deserves further study.

Regional variation in parasite density

Culicoides (a *Haemoproteus* vector) density is typically higher in wetter and warmer areas (Kettle, 1956). This suggests that the higher parasite density observed in birds inhabiting coastal regions (Fig. 4) may be due to increased vector abundance, resulting in increased exposure to and infection with haemosporidians. Alternatively, regional differences may be generated by differences in the physiology or behavior of the most prevalent crossbill types (e.g. captures in the coastal regions were almost entirely Type 3 individuals). A type-specific analysis within the Teton Mountain region revealed no statistical differences in parasite density between different types (Fig. 5), lending support to the hypothesis that geographical conditions contribute to differences in infection intensity. Interestingly, however, the few samples collected from infected Type 3 individuals in the Teton Mountain region had levels similar to those from the coastal regions, suggesting that some aspect specific to Type 3 behavior or physiology contributes to regional differences or that they were inoculated in a coastal region prior to migration.

Conclusions

In summary, we found strong seasonality in parasite prevalence; however, we found no evidence linking the timing of the spring emergence in haemosporidian infections in red crossbills to reproductive or stress physiology. Infections appeared earlier in adults relative to juveniles, suggesting that the spring peak reflects a relapse of chronic infections in addition to new vector-borne infections. Infection intensity, however, was affected by both season and individual physiology: parasite density correlated positively with testosterone, CBG and free CORT. The positive correlation with testosterone but lack of correlation with reproductive condition suggests a direct mechanistic link that may be dissociated from reproductive activity or investment. The lack of relationship between parasite load and body condition, total baseline CORT or induced CORT levels suggests that infections are not affecting allostatic load in red crossbills. Instead, CBG may directly influence free CORT if binding capacity is increased during the inflammatory response to infection. Together, these data suggest that *Haemoproteus* parasites may not be particularly costly for their red crossbill hosts. While aspects of reproductive and stress physiology do impact the severity of acute infections, they do not appear to underlie the spring shift from latent to active infections observed across avian species. Instead, we suggest that more direct signals of season (e.g. melatonin) or some calendar intrinsic to the parasite may play important roles in the emergence of latent infections.

MATERIALS AND METHODS

We captured 549 free-living red crossbills with mist nets in Washington, Oregon, Wyoming and California from 2003 to 2012. No individuals were re-captured. Capture sites were in five distinct geographic regions: Olympic Peninsula (OP; 47°N, 123°W), Columbia River (CR; 46°N, 123°W), Northern California Coast (CA; 41°N, 124°W), Warner Mountains (WM; 42°N, 120°W) and Teton Mountains (TM; 43°N, 110°W). There are at least 10 vocal 'types' of red crossbills in North America, in four general size classes (Benkman, 1993; Groth, 1988; Groth, 1993; Irwin, 2010). Variation in body size and bill morphology reflects adaptation to feeding on different conifer species (Benkman, 1993; Benkman, 2003) and geographic distributions of different types vary accordingly (Benkman, 2003; Groth, 1988; Groth, 1993). In this study, we captured Type 2, 3, 4 and 5 red crossbills. Crossbill types were not randomly distributed with respect to capture regions (χ^2=465, P<0.0001, R^2=0.71); we used region in our main analyses rather than type and performed a separate analysis on type where appropriate (see below) because mode of transmission for infection is probably not type-specific.

Crossbills were removed from mist nets immediately upon capture for blood collection. A 26 gauge needle was used to collect a 10 µl sample from the alar vein for detection of blood parasites (N=527) and, for those individuals sampled within 3 min of capture, a 60 µl sample was collected for baseline hormone levels (CORT N=302 and testosterone N=132). Testosterone levels were only measured in male crossbills. Birds sampled for CORT were subjected to a standard handling stress protocol, where additional 70 µl samples were collected at 10, 30 and 60 min. 'Induced CORT' was determined for each individual as the highest CORT level measured across this time series during the handling stress protocol. CORT exists in the plasma either in a free state or bound to CBG. The binding capacity of CBG can change during a handling stress protocol and is known to decline in red crossbills after 30 min of handling stress (Breuner et al., 2006). The physiological role of CBG is not yet certain; however, it is suspected to regulate the amount of hormone available to tissues (Breuner and Orchinik, 2002). We determined binding capacity for CBG in baseline blood samples but not for induced samples. We therefore report 'total baseline' (e.g. the total concentration of CORT detected in the baseline sample) and 'free baseline' (e.g. the concentration of CORT that is unbound, or 'free' of CBG in the baseline sample), as well as induced total CORT. Although these measures cannot be treated as independent, total and free CORT levels may have different physiological actions and are, therefore, both reported in this paper with appropriate statistical considerations.

Age and sex were determined using plumage and skull characteristics. Juveniles were defined by streaky brown plumage or by incomplete skull pneumatization and adults were defined by adult plumage and complete skull pneumatization. Birds were weighed to the nearest 0.5 g with a 50 g Pesola spring scale. Subcutaneous fat was scored on an arbitrary scale from 0.0 (no fat evident) to 5.0 (gross bulging fat deposits) as described previously (Hahn, 1998; Helms and Drury, 1960; Nolan and Ketterson, 1983). Furcular and abdominal regions were scored separately and summed for analysis. Length (mm) of tarsus, wing chord and keel were taken as measures of body size. PC1 of a principal components analysis of these three measures described 76% of the variation and was used as a measure of body size (Y_1=0.61×Wing+0.57×Tarsus+0.55×Keel). To estimate body condition, we saved the residuals from the best-fit regression of PC1 and body mass. Although body mass residuals and fat score positively covaried in this study (P<0.0001), the fit was not strong (R^2=0.06). This is probably due to mass of other tissue (e.g. muscle), which may change independently of fat. We use size-corrected body mass residuals as well as fat scores as two measures of body condition.

Cloacal protuberance (CP) length in males and brood patch (BP) condition in females can be used to assess reproductive condition in passerines (Bailey, 1952; Wolfson, 1952). We used these measures to assign individuals to broad categories of high or low reproductive condition, as described previously (Cornelius et al., 2012). BPs are used by female red crossbills to incubate eggs and young nestlings and are scored on a scale of 0 to 4 (see description below); however, the most useful information for predicting ovary condition is the distinction between a BP score of 0 and one greater than 0. Females in this study were therefore given a reproductive condition that was high if BP>0 or low if BP=0. BP stage was scored as 0 if the breast was dry and fully feathered and was scored as >0 if there was feather loss, vascularization or edema [see Cornelius et al. (Cornelius et al., 2012) for more detailed descriptions].

CP length is dependent on testosterone and varies with reproductive condition in male red crossbills (Cornelius et al., 2012). Despite size differences in red crossbill types, type does not affect the relationship between CP length and testis size in red crossbills (T.P.H., unpublished data). CP length was used to assign each male a breeding condition: males with a CP of 5 mm or longer were assigned a high reproductive condition and males with a CP less than 4 mm were assigned a low reproductive condition. Males with a CP between 4 and 5 mm (i.e. medium reproductive condition) were not included in statistical analyses to remain consistent in categorization across sexes (i.e. no medium reproductive condition in females) and to reduce ambiguity in data for males (Cornelius et al., 2012), although inclusion does not change the reported patterns. CP length was

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measured with dial calipers from the point of emergence from the abdomen to the tip of the fleshy portion.

Blood parasite scoring

Immediately following the baseline blood collection, a drop of blood was placed on a glass slide and a thin smear was made. Blood smears were then allowed to air dry before being fixed with 100% methanol and stored for later staining with a Hema 3 manual staining system (Fisher Scientific, Pittsburgh, PA, USA). Smears were scored at 1000× under oil immersion. Approximately 10,000 erythrocytes were examined for each smear and the number of cells infected with *Plasmodium* spp. or *Haemoproteus* spp. was counted; parasites were identified using morphological characteristics. Of 549 red crossbills, seven were infected with *Plasmodium* spp. and 226 were infected with *Haemoproteus* spp. Because *Plasmodium* infection was relatively rare, we focused all further analyses on *Haemoproteus* infections. Fields scored for parasites were evenly distributed over the entire surface of the smear to avoid oversampling a single area.

CORT and CBG assays

An enzyme immunoassay kit from Enzo Life Sciences (cat. no. ADI-901-097, Farmingdale, NY, USA) was used to measure CORT in red crossbills as described previously (Cornelius et al., 2010). Plasma dilution and steroid displacement buffer (SDB) concentrations were optimized for red crossbills at 1:40 dilution and 1% (per raw plasma volume) SDB. Samples were run in duplicate and randomized across 32 plates, each with a separate standard curve and hormone standard. Inter-plate variation was 12%, intra-assay variation was 7.3% and minimum detectability was 1.9 ng ml^{-1} (range: 0.6–4 ng ml^{-1}). Detectability was determined separately for each plate as two standard deviations from the mean of blank wells. Values that were below the limit of detection for each plate were assigned the minimum sensitivity of that plate.

CBG capacity was measured using a tritiated CORT ligand-binding assay as described in Breuner and Orchinik (Breuner and Orchinik, 2002) and Lynn et al. (Lynn et al., 2003). Pooled plasma from free-living red crossbills was used to optimize specific binding at 2 h of incubation, 4°C and 1:900 plasma dilution. All samples were run in triplicate and free radioligand was separated from bound by rapid vacuum filtration (Brandel Harvester, Gaithersburg, MD, USA) over glass fiber filters (Whatman GF/B) soaked in 25 nmol l^{-1} Tris and 0.3% polyethyleneimine for 1 h prior to filtration. Radioactivity was measured using standard liquid scintillation spectroscopy. Specific binding was determined using pooled plasma incubated with 0.9 to 12 nmol l^{-1} ^{3}H-CORT. The affinity (K_d) estimate (i.e. how strongly CORT binds to CBG) for red crossbills was 2.27±0.17 nmol l^{-1} (Cornelius et al., 2011). Individual samples were incubated with 20.4 nmol l^{-1} ^{3}H-CORT, which should occupy ~90% of the total binding sites (B_{max}). Thus capacity values were adjusted to 100% for free CORT calculations. All point samples were run in a single assay on 33 filters. Inter-filter variation was determined using plasma standards and was 14%. Within-triplicate variation was less than 7%. Free CORT concentrations were estimated using the equation of Barsano and Baumann (Barsano and Baumann, 1989) as in Lynn et al. (Lynn et al., 2003).

Testosterone assay

An enzyme immunoassay kit from Enzo Life Sciences (cat. no. ADI-901-065) was used to measure testosterone. Plasma dilution and SDB concentrations were optimized for red crossbills at 1:20 dilution and 1% (per raw plasma volume) SDB. Samples were run in duplicate and randomized across five plates, each with a separate standard curve and hormone standard. Inter-plate variation was 6.9%, intra-assay variation was 9.6% and detectability was 0.4 ng ml^{-1}. Detectability was determined separately for each plate as two standard deviations from the mean of blank wells. Values that were below the limit of detection for each plate were assigned the minimum sensitivity of that plate.

Statistical analysis

We describe infections in two ways: parasite density [i.e. the number of parasites detected per 10,000 RBCs (Bush et al., 1997)] and individual infection status (i.e. infected or uninfected). Individual infection status allows for evaluation of factors affecting likelihood of infection between individuals, and is further used to determine prevalence (i.e. the proportion of the population that is infected at a given time), allowing for the evaluation of population-level patterns of parasite emergence across time (Bush et al., 1997). Examining parasite density allows for evaluation of factors affecting intensity of infection between individuals. All statistics were performed using JMP® 8.0.2 (SAS Institute Inc., Cary, NC, USA) and R (R Development Core Team, 2010). Means ± s.e.m. are reported.

Infection status is a binary variable, thus we used a logistic regression model to determine significant contributors. Hormone data were collected from a subset (CORT and CBG: N=302; testosterone: N=132) of the total data set (N=549) and were square root transformed. To preserve predictive power, we ran our model using month, region, age, sex, reproductive condition, body condition, fat deposit and all biologically relevant interactions as predictor variables with the larger dataset first. Year may be an important predictor of parasite abundance or density. Given our unbalanced sampling between months in each year, we tested year as a random intercept in our model. This initial test was used to decide whether either generalized linear mixed models (GLMMs) or generalized linear models (GLMs) were most appropriate to model our data (Bolker et al., 2009). To do so, we fit a saturated model with the same fixed-effect structure, using the lme4 package for GLMM including the random intercept term for year and ordinary logistic regression excluding this random effect, i.e. the GLM model was nested within the GLMM model. We then used a likelihood ratio test (LRT) to investigate whether the inclusion of a random intercept was warranted. The LRT suggested that the inclusion of the random intercept did not significantly improve the model (χ^2=3.2, d.f.=1, P=0.07), which prompted the use of a GLM for the remainder of the analysis.

After removing non-significant predictor variables, we ran a second iteration with the addition of HPA-axis measures (i.e. free and total CORT measures and CBG capacity) to determine whether infection status varies with stress physiology. Finally, we ran a third iteration that further restricted the analysis to the subset of males for which we took testosterone samples (N=132). Significant effect variables were evaluated *post hoc* using contingency analysis (chi-square) and the relationships between categorical groups were further assessed using Mann–Whitney U-tests. Alpha levels were adjusted using Holm–Bonferroni corrections for multiple comparisons given that CORT measures are not independent of each other (Holm, 1979).

Lack of infection in an individual can be due to some aspect of physiology (i.e. the individual was able to clear an infection), lack of exposure to the parasite, or our inability to detect particularly low-level or dormant infections. The inability to distinguish between these scenarios may confound relationships between our predictor variables and parasite density. Therefore, we restricted our analysis of parasite density to infected individuals only. Parasite density varied from 1 to 990 *Heamoproteus* per 10,000 RBCs (mean=27, s.d.=76) and was log transformed to reduce heteroscedasticity in the data.

As described above for infection status, we used an LRT to investigate whether the inclusion of a random intercept for year was warranted. The LRT strongly suggested that the inclusion of the random intercept did not improve the model (χ^2=0, d.f.=1, P=1.0), which again prompted the use of a GLM for the remainder of the analysis. Sample sizes were smaller for the model with parasite density as response (N=222), precluding inclusion of multiple interaction effects. To determine which biologically significant interactions should be included in the full GLM, we first ran independent GLMs for each interaction term. Reproductive condition × sex was the only significant interaction in these reduced, interaction-specific models, and was therefore included in the full model (F_3=2.42, P=0.02). We used a GLM to determine whether month, region, age, sex, fat, body condition, reproductive condition or reproductive condition × sex interaction affected parasite load. As described above, we added hormone measures in a stepwise manner after removing non-significant predictor variables from the full model. For these analyses, there were 222 infected individuals included in the first model, 104 infected individuals included in the HPA model (i.e. baseline total CORT, baseline free CORT, induced total CORT, and CBG capacity) and 52 infected individuals included in the HPG model (testosterone). Model fit (R^2) was assessed as the coefficient of determination between the actual and predicted response. *Post hoc* tests were Holm–Bonferroni adjusted.

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Competing interests
The authors declare no competing financial interests.

Author contributions
J.M.C. performed all of the fieldwork and hormone assays, contributed to statistical analyses, and wrote/edited the manuscript. M.Z. prepped and scored blood smears for parasites and contributed substantially to writing/editing of the manuscript. C.W.B. provided resources, partial funding and laboratory space for hormone assays and participated in editing of the manuscript. A.C.G. performed statistical analyses and participated in editing of the manuscript. T.P.H. provided funding for all aspects of the study, contributed field work and participated in writing/editing of the manuscript.

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