Consequences of nest reuse for parasite burden and female health and condition in blue tits, *Cyanistes caeruleus*

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Abstract

Research on the costs of nest reuse is central to understanding the population ecology and evolution of cavity nesting birds. We explored the consequences of nest reuse by offering blue tits three different types of nest-boxes to breed: nest-boxes with an old nest (O), empty nest-boxes (E) and nest-boxes with an old nest fumigated with insecticide (F). The experimental groups differed in ectoparasitism level; the O nest-boxes held the highest and the F nest-boxes the lowest level of ectoparasitism. Our results show that blue tits reusing cavities containing old nests from the previous season pay a cost caused by the presence of nest ectoparasites. This cost was expressed in terms of reduced reproductive success and female body mass at the end of the nestling period. Interestingly, an additional cost arose as haematozoan infections in females increased with level of ectoparasitism. The costs of nest reuse would be reduced in areas and / or seasons with low incidence of ectoparasitism, as shown by the results from the group with fumigated old nests.

For cavity nesting passerines, nest site availability is one of the main factors constraining reproduction (Newton 1994), and due to scarcity of appropriate cavities birds often reuse those that have been occupied in the previous season, incurring in costs caused by the presence of ectoparasites (Loye & Carroll 1998). Research on the costs of nest reuse is central to understanding the population ecology and evolution of cavity nesting birds (Møller & Erritzøe 1996; Aitken et al. 2002). Parasites represent an important selective force for animals in the wild (Loye & Zuk 1991; Clayton & Moore 1997, and references therein). Consequently, ecologists in the last decades have paid more attention to the direct and indirect pressures that parasites may exert on the life histories of their hosts (Møller 1997). In this respect, bird-ectoparasite associations are one of the most fruitful model systems, having provided many influential examples of parasite-mediated evolution and ecology (Møller et al. 1990; Møller 1991a; Richner 1998; Proctor & Owens 2000). Various arthropods, including fleas, adult and larval dipterans, mites and ticks feed on the blood of adult and nestling birds while on their nests (e.g. Møller et al. 1990; Loye & Zuk 1991 and references therein). However, most studies have been performed on model systems in which a single (less frequently two) ectoparasite species is involved (see Martin et al. 2001). In addition, most of these studies have been carried out on colonial birds, and available information about the effects of ectoparasites on solitary nesters is comparatively scarce (Loye & Carroll 1998; Merino et al. 2001; see Fessl et al. 2006).

Many observational and experimental studies have demonstrated that ectoparasites can have severe negative impacts on host condition, reproductive performance and survival (reviewed in Loye & Zuk 1991; Lehmann 1993; Clayton & Moore 1997). Besides the relatively well documented direct effects on their hosts, haematophagous ectoparasites can transmit microparasites (e.g. protozoa, virus,

bacteria) among birds (Macfie & Thomson 1929; Price 1980; Marshall 1981). However, little is known about the realized role of nest ectoparasites as vectors of disease in wild avian populations (Allander & Bennett 1994; Proctor & Owens 2000; Votýpka et al. 2002). This remains as one of the less explored potential effects of ectoparasitism to date, despite the known pathogenic consequences of vector-transmitted endoparasites in wild birds (e.g. Merino et al. 2000). Thus, more information is clearly needed to fully understand its ecological, behavioural and evolutionary implications in the context of host-parasite interactions.

In the present experimental study, we explored the consequences of nest reuse by offering blue tits (*Cyanistes caeruleus*) three different types of nest-boxes to breed: i) Nest-boxes with an old nest that simulate the natural situation in which cavities are reused during several years (natural levels of ectoparasitism); ii) Emptied nest-boxes reflecting the common practice of nest-box studies in which the nest material is removed after each breeding season, hence eliminating most ectoparasites (Møller 1989); iii) Nest-boxes with an old nest treated with insecticide that simulate the natural situation but without ectoparasites.

We predict that nest-boxes with old nests should present a higher number of ectoparasites than empty nest-boxes, as part of their populations might overwinter in the nest material waiting for new hosts in the following season (Burtt et al. 1991; Harper et al. 1992; Møller 1992; but see Mappes et al. 1994). Obviously, fumigated nest-boxes should hold the lowest ectoparasite loads. Optimal clutch size in the presence of ectoparasitism has been suggested to vary depending on whether ectoparasites have short or long life-cycles (see Richner & Heeb 1995 and references therein). However, since our blue tit population suffers from infestation by both long-cycled (fleas and blowflies) and short-cycled ectoparasites (mites), we have no a priori prediction on how

clutch size should differ among experimental groups. In addition, we predict: 1) Delay of laying in more infested nests (e.g. Oppliger et al. 1994; but see Møller 1990), 2) detrimental effects of ectoparasites on hatching success (e.g. Møller et al. 1990; Oppliger et al. 1994; but see Richner et al. 1993), reproductive success (e.g. Møller 1990; Fitze et al. 2004), female body mass at the end of the nestling period (e.g. Christe et al. 2002; but see Tripet & Richner 1997a) and nestling body size and mass (e.g. Merino & Potti 1995a; Hurtrez-Boussès et al. 1998), and 3) as blood parasites are often transmitted by ectoparasites (Price 1980; Marshall 1981), we predict that female blood parasite intensities (females spend prolonged periods in the nest while incubating and brooding) and prevalences should be higher as ectoparasitism increases (Møller 1989). Intriguingly, this last prediction has seldom been tested in the study of host-ectoparasite interactions at the among-nest scale (but see Christe et al. 2002).

MATERIALS AND METHODS

Study Area and Species

The study was carried out during the breeding season of 2002 in a deciduous oak (*Quercus pyrenaica*) forest near La Granja, Segovia province, central Spain (40º53'N, 4º01'W, 1200 m a.s.l.). Nest-boxes in the study area have been available for hole-nesting passerines since 1991 (e.g. Sanz 2002). Old nests are yearly removed after the breeding season, except occasionally. The blue tit is a small (10 – 12 g) hole-nesting passerine quite common in mixed and deciduous forests throughout the Western Palaearctic region (Cramp & Perrins 1998) that readily accepts nest-boxes when provided. Egg laying in the population under study typically begins in early May, females laying a single clutch per year averaging 9.14 eggs (Sanz 2002).

90 The hen flea *Ceratophyllus gallinae* (Siphonaptera: Ceratophyllidae) is a
91 common ectoparasite of birds particularly abundant in nests of tits (Paridae) species, the
92 blue tit being its main host (Harper et al. 1992; Tripet & Richner 1997b). Adult hen
93 fleas use adult birds and nestlings for regular blood meals and often complete one or
94 two generations within the nestling period of the host (Tripet & Richner 1999a, b).
95 From Berlese counts (see below) we also distinguished larval stages, which grow in the
96 nest material and feed on detritus and blood faeces excreted by adult fleas (e.g. Lehane
97 1991).
98 Larvae of the blowfly *Protocalliphora azurea* (Diptera: Calliphoridae) are
99 common ectoparasites of Holarctic birds (Bennett & Whitworth 1992). Adults are free-
100 living flies that lay their eggs in the nest material when nestlings are one-quarter to one-
101 third grown (Bennett & Whitworth 1991). The three larval stages dwell in the nest and
102 feed on chicks by sucking their blood, completing a single generation within the
103 nestling period.
104 The nest-dwelling mite *Dermanyssus gallinoides* (Acari: Dermanyssidae) is a
105 fast-moving mite in which both adult and some nymphal stages are haematophagous.
106 This mite has short generation times and can readily build up huge populations (Richner
107 & Heeb 1995; Proctor & Owens 2000).
108 *Haemoproteus majoris* (Haemosporida: Haemoproteidae) is an intracellular
109 blood parasite which infects ectoparasitic arthropods as vectors and birds (among them
110 Paridae) as vertebrate hosts (Peirce 1981). The period between initial infection and
111 release of gametocytes into the blood is ca. 12-13 days (Fallis & Bennett 1961; authors'
112 unpublished data).
113 *Leucocytozoon majoris* (Haemosporida: Leucocytozoidae) is an intracellular
114 blood parasite widespread in a great number of passerines from almost all geographic

115 zones (Valkiūnas 2005). The prepatency period for this parasite is 5-6 days (Desser &
116 Bennett 1993).
117 *Trypanosoma avium* (Kinetoplastida: Trypanosomatidae) is a flagellated
118 extracellular parasite that divides and circulates into the bloodstream, with a prepatency
119 period of 24-48 h (Bennett 1970). It is transmitted by various blood-sucking
120 invertebrates and is a widespread parasite currently considered as a species complex
121 (Votýpka et al. 2002).
122

123 **Experimental Protocol**

124 A total of 99 nest-boxes arranged in a grid with ca. 25 m between adjacent boxes
125 were assigned alternatively to one of the following treatments (33 nest-boxes each) two
126 weeks before the onset of blue tit reproduction: i) Empty nest-boxes (E), where all the
127 old nesting material and debris was carefully removed; ii) Nest-boxes with an old tit
128 nest (O), where tit nests from an adjacent area that were not removed in the previous
129 breeding season were translocated in sealed plastic bags; iii) Nest-boxes with fumigated
130 nests (F), where old tit nests from the previous season as in the O-group were employed,
131 but an insecticide solution (Stockade ©, Fort Dodge Veterinaria, S.A., Vall de Bianya,
132 Girona, Spain) comprising 0.5 % Permethrin and 1 % Piperonil butoxide was applied
133 during transport in bags (about 15 min). Permethrin is a synthetic pyrethroid similar to
134 the natural insecticide pyrethrum which comes from the chrysanthemum plant, but it
135 remains effective for longer time periods, whereas piperonyl butoxide acts as a synergist
136 (Jackson 1985). Pyrethroids kill many arthropods by quickly paralyzing their nervous
137 system, being effective against all stages of growth, and having also repellent effects
138 (WHO 1990). Permethrin is of low toxicity to birds (WHO 1990), and did not seem to
139 affect immune or stress responses in adult blue tits (authors' unpublished data). Water

140 was sprayed in nest-boxes from the E- and O-groups to avoid differences in humidity
141 that otherwise may have affected ectoparasite populations (Bennett & Whitworth 1991;
142 Chilton et al. 2000; Heeb et al. 2000). Some ectoparasites overwinter within the nest
143 material and become active when receiving mechanical stimuli (e.g. fleas, Humpries
144 1968). Thus, in order to minimise parasite emigration or death due to pre-optimal
145 emergence or activation, we attempted to initiate the experiment as close as possible to
146 the onset of blue tit reproduction, but before birds had become established in nest-
147 boxes.

148 We considered as occupied nests those where laying began. Through periodic
149 inspections of nests, clutch sizes as well as laying and hatching dates were determined
150 for every occupied nest. Those nests belonging to the F-group were re-fumigated when
151 nestlings were 7 days old (hatching date = day 0) as our objective was to maintain this
152 group of nests with a low number of ectoparasites. Nestlings were briefly removed from
153 the nests before fumigation and then returned. The other nests were manipulated in the
154 same way at that date, spraying water instead of insecticide.

155 Female blue tits were trapped in nest-boxes when feeding 13 days old nestlings,
156 aged as yearlings or older according to Svensson (1992) and banded individually with
157 numbered aluminium rings when necessary. Body mass of females was recorded to the
158 nearest 0.05 g with a Pesola (Baar, Switzerland) spring balance, tarsus length was
159 measured with a digital calliper to the nearest 0.01 mm and wing length was measured
160 with a ruler (precision of 0.5 mm). A drop of blood was obtained from the brachial vein
161 of females with the aid of a capillary tube and smeared on a slide. Blood smears were
162 immediately air-dried and later fixed with ethanol (96%) and stained with Giemsa (1/10
163 v/v) for 45 min. Half of the symmetrical smear was scanned at x200 magnification in
164 search of large blood parasites such as *Trypanosoma*, *Leucocytozoon* or microfilaria,

whereas small intra-erythrocytic parasites such as *Haemoproteus* or lankesterellids were detected on the other half of the smear using x1000 magnification (Merino & Potti 1995b; Merino et al. 1997). Intensity of infection (abundance) by *Haemoproteus* parasites was estimated as the number of infected cells per 2000 erythrocytes (Godfrey et al. 1987). We use presence/absence indexes for *Trypanosoma* and *Leucocytozoon* parasites due to their low intensities of infection. Lankesterellids and microfilaria prevalences were not analysed in relation to treatment due to the small number of infected birds, but were considered for the number of haemoparasite species infecting females.

Tarsus length of all nestlings was measured at 13 days of age and nestling mass was recorded as for females. Nestlings successfully fledged in 11 nests from the F-group, 14 nests from the E-group and 8 nests from the O-group. Just after fledging, i.e. 20 days post-hatching, nests were collected from nest-boxes in individually labelled sealed plastic bags. Nests were stored at 4 ºC until examined for nest ectoparasites. Within a month after collection, nests were defaunated in Berlese funnels for 48 h, under constant temperature and illumination conditions provided by a 60 W lamp placed 20 cm above the nests. A thin cloth was placed sealing the upper part of the funnels to avoid possible escapes of ectoparasites. This approach is one of the most rigorous for counting some nest ectoparasites such as mites (Proctor & Owens 2000). Thus, small nest ectoparasites (mites and adult and larval fleas) were collected in vials containing 70% ethanol and counted under a binocular microscope. Then nests were carefully dismantled in search of blow fly pupae or larvae as well as mites, fleas or other ectoparasites not obtained by this method (see Merino & Potti 1995a). Nest-containing bags were inspected as well.

Statistical Analyses

Sample sizes differ among analyses (Table 1) as data from three deserted nests were included only until desertion and some captures of females were not attempted due to chilling weather. Abundances of parasites were logarithmically transformed. Hatching (hatchlings / eggs laid) and reproductive success (fledglings / eggs laid) were arcsin-square root transformed before analyses. As we have no a priori prediction on how clutch size might vary in relation to treatment, ANOVA was used to explore differences among groups. There is a clear directional hypothesis that the ectoparasite load in nests should increase in the order *F-group* < *E-group* < *O-group* (see Results), and that ectoparasite effects should consistently operate in the same order of strength. Hence, there are obvious derived directional hypotheses that female post-breeding body mass, nestling body size and hatching and reproductive success should increase in the opposite order (*F-group* > *E-group* > *O-group*). In addition, we expect that laying date should differ among groups in the order *F-group* < *E-group* < *O-group*. Finally, we predict that female blood parasite intensities and prevalences should increase in the order *F-group* < *E-group* < *O-group* (see Introduction for the rationale of predictions). We therefore tested these effects of the three treatments using isotonic regressions (Gaines & Rice 1990). When isotonic regression could not be applied (variables with a binary response, e.g. prevalences), we employed ordered heterogeneity (OH) tests following the recommendations of Rice & Gaines (1994a). All OH tests are one-tailed because we have a clear directional prediction of the effect of the treatment. However, the use of two-tailed instead of one-tailed tests does not change the main results. The heterogeneity tests underlying the OH tests were ANOVAs with binomial errors and a logit link. Both the isotonic regression and the OH test are more appropriate and provide a substantial gain in power (i.e. reduce type-II errors) when testing ordered predictions

(Gaines & Rice 1990; Rice & Gaines 1994a, b, c). In both analyses, the *P* value depends on the direction as well as the magnitude of the deviation among the parameter estimates from the populations (Gaines & Rice 1990; Rice & Gaines 1994a).

To further explore relationships among ectoparasite loads and female hematozoan infections, GRM (for *Haemoproteus* intensity) or GLZ (for *Trypanosoma* and *Leucocytozoon* prevalences) models (Statsoft 2001) applying backward stepwise procedures were performed with adult fleas, mites and blowflies as explanatory variables. Including treatment as a categorical factor or applying forward stepwise procedures did not change these results. In addition, we further explored the possible relation between blood parasites and reproductive success and female body mass by GRM models applying backward stepwise procedures, with either reproductive success or female body mass as dependent variable. Treatment, *Haemoproteus* intensity and *Leucocytozoon* and *Trypanosoma* prevalences were included as predictor variables. Applying forward stepwise procedures did not change these results.

RESULTS

Treatment and Ectoparasite Load

Overall, 31 of the 33 (93.9 %) nests that successfully fledged young were infested by ectoparasites. We found three ectoparasite species in blue tit nests collected just after nestlings fledged. Mites were found in 27 (81.8 %) nests, fleas in 20 (60.6 %) nests and blowflies were found in 19 (57.6 %) nests. Treatment successfully created differences in the infestation by every ectoparasite species recorded in the predicted direction (Table 2). Overall, F-group nests supported the lowest levels of parasites, while the E-group had intermediate levels and the O-group had the highest levels. In

addition, the mean number of ectoparasite species present also differed significantly among groups in the same predicted direction (Table 2). The powerful nature of the isotonic regression allows detecting significant differences among groups in the predicted direction for mite abundance, despite the fact that mean mite abundance was lower in the O-group than in the E-group. Prevalences of adult fleas, total (adult + larvae) fleas and blowflies significantly differed among groups in the predicted direction (OH tests: $P = 0.028$, 0.001, 0.010, respectively). In all cases, the F-group showed the lowest prevalence and the O-group showed the highest prevalence. Prevalences of flea larvae and mites showed a similar pattern, although differences among groups in the predicted direction were not statistically significant (OH tests: $P > 0.20$).

Treatment and Breeding Performance

There were no significant differences in the occupation rate of nest-boxes from different experimental treatments by blue tits (Pearson χ^2: $P > 0.30$). Females of different age classes (yearlings or older) did not occupy nest-boxes differentially in relation to treatment (Pearson χ^2: $P > 0.50$), nor were there differences in tarsus and wing length of females occupying nest-boxes from different treatments (ANOVA: all $P > 0.50$). We did not find significant differences among groups for laying date in the predicted direction (Table 3). Clutch size did not differ among experimental groups (Table 3). Hatching success showed a tendency to differ among groups in the predicted direction *F-group* > *E-group* > *O-group* (Table 3). We did not find significant differences in the predicted direction for the average nestling measurements on day 13 (Table 3). However, there was an effect of treatment on reproductive success in the predicted direction (Table 3, Fig. 1a), especially due to the reduced success for the O-

group. Furthermore, there was a significant difference among the three groups in the same predicted direction for female body mass at the end of the experiment (Table 3, Fig. 1b).

Treatment and Female Blood Parasite Load

At the nestling age of 13 days, 96 % (24 out of 25) of the captured females were found infected by blood parasites. The more common blood parasite was *Haemoproteus majoris* (68 %, $N = 17$), followed by *Leucocytozoon majoris* (56 %, $N = 14$), *Trypanosoma avium* (40 %, $N = 10$) and a lankesterellid and microfilaria (both 20 %, $N = 5$). *Leucocytozoon* and *Trypanosoma* prevalences were clearly affected by the treatment in the predicted direction: females from the F-group showed the lowest prevalences and females from the O-group the highest prevalences (Table 3, Fig. 2). *Haemoproteus* infection intensity showed a similar pattern, although differences among groups in the predicted direction were not significant (Table 3). In addition, there was a significant difference among groups in the predicted direction for the mean number of blood parasite species infecting females (Table 3, Fig. 3). On average, females from the F-group were infected by the lowest number of blood parasite species, and females from the O-group were infected by the highest number.

Leucocytozoon prevalence was not significantly associated to any ectoparasite species (all $P > 0.05$). However, *Trypanosoma* prevalence was significantly related to the abundance of blowflies (Wald Statistic = 3.90, $P = 0.048$). *Haemoproteus* intensity was significantly related to the abundance of adult fleas ($F_{1,23} = 7.71$, $P = 0.011$). When exploring reproductive success in a GRM including blood parasitaemias together with treatment as potential predictors, no term remained significant. When exploring female body mass in the same way, only treatment was associated with female body mass as

290 previously reported (Model $R^2 = 0.31$, $F_{2,21} = 4.82$, $P = 0.019$), while no blood parasite
291 species was retained in the model.
292
293 # DISCUSSION
294
295 ## Treatment and Ectoparasite Load
296 Our experimental treatment was successful in creating differences with respect
297 to all ectoparasite species we recorded infesting blue tit nests. Nests from the F-group
298 held the lowest ectoparasite loads, whereas the E-group held intermediate loads and the
299 O-group the highest ectoparasite loads. Although we did not directly assess nest
300 material removal by blue tits, we found more or less intact old nests below the new
301 materials, suggesting that blue tits in our population did not substantially remove old
302 nest material. Breeding in a nest-box containing an old nest may be advantageous if it
303 reduces nest building effort (Collias & Collias 1984; Møller 1990; Hansell 2000). This
304 could be more likely to occur in areas with reduced detrimental effects of ectoparasitism
305 (e.g. Orell et al. 1993) or in those seasons when ectoparasite pressure is expected to be
306 low. In any case, birds from the E-group had to build completely new nests and may
307 have paid an additional cost in comparison with the other groups. Besides, an entire
308 fresh nest may hold a larger number of some ectoparasites, and perhaps this might
309 explain the high mite abundance in the E-group. Remarkably, that the prevalence and
310 abundance of blowflies were higher in the O-group than in the E-group is a quite
311 surprising result, given that it is supposed that blowflies do not or rarely overwinter
312 within the nest material (Bennett & Whitworth 1991). Alternatively, more intense odour
313 cues in nest-boxes with old nests may facilitate its detection by adult blowflies.
314 Whatever the case, the possibility exists that removal of old nests by researchers might

affect blowfly populations in a similar manner as shown for fleas (Rendell & Verbeek 1996; this study) and mites (this study), for which it is better known that part of their populations overwinter in nest-boxes (Burtt et al. 1991; Harper et al. 1992).

Treatment and Breeding Performance

Delayed egg laying dates have been reported in great tits *Parus major* as a host response to avoid ectoparasites (Oppliger et al. 1994). The reasoning is that some ectoparasites (e.g. fleas, Humphries 1968; Tripet et al. 2002a) start emigrating from a given nest-box as the breeding season progresses if it is not occupied. Hence, hosts could have to trade off the reduction in reproductive success caused by delayed egg laying (e.g. Barba et al. 1995) against the cost of breeding earlier with a higher load of ectoparasites (Oppliger et al. 1994; Richner 1998). Nevertheless, other ectoparasites might be more harmful for the birds with the advancing season (e.g. blowflies, Merino & Potti 1995a). Counterbalancing different pressures infringed by different ectoparasites, together with other factors such as the seasonal decline in food availability, may be the reason why we did not detect differences in laying date among experimental groups.

We did not find significant differences in clutch size among groups. This fact may indicate that female blue tits are unable to assess and forecast parasite loads by the time of laying to adjust clutch size, which is in agreement with previous studies for swallows (Møller 1991b, 1993). On the other hand, Richner & Heeb (1995) proposed that different optimal clutch sizes may be expected depending on the cycle length of the ectoparasite. As our blue tit population is infested by both long-cycled (fleas and blowflies) and short-cycled ectoparasites (mites), this may explain why there were not differences in clutch size among experimental groups.

340 Ectoparasites may affect the ability of parents to incubate their eggs, potentially
341 causing lowered hatching success (e.g. Møller et al. 1990; De Lope et al. 1993;
342 Oppliger et al. 1994). Our results show a tendency for hatching success to decrease as
343 ectoparasite pressure increases, although it was not statistically significant.
344 This study shows that ectoparasites can have dramatic detrimental effects on the
345 reproductive success of blue tits, which is in agreement with previous studies on this
346 (Bańbura et al. 2004) and other species (e.g. Møller 1990, 1993; De Lope et al. 1993;
347 Merino & Potti 1995a; Fitze et al. 2004). It is known that some ectoparasites increase
348 the energy requirements of nestlings, which indeed beg more (Tripet & Richner 1997a)
349 and receive more food from the adults (Tripet & Richner 1997a; Hurtrez-Boussès et al.
350 1998). Merino et al. (1998) showed that female pied flycatchers *Ficedula hypoleuca*
351 increase their energy expenditure to buffer the detrimental effects of ectoparasitism on
352 nestlings, but only to a certain level to avoid severe costs of ectoparasitism on adults.
353 Hence, female parents may have compensated at their own cost for the effect of
354 ectoparasites on its offspring, and thus, in addition to a reduced reproductive success,
355 we have shown that female blue tits paid added costs in terms of reduced body mass at
356 the end of the nestling period and higher haematozoan infections. Our results also show
357 that the reduced reproductive success is not likely to be caused by female blood
358 parasitaemias.
359 The observed differences among treatments in female body mass at the end of
360 the nestling period may be the result of birds working harder in more infested nests by
361 increasing provisioning rates (Hurtrez-Boussès et al. 1998; Tripet et al. 2002b) or nest
362 sanitation activities (Tripet et al. 2002b; see also Merino et al. 1998). However, these
363 differences could also be attributed directly to a detrimental effect of ectoparasites on
364 adult body mass (Christe et al. 2002), or indirectly to the drain imposed by blood

parasites (Merino et al. 2000; Tomás et al. 2005). However, this latter possibility is not supported by our results, as female blood parasitaemias were not associated to female body mass.

Treatment and Female Blood Parasite Load

At small ecological and geographical scales the prevalence of blood parasites is associated with the abundance of their ectoparasite vectors (e.g. Van Riper et al. 1986; Garvin & Remsen 1997; Apanius et al. 2000; Sol et al. 2000). We have shown that as ectoparasitism increases, females are more likely to become infected by *Trypanosoma* and *Leucocytozoon* parasites, and to host a larger number of haematozoan species. *Haemoproteus* intensity showed the same pattern, although differences were not statistically significant. In a similar experiment, Christe et al. (2002) did not find an effect of ectoparasitism on prevalence and intensity of blood parasites in adult house martins (*Delichon urbica*), although only one species (*Haemoproteus prognei*) was reported infecting adult birds and the prevalence of infection was lower than in our blue tit population. Prepatency periods for *Trypanosoma* and *Leucocytozoon* parasites are short enough to detect new infections during the duration of the experiment (see Methods). In contrast, the period between initial infection and release of gametocytes into the blood for *Haemoproteus* is longer, and this may obscure results concerning this parasite.

Alternatively, the differences we have shown in blood parasitaemias among groups might be related not only to differences in ectoparasite loads but also to differences in parental effort (Norris et al. 1994; Richner et al. 1995; Oppliger et al. 1996; Fargallo & Merino 1999) or even a combination of both factors. The idea that differences in blood parasitaemias are a direct consequence of differences in the

abundance of ectoparasites is supported by the observed relationships between blowflies and *Trypanosoma* prevalence and between fleas and *Haemoproteus* intensity. Therefore, our results might suggest that some of the ectoparasite species studied here might act as vectors of the blood parasites detected (see Macfie & Thompson 1929). However, it should be noted that the method we have employed for ectoparasite quantification does not allow detecting other potential insect vectors such as biting midges (Diptera, Ceratopogonidae), that are known vectors of blood parasites (Fallis & Wood 1957; Votýpka et al. 2002) and are common in blue tit nests in our study area (authors' unpublished data). Nevertheless, it is possible that biting midges were affected by the treatment in a similar manner as the other ectoparasites, in which case the observed relationships among ectoparasites and haematozoa might not be due to the abundance of fleas, blowflies and mites, but of correlated levels of biting midges. Whatever the case, our results show that female haematozoan infections are related to nest ectoparasite infestation, implying an added indirect cost of nest reuse.

It could be argued that the observed differences in reproductive success, female body mass and female haematozoa were caused by a difference in quality or dominance of birds nesting in nest-boxes from different treatments. In blue tits body size or age could reflect measures of individual quality (Kempenaers et al. 1992) or social dominance (Braillet et al. 2002). A comparison of tarsus and wing length of females among treatments showed no difference. We also found no difference in age of females or occupation rate among treatments. It seems therefore unlikely that the observed differences are caused by factors related to phenotypic differences among breeding birds (see Oppliger et al. 1994). However, as blue tits are territorial birds with small territories (Cramp & Perrins 1998), we cannot completely rule out the possibility that competitive exclusion from the best nest-boxes have occurred. It could be interesting to

perform an experiment under more controlled conditions, for example with captive birds assigned at random to the different treatments.

Validity of results derived from studies using nest-box populations of birds was called into question by Møller (1989, 1992). Møller argued that the common practice for researchers to yearly remove old nests from nest-boxes might bias the results of many of these studies, in part because ectoparasite loads might be reduced and hence their population dynamics artificially modified. Our results are in agreement with Møller's assumption, and emphasize that blood parasite populations can also be modified, as Møller (1989) suggested. In addition, if ectoparasite populations are artificially larger in nest-boxes in comparison with natural holes as shown by Wesołowski & Stańska (2001), and blood parasite infections are related to nest ectoparasite loads at a among-nest scale (this study), then bird populations breeding in nest-boxes could be exposed to artificially increased blood parasite loads. This fact would imply both that nest-box studies of birds represent an exceptional opportunity to test predictions related to host-blood parasite interactions and that extrapolations to natural breeding conditions should be drawn with caution.

To conclude, the reported costs of nest reuse would be reduced in areas and / or seasons with low incidence of ectoparasitism, as is shown by the results from the group with fumigated old nests. Knowledge about the actual impact of ectoparasites as vectors or inductors of disease in wild birds is still scarce (Proctor & Owens 2000; Votýpka et al. 2002), and more research effort should be devoted to elucidate the importance of this indirect effect of ectoparasitism on hosts. Future studies in this direction will help to better understand the ecological and evolutionary implications of the interaction among blood parasites, ectoparasites and their avian hosts.

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Table 1. Sample sizes available for every variable analysed for each experimental treatment. F-group: nest-boxes with a fumigated nest; E-group: empty nest-boxes; O-group: nest-boxes with an old nest

	F-group	E-group	O-group
Laying date	12	15	9
Clutch size	12	15	9
Hatching success	12	14	9
Nestling measurements	11	14	8
Reproductive success	11	14	8
Ectoparasites	11	14	8
Female age	9	14	5
Female measurements [a]	9	11	5
Female haematozoa	9	11	5

[a] One female from the E-group escaped before weighing, so actual sample size for female mass was $N = 10$.

Table 2. Number of ectoparasites (mean (SE)) in blue tit nests in relation to experimental treatment. F-group: nest-boxes with a fumigated nest; E-group: empty nest-boxes; O-group: nest-boxes with an old nest

	F-group	E-group	O-group	E^2_3	P [a]
Fleas (adults)	0.27±0.19	7.50±6.15	41.75±34.34	0.19	0.013
Fleas (larvae)	0.00±0.00	295.43±91.12	546.00±281.46	0.44	<0.001
Fleas (total)	0.27±0.19	302.93±94.88	587.75±276.40	0.42	<0.001
Blowflies	3.36±2.07	10.07±2.48	12.75±4.51	0.19	0.016
Mites	14.55±6.55	1017.14±432.08	493.75±344.84	0.25	0.004
# species	1.18±0.23	2.36±0.17	2.50±0.19	0.46	<0.001

[a] *P*-values are for isotonic regression (E^2; Gaines and Rice 1990) testing the directional prediction that the number of ectoparasites and the number of ectoparasite species vary between treatments as *F-group* < *E-group* < *O-group*. Tests are performed on logarithmically transformed data except for number of ectoparasite species.

Table 3. Mean (SE) laying date, clutch size, hatching and reproductive success, nestling measurements, female body mass and female blood parasite infections for blue tit nests in relation to experimental treatment. F-group: nest-boxes with a fumigated nest; E-group: empty nest-boxes; O-group: nest-boxes with an old nest

	F-group	E-group	O-group	E^2_3	P [a]
Laying date (1 = 1 April) [b]	29.00±0.58	28.53±0.76	29.44±0.53	0.02	0.351
Clutch size	9.33±0.43	9.07±0.25	9.22±0.40	0.16	0.851
Hatching success [c]	0.92±0.02	0.96±0.02	0.85±0.06	0.11	0.060
Nestling body mass (g) [c]	10.38±0.23	10.21±0.14	10.24±0.29	0.01	0.417
Nestling tarsus length (mm) [c]	16.10±0.14	16.34±0.12	16.04±0.12	0.04	0.248
Reproductive success [c]	0.93±0.02	0.95±0.02	0.81±0.56	0.21	0.010
Female body mass (g) [c]	10.63±0.12	10.17±0.14	10.00±0.18	0.32	0.006
Leucocytozoon prevalence [b]	0.33±0.17	0.64±0.15	0.80±0.20	-	0.036
Trypanosoma prevalence [b]	0.11±0.11	0.55±0.16	0.60±0.24	-	0.023
Haemoproteus intensity [b]	7.50±3.06	27.42±11.48	28.98±9.10	0.11	0.107
# blood parasite species [b]	1.56±0.24	2.09±0.28	2.80±0.58	0.20	0.029

[a] *P*-values are for isotonic regression (E^2; Gaines and Rice 1990), except for clutch size that were tested with ANOVA, and parasite prevalences that were tested with OH tests (Rice and Gaines 1994a). Tests are performed on logarithmically transformed data for *Haemoproteus* intensity and on arcsin-square root transformed data for hatching and reproductive success.

[b] The directional prediction tested was *F-group* < *E-group* < *O-group*.

[c] The directional prediction tested was *F-group* > *E-group* > *O-group*.

Figure legends

Fig. 1. Mean reproductive success (a) and mean body mass of female blue tits on day 13 of nestling age (b) in relation to experimental treatment. F-group: nest-boxes with a fumigated nest; E-group: empty nest-boxes; O-group: nest-boxes with an old nest. Bars are SE.

Fig. 2. Prevalences (%) of *Trypanosoma* and *Leucocytozoon* in female blue tits on day 13 of nestling age in relation to experimental treatment. F-group: nest-boxes with a fumigated nest; E-group: empty nest-boxes; O-group: nest-boxes with an old nest.

Fig. 3. Mean ($\pm$SE) number of blood parasite species infecting female blue tits on day 13 of nestling age in relation to experimental treatment. F-group: nest-boxes with a fumigated nest; E-group: empty nest-boxes; O-group: nest-boxes with an old nest.

Fig. 1. Tomás et al.

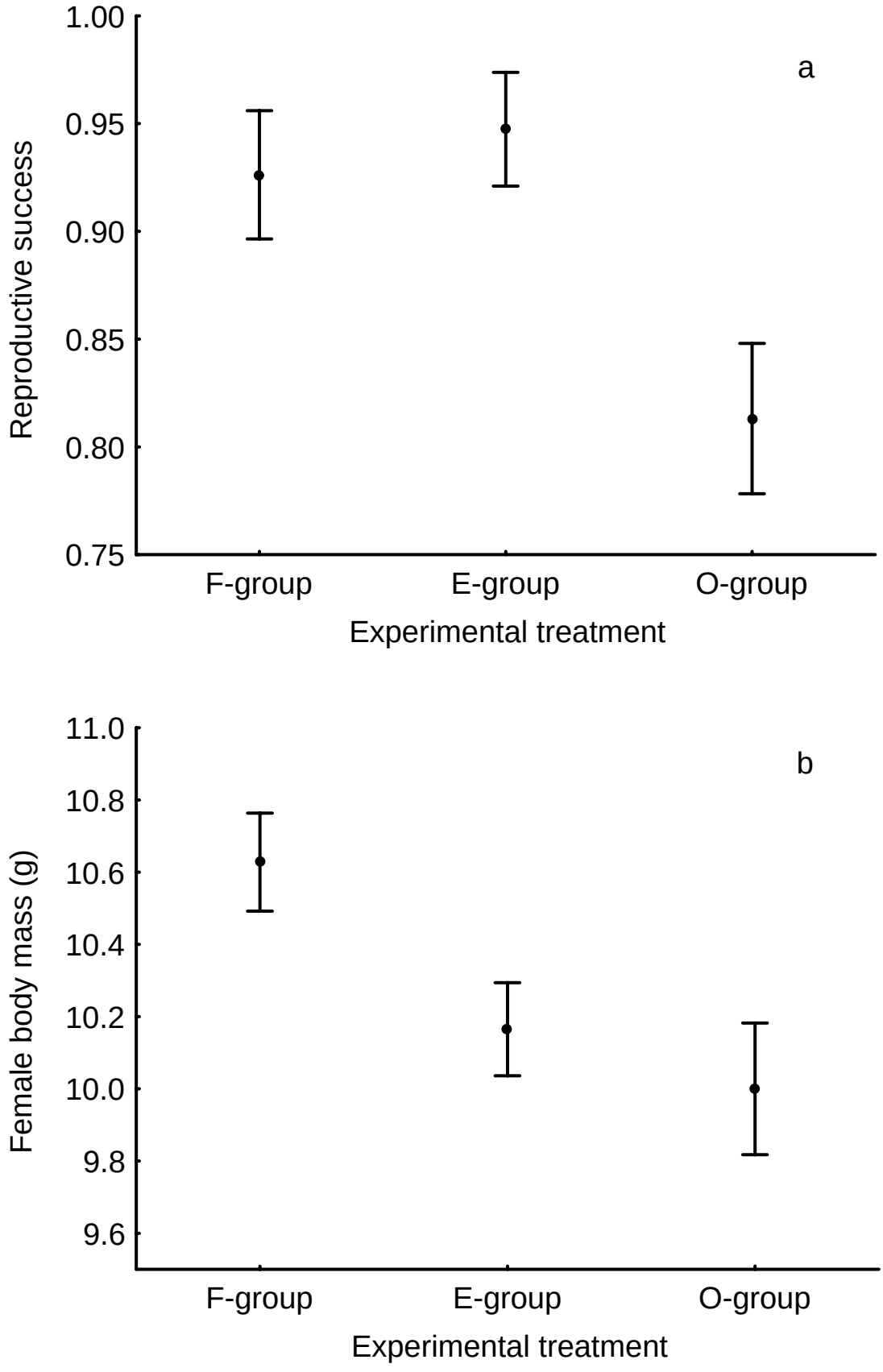

Fig. 2. Tomás et al.

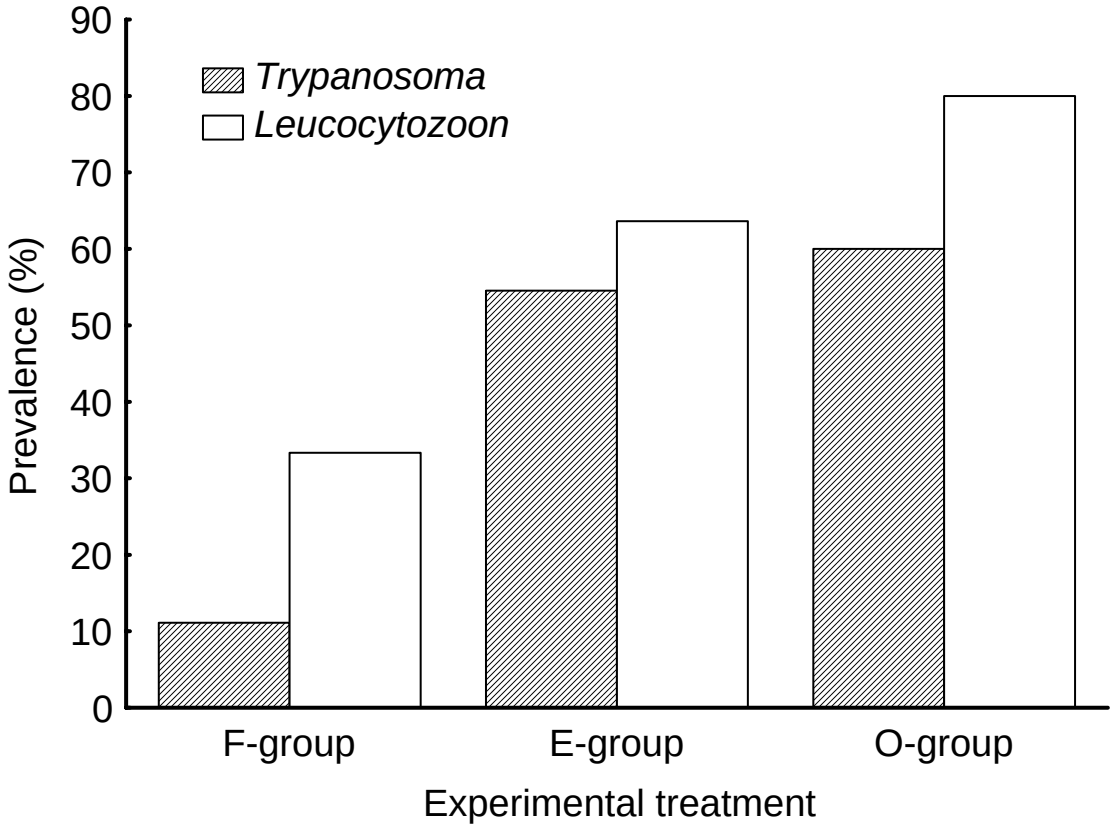

Fig. 3. Tomás et al.