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Relative fitness of a generalist parasite on two alternative hosts: a cross-infestation experiment to test host specialization of the hen flea *Ceratophyllus gallinae* (Schrank)

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Abstract

Host range is a key element of a parasite's ecology and evolution and can vary greatly depending on spatial scale. Generalist parasites frequently show local population structure in relation to alternative sympatric hosts (i.e. host races) and may thus be specialists at local scales. Here, we investigated local population specialization of a common avian nest-based parasite, the hen flea *Ceratophyllus gallinae* (Schrank), exploiting two abundant host species that share the same breeding sites, the great tit *Parus major* (Linnaeus) and the collared flycatcher *Ficedula albicollis* (Temminck). We performed a cross-infestation experiment of fleas between the two host species in two distinct study areas during a single breeding season and recorded the reproductive success of both hosts and parasites. In the following year, hosts were monitored again to assess the long-term impact of cross-infestation. Our results partly support the local specialization hypothesis: in great tit nests, tit fleas caused higher damage to their hosts than flycatcher fleas, and in collared flycatcher nests, flycatcher fleas had a faster larval development rates than tit fleas. However, these results were significant in only one of the two studied areas, suggesting that the location and history of the host population can modulate the specialization process. Caution is therefore called for when interpreting single location studies. More generally, our results emphasize the need to explicitly account for host diversity in order to understand the population ecology and evolutionary trajectory of generalist parasites.

Introduction

Host range is a key trait that shapes the distribution and evolution of parasites (Combes, 2001; Lajeunesse & Forbes, 2002; Krasnov *et al.*, 2005). Many presumed generalist parasites have been shown to consist of complexes of local populations, with genetically distinct populations infecting different sympatric host species (Linn *et al.*, 2003; McCoy *et al.*, 2005; Le Gac *et al.*, 2007; Magalhães *et al.*, 2007; Peccoud *et al.*, 2009; De Meeûs *et al.*, 2010; Kempf *et al.*, 2011; Dietrich *et al.*, 2014a). These distinct populations span the continuum between host races (e.g. Kempf *et al.*, 2011) and cryptic species (e.g. Le Gac *et al.*, 2007) and suggest that the interaction between supposedly generalist parasites and their hosts is often more complex than initially thought. These cryptic divergence patterns can profoundly impact the epidemiology and evolution of parasites within host communities and require explicit consideration when modelling host–parasite interactions (McCoy *et al.*, 2013; Dietrich *et al.*, 2014a).

Intraspecific host-related divergence of parasites may be the result of several processes: (i) selection on

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parasites to adapt to different host species, (ii) drift due to parasite population isolation (i.e. the incapacity of parasites exploiting one host to maintain gene flow with parasites on a different host) or (iii) a combination of both processes. Specialization due to adaptation may be particularly likely for parasites infesting hosts with high density and/or highly predictable spatial distributions (Norton & Carpenter, 1998; Sasal *et al.*, 1999; Soler *et al.*, 2009). Experimentally transplanting parasites from their host of origin to alternative host species can help us discriminate among these phenomena by overcoming natural constraints on parasite dispersal and revealing possible host-associated adaptations of the parasite (Dietrich *et al.*, 2014b).

Several parasite transplant experiments have been performed in the past with different outcomes depending on the experimental design and the biological system. In some cases, apparent parasite population structure was not associated with differential performance on alternative hosts (Little *et al.*, 2006; Ramírez *et al.*, 2014; Van Oosten *et al.*, 2014; Vrba & Pakandl, 2015). In others, parasites performed better, with higher feeding or breeding success, on their host of origin than on the alternative host (Little *et al.*, 2006; Dietrich *et al.*, 2014b; Ramírez *et al.*, 2014; Vrba & Pakandl, 2015). Most of these parasite transfer experiments were carried out under laboratory conditions with artificially maintained populations (Little *et al.*, 2006; Poulin & Keeney, 2008; Ramírez *et al.*, 2014; Vrba & Pakandl, 2015). These experiments typically control for spatiotemporal environmental variation, which can help identify the specific host and parasite traits involved in the adaptations. However, variation in environmental conditions and host infectious history are known to modulate host tolerance and resistance to parasites under natural conditions (De Neve *et al.*, 2007; Adelman *et al.*, 2013; Budischak *et al.*, 2015). Thus, field-based experimental approaches performed on hosts and parasites showing natural levels of genetic diversity are more likely than laboratory experiments to provide information about the relative ecological impact of potential adaptations in the wild and whether they may, or may not, lead to parasite diversification. Parasite transfer experiments under field conditions are thus scarce and, due to constraints inherent to the biological systems, often consist in either transferring different parasite populations to a common host (e.g. Dietrich *et al.*, 2014b) or a single parasite population to different hosts (e.g. Van Oosten *et al.*, 2014) and therefore do not explore reciprocal associations. Reciprocal parasite transfers among alternative hosts under natural conditions are nevertheless required to discriminate between differences in host profitability (i.e. one host is always better) and host-associated adaptations and therefore to fully understand the mechanisms of host specialization in parasites and their ecological and evolutionary consequences.

Using reciprocal transfers of parasites, we experimentally explored whether specialization to alternative host species, as reflected by increased parasite fitness on the host of origin, occurred in a common nest-based avian parasite, the hen flea *Ceratophyllus gallinae* (Schrank). To do so, we used two abundant avian species that are frequently infested by this ectoparasite in our study area: the great tit *Parus major* (Linnaeus) and the collared flycatcher *Ficedula albicollis* (Temminck). These two hole-nesting passerines use the same breeding sites (Gustafsson, 1987), but differ in several key life-history traits that may impose divergent selective pressures on hen fleas: migratory strategy (Nowakowski & Vähätalo, 2003; Weidinger & Kral, 2007), breeding phenology (i.e. onset and duration of breeding), clutch size, probability of a second clutch (Cramp & Perrins, 1994) and the composition of nesting material (Lemoine *et al.*, 2011). To test for host specialization in fleas, and examine its impact on both host and parasite fitness, we performed a reciprocal cross-infestation experiment of fleas between the two host species and subsequently monitored host and parasite reproductive success. We evaluated the degree of parasite adaptation to alternative hosts by measuring its relative reproductive performance on each host type and parasite virulence by measuring host fitness components (reproductive success and body condition) when exposed to alternative parasite populations. We tested three hypotheses, which are mutually exclusive at the spatial scale considered: (i) the hen flea is a true generalist and consists of a single local population exploiting both host types equally well; (ii) the hen flea is a single population specialized to exploit one host type (great tits; Tripet & Richner, 1997) and simply spills over to alternative host species; and (iii) the hen flea consists of a complex of populations locally specialized on different hosts. Under the first hypothesis, we expected no difference in flea performance on the two hosts, regardless of the origin. Under the second, we predicted that fleas should always perform better on great tits (i.e. the host with the higher infestation prevalence and intensity) than on collared flycatchers, regardless of the host of origin. Finally, under the third hypothesis, we expected fleas to perform better on their original host compared with the alternative host, reflecting adaptation to each host species.

Materials and methods

Study site

This study was conducted from March to July 2013 on the Swedish island of Gotland (57°10′N, 18°20′E), characterized by a fragmented rural landscape. We used a total of 17 wood patches located in two different areas of the island (A1 and A2) for our cross-infestation experiment (Fig. 1). Each patch was equipped with

standard wooden nest boxes (~10 × 10 cm inner base). The two areas are separated by approximately 35 km and have been used differently in previous years: most nest boxes in area A1 were set up for our experiment and this area was never involved in previous experimental work, whereas nest boxes in area A2 were set up in 2004–2005 and were used for experimental work during 5–6 years after which time they remained unused. For flea collection, we sampled a larger area, including 14 supplementary patches within the two areas (Fig. 1).

Biological system

On Gotland, great tits and collared flycatchers are the two most abundant hosts for the hen flea and are present at similar densities. They belong to the two main host families used by hen fleas, the Paridae and the Muscicapidae, respectively (Tripet & Richner, 1997). Collared flycatchers are the only hole-nesting representative species of the Muscicapidae family, because the presence of pied flycatchers (*Ficedula hypoleuca*) on the island is nowadays only accidental, whereas Paridae species are also represented by blue tits (*Cyanistes caeruleus*), and coal tits (*Periparus ater*) at a significantly lower abundance. Here, we experimentally tested the possible specialization of the parasite between hole-nesting Muscicapiadae and Paridae species using the most abundant species of each family. Great tits are residents or short-distance migrants and start breeding in mid-April, whereas flycatchers are trans-Saharan migrants and start breeding early May (Gustafsson, 1987; Cramp & Perrins, 1994). The material used for nest construction differs between the two species: moss and hair for great tits, and dry grass and leaves for flycatchers. Clutch size also differs between the two species, with an average of 8–9 eggs for great tits and 6–7 for collared flycatchers on Gotland (Lemoine *et al.*, 2011). The nestling period is also 3 days shorter on average for collared flycatchers compared with great tits (Cramp & Perrins, 1994).

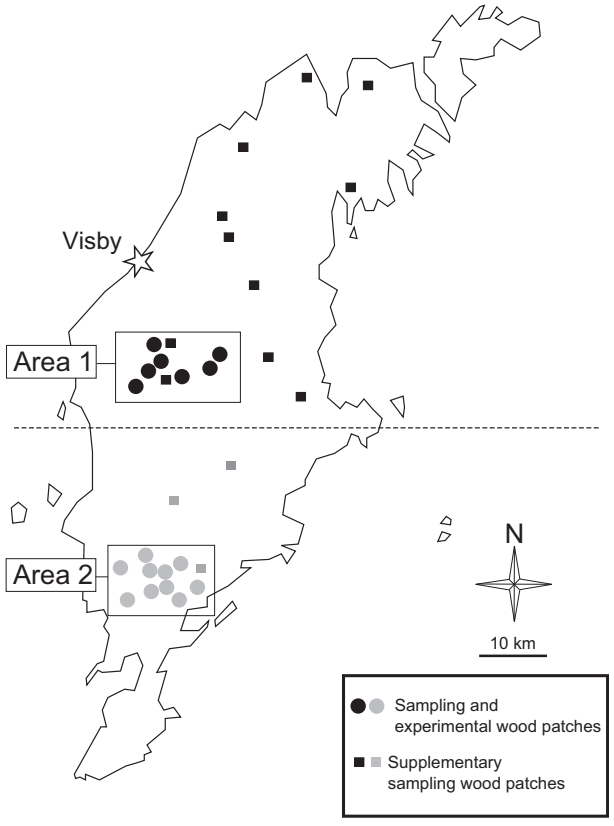

Fig. 1 Location of flea sampling wood patches (both symbols) and experimental wood patches (circles only) in the two study areas (black and grey) on the island of Gotland (Sweden).

The hen flea is a common avian ectoparasite, with infestations reported from at least 72 bird species of 36 families (Tripet & Richner, 1997). It is therefore considered as a generalist parasite. Nevertheless, the prevalence and intensity of infestation tend to be the highest on hole-nesting birds, and particularly so for the Paridae (Tripet & Richner, 1997). As a consequence, it has been suggested that the hen flea may have coevolved with tits and that other bird species play a secondary role as hosts (Tripet & Richner, 1997). The Muscicapidae family experiences similar prevalence of hen flea infestations as the Paridae, but lower intensities (Tripet & Richner, 1997). This parasite probably exerts an important selective pressure on both bird species due to its negative impact on reproductive success (Fitze *et al.*, 2004; Lemoine *et al.*, 2011).

The hen flea completes its reproductive cycle within host nests during the host breeding period. Its life cycle comprises three larval stages that feed on keratin and dust shed by the nestlings and blood crystals excreted by the blood-sucking adult fleas, followed by a non-feeding nymphal stage and finally, the imaginal and parasitic stage. A single cycle can last for 2 to 5 weeks, and up to two parasitic cycles can be completed during one host breeding season (Tripet & Richner, 1999). The nonfeeding nymphal stage is typically the overwintering stage. The duration and success of bird breeding are important determinants of flea fitness, because the nest is maintained at optimal developmental temperatures by the presence of the birds (Cotton, 1970; Harper *et al.*, 1992; Tripet & Richner, 1999; Lemoine *et al.*, 2011). Nest materials, which constitute the microhabitat for larval stages, may also affect parasite success (Lemoine *et al.*, 2011).

Cross-infestation experiment

We performed a reciprocal cross-infestation experiment to explore differences in flea performance depending

on the origin of fleas and the host species they infest. Before the start of the 2013 breeding season, we collected old nests from nest boxes in the two areas (Fig. 1) to extract fleas for experimental infestations. To ensure a sufficient number of fleas, we collected old nests from 14 additional patches (Fig. 1). Old infested nests were pooled by area (A1 vs. A2) and host of origin based on the material left in the nest box (tit vs. flycatcher), pooling together old nests from all tits (mostly great tits, but also some blue tits *C. caeruleus*), which are very similar in composition and cannot always be readily distinguished. Because our experiment aimed at exploring flea specialization between the two host families, such pooling should not be a problem. The four pools of old nests obtained, that is 'Tit-A1', 'Tit-A2', 'Flycatcher-A1' and 'Flycatcher-A2' (Fig. 2), were stored in hermetically closed buckets at ambient temperature from the end of February to the end of May. We collected a total of 650 nests, in equal number from the two host types, to create the flea pools. Due to the lower number of nest boxes in area A1 at the onset of the experiment, the A2 pools contained three times more nests than the A1 pools. All nests that could not be clearly assigned to one of the two studied bird families were excluded from this experiment.

After the collection of old nests (i.e. end of March), we blocked the entrance of one half of all nest boxes selected at random in each patch to ensure that a balanced number of tits and flycatchers could settle in each patch and to avoid a potential bias in site quality between the two species. We left the first half of nest boxes open for the settlement of great tit pairs and opened the second half in late April/early May to correspond to the arrival of collared flycatchers. During set-up, we carefully scrubbed all the nest boxes and heat-treated them with a blowtorch to eliminate any local invertebrates. After set-up, we visited nest boxes every second day to check for nest building and breeding activity. Towards the end of nest building or during early egg laying, we heat-treated the nest (without the bird eggs) using a microwave (700 watts during 1 to 2 min) to kill any naturally present fleas. We then left nests to cool down to ambient temperature before putting them back in the boxes and then added 20 adult fleas from one of the four pools (Fig. 2). We used a total of 125 great tit and 134 collared flycatcher pairs in this experiment. Flea geographic and host origin (i.e. flea pool) was successfully randomized among experimental nests according to laying date (ANOVA: collared flycatchers: $F_{3,130} = 0.460$, $P = 0.71$; great tits: $F_{3,125} = 0.643$, $P = 0.59$) and study area (χ^2 test; collared flycatchers: χ^2_3=1.767, $P = 0.62$; great tits: χ^2_3=0.412, $P = 0.94$). The number of nest boxes infested with fleas from A1 and A2 was proportional to the number of old nests collected in the two areas (i.e. fleas from A1 were used to infest one-fourth of experimental nests, and fleas from A2 the other three-fourth).

Host and parasite monitoring

We checked each nest every second day until the start of incubation. After the 12th day of incubation, we checked nests again on a daily basis for hatching (day 0). During the rearing period, we visited nests twice to record nestling number and growth. On the first visit (day 8 for collared flycatchers, day 9 for great tits), we ringed and weighed nestlings. On the second visit (day 12 for collared flycatchers, day 14 for great tits), we weighed nestlings again and measured their tarsus

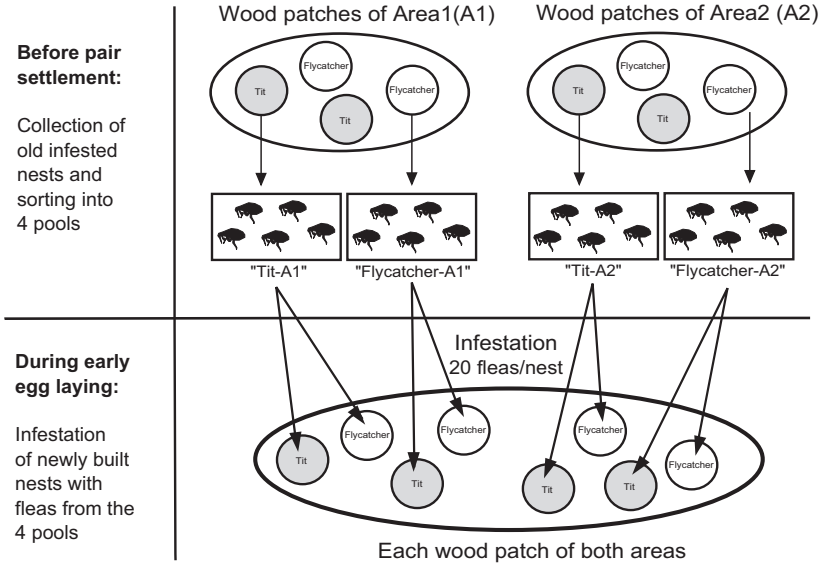

Fig. 2 Experimental design of the cross-infestation experiment. Each breeding pair received 20 adult fleas coming from one of the four pools (two areas and two hosts of origin). Each pair therefore received fleas originating from nests of either its own species or the other species and from either its own breeding area or from the other breeding area.

length (to the nearest 0.1 mm). During the second half of the rearing period, we also captured parents inside nest boxes using swing-door traps. We aged parents (yearling vs. older) according to plumage traits (Svensson, 1992), weighed and measured them (tarsus length to the nearest 0.1 mm). From day 17 for collared flycatchers and day 20 for great tits, we visited nests daily until fledging. After fledging, we collected the empty nests in sealed bags and placed them the same day in a Berlese funnel for 24 h to extract flea larvae (see Appendix S1; Lemoine *et al.*, 2011). These larvae were then stored in 70% alcohol and brought to the laboratory for purification, using a sucrose flotation, and quantification (see supplementary Appendix S1; Lemoine *et al.*, 2011). We quantified the total number of flea larvae, as well as the proportion of third-instar larvae as a measure of parasite developmental success. Because nymphal and imaginal stages are the overwintering and dispersive stages, respectively (Tripet & Richner, 1999), larval stage and size at fledgling of the birds can determine the parasite reproductive success and local persistence. Third-instar larvae can only be distinguished from earlier stages based on larvae size (i.e. equal or longer than 5 mm; (Sikes, 1930). Thus, we estimated the number of third-instar larvae by counting all larvae that were 5 mm or more in length (Kiryakova, 1965). As larval body size can overlap slightly among larval instars (Moser *et al.*, 1991), a single observer estimated the number of third-instar larvae for all experimental nests to reduce measurement error.

To record the long-term consequences of the cross-infestation on host local survival and reproductive success, we monitored bird populations of our experimental patches again in the following year (spring 2014). We thus obtained estimates of local return rates and reproductive success for the experimental adult birds after 1 year of the experiment.

Statistical analyses

We investigated the combined influence of the host of origin of fleas (nest of flycatchers vs. nest of tits), the host species (collared flycatcher vs. great tit) and the study area (A1 vs. A2) on measures of host and parasite reproductive performance. We assessed host reproductive success by (i) total failure probability (combining nest desertion before egg hatching and full brood mortality when at least one egg hatched; cases of nest predation were excluded), (ii) fledging success (i.e. the probability for the young to fledge) in successful nests (i.e. where at least one nestling fledged) and (iii) nestling body condition at fledging (calculated here as the ratio of body mass on tarsus length at day 12 or 14 depending on host species). In addition, we analysed the return rate of experimental adults in the following year and, for individuals that returned, their nesting success (fledging probability). We measured parasite reproductive success in nests where at least one nestling fledged by (i) the total number of flea larvae in a nest at the end of the host breeding period and (ii) the probability for a larva to be in the third-instar stage. We considered here only successful host nests because nests that failed early did not allow fleas to develop over the same period of time. Summary statistics of the main response variables are given in Appendix S2 and S3.

We analysed binary variables (total failure probability, fledging probability, adult bird return rate and probability for flea larvae to reach the third instar) using generalized linear models (GLM) with binomial error structures (or quasi-binomial when required due to overdispersion; logit link function). We analysed the final number of flea larvae as a count variable using a GLM with quasi-Poisson error structure (log link function). Finally, we analysed average nestling body condition per nest using a linear model (LM).

All models included flea host of origin (reference level: nests of flycatchers), host species (reference level: collared flycatchers) and study area (reference level: A2) as fixed factors, as well as all two- and three-way interactions between these variables, except for total failure probability for which the low sample size prevented us from investigating all interactions. Although we had no specific predictions on between-area differences, we chose to take into account this factor because of the possible confounding effects of their contrasted experimental histories on our results. The three-way interaction in the models therefore tests for a differential effect of flea host of origin on host species depending on area. When an effect of area was detected (in all cases, in interaction with other factors), we explored the potential influence of the area of origin of fleas for explaining observed spatial differences (see Discussion). In addition, except for adult return rate, we controlled for the effect of breeding phenology on reproductive performance by including laying date as a covariate. Because of the difference in timing between the two host species, we standardized laying date within each host species (i.e. [x-mean]/SE). Finally, because host and parasite reproductive performance may depend on host investment and the cost of parasitism, we included as covariates (i) clutch size (except for fledging probability as the response variable included clutch size) and final number of flea larvae (except in the case total failure probability as larvae were not quantified in unsuccessful nests), and (ii) clutch size for parasite performance measures. Because of the difference in clutch size between the two host species, we standardized clutch size within each host species (i.e. [x-mean]/SE). We also log-transformed the final number of flea larvae (i.e. log[x + 1]) because of high dispersion of the data. As 85% of adult birds paired with a new partner in 2014, nest identity was not included as random factor for models examining bird return rates and second-year fledging success.

We performed all statistical analyses using R (R Core Team, 2013) version 3.0.2 and with the package lme4 (Bates, 2010). For each model, we used a backward stepwise selection procedure based on a probability threshold of 10%. Backward selection based on *P*-values was used because model selection based on Akaike information criterion (AIC) was not possible for all models. For models where both procedures were possible, results obtained were not influenced by the model selection method. The validity of all models was checked and adjusted accordingly (data distribution and error structures). For nonsignificant variables, the test statistics and associated *P*-values reported correspond to the values calculated just before their removal from the model.

Results

Host reproductive success

Breeding success

A total of 26 of 125 (i.e. 20.8%) great tit pairs and 42 of 134 (i.e. 31.3%) collared flycatcher pairs failed to fledge any young. Total failure did not depend on the flea host of origin ($Z = -0.272$, $P = 0.78$), study area ($Z = -0.196$, $P = 0.84$) or laying date ($Z = 1.442$, $P = 0.15$). Between-host species differences in total failure probability was not significant, although great tits tended to experience less failure than collared flycatchers ($Z = -1.916$, $P = 0.055$).

Among successful nests (i.e. that fledge at least one young), the probability of fledging differed between host species depending on the host of origin of fleas and the study area (three-way interaction for host species, flea host of origin and study area: $N = 175$, $t = -2.894$, $P = 0.004$; Fig. 3). In area A1, great tits experienced lower fledging probability when infested with fleas sampled from old nests of their own species ($N = 39$, estimate $\pm$ SE $= -1.210 \pm 0.421$, $t = -2.873$, $P = 0.007$; Fig. 3b). This trend was not significant for collared flycatchers (collared flycatchers: $N = 34$, $t = 1.889$, $P = 0.068$; Fig. 3a). In area A2, however, fledging probability did not differ with the flea host of origin for either host species (collared flycatchers: $N = 53$, $t = -0.368$, $P = 0.715$; great tits: $N = 48$, $t = 0.435$, $P = 0.665$; Fig. 3c, d). In addition, fledging probability decreased with increasing laying date (estimate $\pm$ SE $= -0.589 \pm 0.0.126$, $t = -4.665$, $P < 0.001$) , but was not linked to the final number of fleas ($t = -0.166$, $P = 0.868$).

Nestling body condition

Average nestling body condition per nest did not depend on interactions between the host species, the flea host of origin and the area ($N = 175$, three-way interaction: $t = -0.697$, $P = 0.487$; all two-way interactions: $-1.554 < t < 0.344$, $0.122 < P < 0.732$). It also did not depend on flea host of origin alone ($t = 0.279$, $P = 0.781$) or area alone ($t = -0.857$, $P = 0.393$). Nestling body condition was, however, higher for great tit nestlings than for collared flycatcher nestlings (estimate $\pm$ SE $= 0.06 \pm 0.01$, $t = 6.065$, $P < 0.001$). In addition, it decreased with increasing clutch size (estimate $\pm$ SE $= -0.01 \pm 0.005$, $t = -2.685$, $P = 0.008$). No variation with the laying date was observed ($t = 1.338$, $P = 0.183$). Finally, nestling body condition was positively correlated with the final number of flea larvae in the nest (Estimate $\pm$ SE $= 0.01 \pm 0.003$, $t = 3.437$, $P = 0.001$).

Return rate and breeding success in the following year

Sixty-one of 182 adult collared flycatchers and 54 of 129 great tits were recaptured as breeders in the experimental patches in spring 2014. Overall, return rates were not influenced by the flea host of origin, depending on the study area (two-way interaction between flea host of origin and study area: $Z = 1.680$, $P = 0.093$), but no significant difference in return rates between birds infested with fleas of different hosts of origin was observed when each area was tested separately (A1: $N = 142$, $Z = 0.841$, $P = 0.401$; A2: $N = 169$, $Z = -1.559$, $P = 0.119$). No effect of interactions between the flea host of origin, the area and the host species was observed (three-way interaction: $z = -0.886$, $P = 0.375$; interaction between host species and area: $z = 0.068$, $P = 0.946$; interaction between host species and host of origin of fleas: $z = 0.870$, $P = 0.384$). The two host species had similar return rates ($z = 1.614$, $P = 0.107$).

Fledging probability in 2014 differed according to the flea host of origin in 2013, but this effect was again dependent on the study area ($N = 115$, two-way interaction between flea host of origin and study area: $t = 2.064$, $P = 0.041$). However, within each area, no significant difference was observed between birds infested in 2013 with fleas of different hosts of origin (Area 1: $N = 55$, $t = 1.170$, $P = 0.247$, Area 2: $N = 60$, $t = -1.803$, $P = 0.077$). No other interaction was significant (three-way interaction: $t = 0.143$, $P = 0.887$, interaction between host species and flea host of origin: $t = 0.134$, $P = 0.893$, interaction between area and host species: $t = -1.079$, $P = 0.283$). The fledging probability in 2014 was higher in great tit nests than in collared flycatcher nests (estimate $\pm$ SE $= 1.310 \pm 0.285$, $t = 4.595$, $P < 0.001$).

Parasite reproductive success

Although not significant, the final number of flea larvae tended to be influenced by the flea host of origin, depending on host species and study area ($N = 173$, three-way interaction among flea host of origin, host species and area: $t = -1.713$, $P = 0.089$). In area A1, the final number of flea larvae tended to be higher in collared flycatcher nests infested with fleas from tit nests compared with fleas

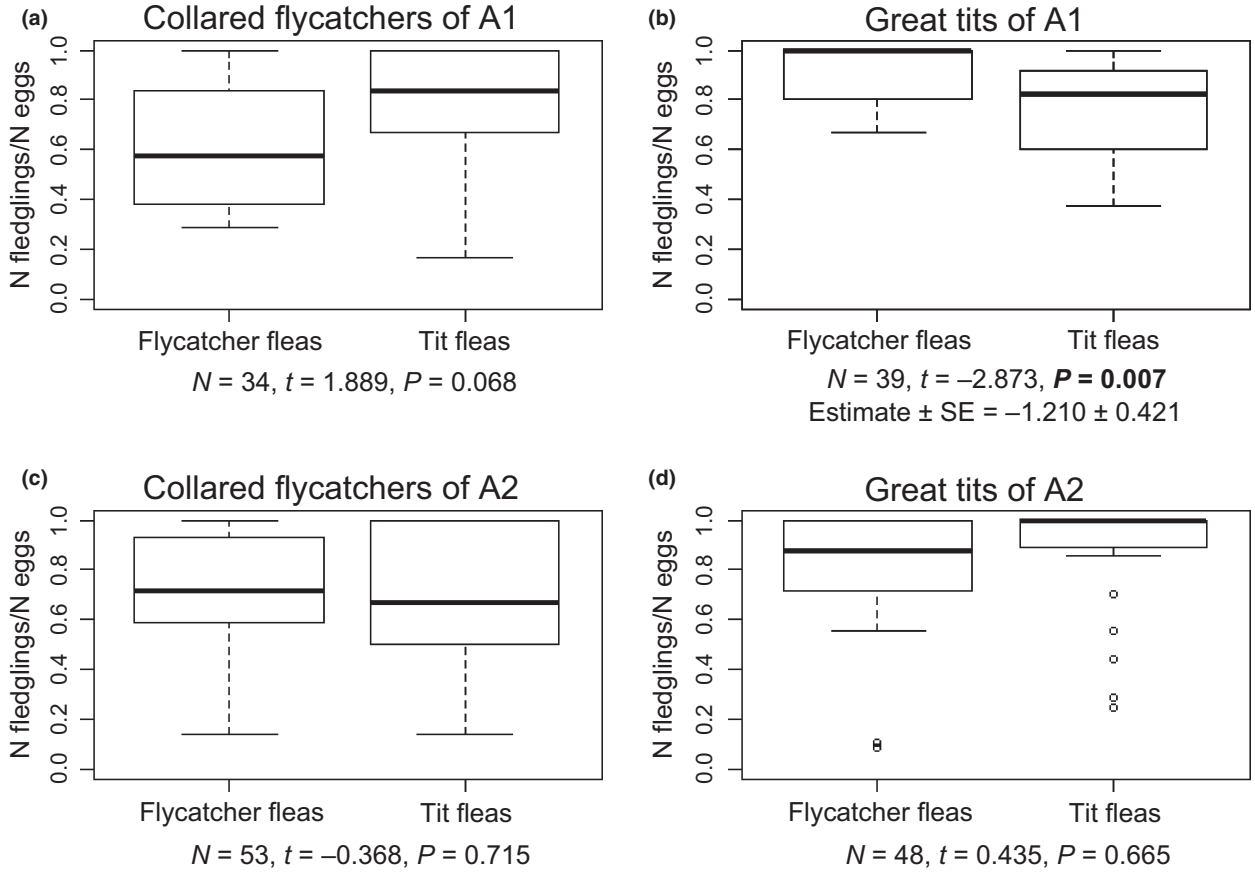

Fig. 3 Fledging success (i.e. number of fledged young divided by clutch size) of successful experimental nests, that is when at least one nestling fledged, presented for each host species (collared flycatcher or great tit), flea host of origin (flycatchers or tits nests) and area (A1 or A2).

from great tit nests ($N = 34$, estimate ± SE = 0.700 ± 0.343, $t = 2.039$, $P = 0.050$). No difference in final flea larvae number was observed among great tit pairs in A1 or for either host species in A2 (effect of flea host of origin in all models: $-0.546 < t < 0.807$, $0.424 < P < 0.899$). The final number of flea larvae was not influenced by laying date ($t = -1.401$, $P = 0.163$), and decreased with increasing clutch size (estimate ± SE = −0.277 ± 0.104, $t = -2.659$, $P = 0.008$). This latter effect can be explained by the negative effect of increasing clutch size on the individual body condition of nestlings.

The probability for a larvae to be in the third-instar stage depended on the combined effects of flea host of origin, host species and area ($N = 145$, three-way interaction: $t = 2.880$, $P = 0.005$). In area A1, the probability of being in the third larval instar was higher in flycatcher nests for flycatcher fleas compared with great tit fleas ($N = 25$, estimate ± SE = −0.597 ± 0.238, $t = -2.507$, $P = 0.021$, Fig. 4a), whereas no difference was observed for fleas in great tit nests ($N = 30$, $t = -0.471$, $P = 0.641$, Fig. 4b). In area A2, no difference was observed for fleas in nests of either host species (flycatchers: $N = 48$, $t = 1.515$, $P = 0.136$; great tits: $N = 42$, $t = -1.191$, $P = 0.256$, Fig. 4c, d).

Discussion

In this study, we used a reciprocal cross-infestation experiment of a common avian ectoparasite, the hen flea, between two of its main avian hosts, the great tit and the collared flycatcher, to test whether this parasite is a true host generalist, is a Paridae specialist that spills over to other species or has specifically adapted to exploit each host type. We compared the relative reproductive success of sympatric great tits and collared flycatchers when exposed to fleas that originated from nests of their own species or from the alternative species. We also quantified the fitness of fleas when exposed to these alternative hosts. Our results show that fledging success was lower when great tit nests were infested by fleas originating from nests of their own species. Moreover, flea larval development was faster in nests of collared flycatchers infested with

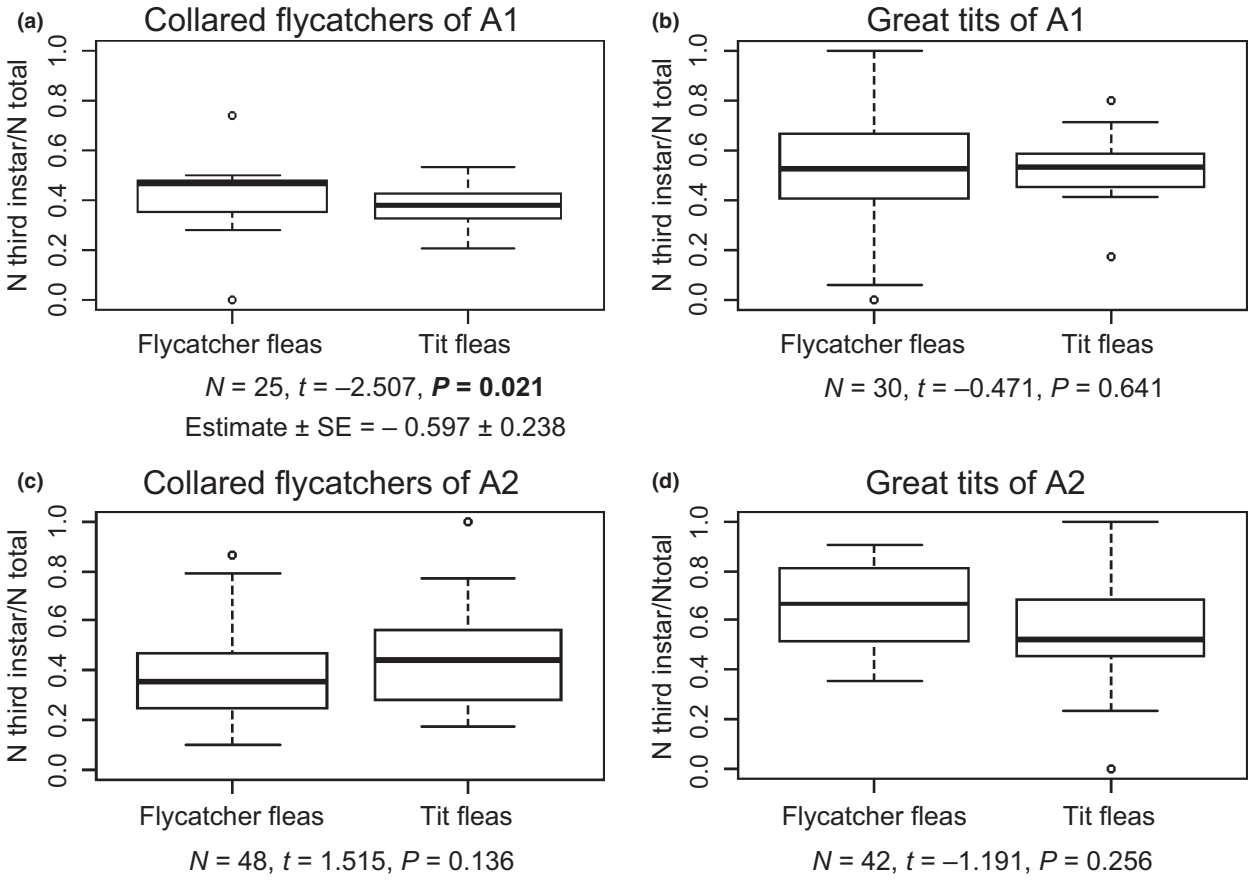

Fig. 4 Proportion of third-instar flea larvae present in nests, presented for each host species (collared flycatcher or great tit), flea host of origin (flycatchers or tits nests) and area (A1 or A2).

flycatcher fleas. However, these results differed between the two study areas. Therefore, under natural conditions, fleas may specialize on alternative host species, but the fitness consequences of this specialization may differ between host species and disappear depending on the host population or environmental context.

The hen flea, a global generalist but local specialist?

In area A1, nestlings from great tit pairs infested with fleas from tit nests had a lower chance of fledging than nestlings from pairs infested with fleas from flycatcher nests. Clutch size and hatching success were not impacted by the host of origin of fleas (Appendix S4); therefore, parasite virulence was exerted on nestlings during the rearing period, through blood loss or toxicity. Host of origin of fleas had no effect on nestling body condition; therefore, the reduction of fledging success was not compensated by a better quality of fledglings. Thus, fleas imposed a higher reproductive cost, as measured by lower final fledging success on hosts when originating from nests of the same species, that is after at least one reproductive season spent in contact with the same host species. In collared flycatcher nests of the same area A1, flea larvae from flycatcher nests were more likely to have reached the third larval instar stage before host fledging compared with collared flycatcher nests infested with tit fleas. Tit fleas seem to be maladapted to collared flycatcher hosts because few of these larvae would survive to overwinter, whereas development of flycatcher flea larvae were more in concert with the shorter reproductive period of flycatchers.

Theoretical studies predict that parasite virulence and transmission are linked and that optimal virulence will depend on where a parasite can maximize its transmission success (Alizon & van Baalen, 2005). Here, in one of the areas, we observed increased virulence (reflected by a decrease in host reproductive success) for tit fleas when infesting great tit nests, but no obvious increase in parasite fitness among the measured parameters. We also found an increased rate of flea larval development for flycatcher fleas when infesting collared flycatcher nests, but no direct impact on host reproductive suc-

cess. At this point, we are therefore unable to draw conclusions about the relationship between virulence and fitness for this parasite. However, the differential results obtained for fleas of different origins on the two host species support the hypothesis of a local complex of parasite populations infesting distinct host species. The observed patterns are therefore in line with the hypothesis of populations of the parasite specialized for different hosts, although we cannot exclude a potential role of other complex metapopulation dynamics. Our results are therefore in line with fleas being global generalists but local specialists, observations that have been made in other host-parasite systems, especially involving ticks (McCoy *et al.*, 2013).

Possible mechanisms of host specialization in fleas

Several mechanisms could explain the specialization of hen flea populations on different hosts. Differences in terms of life-history traits, nest composition and/or maternal effects between hosts could act as a divergent selective pressure on the hen flea and lead to appearance of host-specific adaptations. Moreover, experimental evolution tests on *Xenopsylla ramesis* fleas on two alternative rodent hosts suggested that fleas could evolve rapid specialization on different hosts when between-host transmission was artificially interrupted (Arbiv *et al.*, 2012). Within only nine generations, fleas maintained on one host species experienced an important reduction of their ability to infest an alternative host species as efficiently as their maintenance host (Arbiv *et al.*, 2012). In our system, some mechanisms could reduce the parasite exchanges between host species. In particular, because fleas generally overwinter in nest boxes, the encounter with a host is likely to be largely influenced by host habitat choice. Although sympatric at the scale of the wood patch, great tits and collared flycatchers may show different preferences for breeding sites within wood patches based on, for example, the specific use of tree types or locations (edge vs. centre). These habitat preferences could influence the encounter probability of fleas with alternative host species. However, because hen flea specialization could have a negative impact on host fitness, we cannot exclude that birds may also evolve counter adaptations to avoid infested sites used by their conspecifics. Further studies on host habitat choice and parasite dispersal abilities will now be required to conclude on between host species parasites exchanges.

Spatial variation in host specialization by hen fleas

The difference in host and parasite responses to our manipulation between the two study areas A1 and A2 was an unexpected result, given the short distance between them (35 km on average, Fig. 1). However, coevolutionary processes, including the arms race between hosts and parasites, can be spatially variable, with 'hot spots' (showing strong coevolution) and 'cold spots' (showing weak coevolution), depending on both host and parasite local environments, species community compositions, respective genetic variation, etc. (Thompson, 2005a, b). Fleas were previously suggested to be locally maladapted to great tit populations on Gotland (Lemoine *et al.*, 2012), which may lead to different host responses depending on the spatial origin of parasites. However, differences in host and parasite responses between A1 and A2 could not be attributed to local host–parasite adaptations (Appendix S5). Likewise, this variation was not related to area-specific differences in host population age structure or body condition (Appendix S6). If A2 was more profitable for birds than A1, birds may have been better able to compensate for parasitism costs in this area, removing a signal of adaptation. However, the two areas did not differ obviously in terms of resource availability during breeding: reproductive success and return rates in the next year were similar between areas for both host species. Nest box density was approximately twice as high in A2 compared with A1 (number of nest boxes per ha $\pm$ SE: 2.4 $\pm$ 0.6 in A1 and 5.3 $\pm$ 1.1 in A2), which may favour flea exchange between nests in A2 and thereby attenuate differences between our flea treatments by mixing fleas of different origins between nearby experimental nest boxes. However, in a set of sterilized nest boxes not used for the purpose of this experiment, we observed no difference in probability of nest box colonization between the two areas (χ^2 test, $\chi^2_1 = 0.371$, $P = 0.543$) and larval flea abundance was actually higher in A1 compared with A2 (Wilcoxon–Mann–Whitney test, $W = 519$, $P = 0.026$). Because flea population dynamics is density dependent and competition between flea larvae within nests is strong (Tripet & Richner, 1999), colonization success may be lower in already infested nests compared with uninfested nests, and thus, the results from the sterilized nests may not reflect flea dispersal rates among previously infested nests. Directly controlling for flea immigration during the bird breeding season is not possible. However, molecular tools may help assess the final efficiency of our treatment in each area, if fleas show genetic differences when originating from different hosts. This remains to be investigated. Finally, area A2 has been subjected to more intense nest box monitoring and experiments over time than A1 (see methods), which may have differentially affected evolution within flea and/or host populations. In particular, flea abundance was suggested to be higher in artificial nest boxes than in natural breeding cavities (Wesołowski & Stańska, 2001; Hebda & Wesołowski, 2012). Moreover, the presence of nest boxes may favour a higher host density compared with when only natural holes are available (Mänd *et al.*, 2009), and could therefore increase parasite gene flow between hosts within a wood patch.

Coevolution between hosts and parasites in A2 could therefore have been influenced by the presence of nest boxes in this site during several years before our manipulation. Thus, the outcome of experimental cross-infestations may be scale and context dependent. Variation in environmental conditions, population history and spatial scale may explain, at least partly, the absence of host specialization in previous cross-infestation experiments (Little *et al.*, 2006; Ramírez *et al.*, 2014; Van Oosten *et al.*, 2014; Vrba & Pakandl, 2015). Further experiments focusing at the between-area scale, and including both experimental and natural areas could provide a really interesting insight on the spatial variation of host–parasite coevolution in this system.

Overall, our results support the hypothesis that fleas can show local host specialization, but they also call attention to the complexity of such multi-host–parasite systems, and more particularly to the difficulty in extrapolating results from single population studies. Our results also emphasize the need to explicitly account for host diversity when studying the population ecology and evolution of generalist parasites, as results from the 'main' host may not accurately reflect results on alternative hosts. However, our experiment did not consider the whole diversity of host species available for the parasite, and further work would be needed to explore whether other hosts may also play an important role for the hen flea ecology. Cross-infestation experiments over larger spatial and temporal scales would allow us to assess the influence of interannual variability in environment quality, parasite dispersal and host population history on host and parasite responses and also to identify other ecological and evolutionary factors possibly affecting these responses under natural conditions. Population genetic analyses of parasites at different spatial and temporal scales would also enable us to determine the role of population isolation in the dynamics of these responses and therefore will be key for understanding the reciprocal role of host and parasite selection in the evolution of specialization.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Protocols for extracting and counting flea larvae.

Appendix S2 Summary statistics of the main response variables considered for great tits (GT) and collared flycatchers (CF).

Appendix S3 Summary statistics of the main response variables considered for the hen flea within each host nest.

Appendix S4 Variation in clutch size and hatching probability depending on flea host of origin, host species and area.

Appendix S5 Testing for local adaptation between the hen flea and its hosts.

Appendix S6 Variation in breeding adult quality among areas.

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