Original Article

Parasite- and predator-induced maternal effects in the great tit (*Parus major*)

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Both predators and parasites can elicit behavioral and physiological responses in prey and hosts, respectively. These responses may involve the reallocation of resources and may thus limit each other. We investigated the effects of concurrent pre-laying exposure of great tit females (*Parus major*) to both a simulated predation risk and a nest-based ectoparasite, the hen flea (*Ceratophyllus gallinae*), on nestling growth and development. We manipulated perceived predation risk using models and vocalizations of sparrowhawks (*Accipiter nisus*). At the start of incubation, we swapped whole clutches between treated and untreated nests to separate pre-laying maternal effects from posthatching effects. Since costs and benefits of maternal responses to parasites need to be assessed under parasite pressure, we infested half of the rearing nests with hen fleas. Parasites had negative effects on mass gain and wing growth, both via maternal effects and via direct exposure of nestlings, whereas maternal predation risk had no significant effect. The interaction between predator and parasite treatments was not significant and, thus, suggests the absence of a trade-off between the 2 stressors operating at the level of maternal effects. Alternatively, the complexity of the design, despite a relatively large sample size, may have limited the power for detection of this expected trade-off.

Key words: **growth, predation risk, resource allocation, stress, trade-off.**

INTRODUCTION

Organisms often face both a risk of predation and simultaneous exposure to parasites (Rigby and Jokela 2000), which may induce costly defenses and thereby affect animal behavior and physiology (Eggers et al. 2008; Martin and Briskie 2009; Hawlena and Schmitz 2010). Allocation trade-offs may arise if these 2 types of defenses require resources from a common pool (Martens and Schon 2000; Gallizzi et al. 2008; Hesse et al. 2012). For example, investing more in a specific morphological structure (e.g., wing growth) may lower available resources required for other functions, such as immune defense, mass, or skeletal growth, etc. Responses to increased risk of predation have been shown to modify growth rates, to reduce development, or to impair immunocompetence (Hawlena and Schmitz 2010), while parasites can alter antipredator behavior (Luong et al. 2011).

Females can influence developmental trajectories and/or morphological phenotypes of offspring in response to environmental cues (Berthouly et al. 2008; Groothuis and Schwabl 2008; Sheriff et al. 2010) via maternal effects, and thereby enhance maternal fitness

potentially by enhancing offspring fitness and survival (Bernardo 1996; Saino et al. 2005; Storm and Lima 2010). It has been previously demonstrated that both predation risk and parasites can independently induce maternal effects, which then help offspring to cope with predators and parasites after birth. For example, in several bird species, females adjusted incubation behaviors and egg hormone composition if exposed to predators before laying (Hayward and Wingfield 2004; Henriksen et al. 2011; Coslovsky et al. 2012). Exposure of females to parasites before egg laying reduced the negative consequences of the parasites on nestlings (Heeb et al. 1998; Buechler et al. 2002; Tschirren et al. 2004). However, a maternal effect aimed to prepare offspring to specific environments may change the allocation of resources to different traits during growth and development (Gallizzi et al. 2008; Love et al. 2008; McGhee et al. 2012) and, thus, influence trade-offs among traits. How maternal responses to the simultaneous presence of both parasites and predators may affect nestling phenotype, growth rate, and condition remains unknown. Such interdependent effects of concurrent predation risk and parasitism were suggested in a recent study on great tit (*Parus major*) nestlings (Coslovsky and Richner 2012).

Accordingly, we investigated the effects of pre-laying exposure to the simultaneous presence of parasites and increased predation risk on allocation trade-offs in developing great tit nestlings. In order

to separate the maternally induced effects from the posthatching effects on nestlings, we transferred, at the start of incubation, whole clutches into foster nests without any previous treatment. Since the costs and benefits of maternal responses against a parasite may become visible only when nestlings are exposed to parasites (Gallizzi et al. 2008), we infested half of the foster nests with fleas. Perceived predation risk was experimentally increased by exposing great tit mothers before egg laying to models and vocalizations of sparrowhawks (*Accipiter nisus*). Concurrently, we exposed mothers to a common, nest-based ectoparasite, the hen flea (*Ceratophyllus gallinae*). As shown in previous experiments (Heeb et al. 1998; Saino et al. 2005; Coslovsky and Richner 2011), both treatments can elicit maternal responses that influence nestling growth.

The presence of a postfledging predator before egg laying may select for higher investment in traits that enhance escaping abilities of young (Steiner and Pfeiffer 2007; Storm and Lima 2010; Coslovsky and Richner 2011; Giesing et al. 2011), possibly at the expense of a reduced body mass (Hawlena and Schmitz 2010), while a parasite-induced maternal effect may reduce the negative effects of parasites on body mass (e.g., Heeb et al. 1998; Gallizzi et al. 2008). With a potential interaction between the 2 maternal effects, we would expect a trade-off in the allocation of resources for growth of offspring.

MATERIALS AND METHODS

The study was performed on a free-living population of great tits (*P. major*). Field work took place from the middle of March until the middle of June 2011 in the Bremgartenwald forest near Bern, Switzerland (46°57′N, 7°24′E). The area contains about 300 nest boxes distributed over 20 experimental plots with 14–16 nest boxes per plot. Plots were separated from each other by ca. 120 m, corresponding to about 2 great tit territories (Olioso 2004), in order to reduce the influence of a treatment in one plot on birds in a neighboring plot.

Nest boxes were visited every 3 days, from the expected beginning of the breeding season in order to record territory establishment, the start of nest building, the start of egg laying, and the beginning of incubation. From the tenth day of incubation onwards (the first expected day of hatching), nests were visited daily in the late afternoon to determine hatching day (day 0). From day 16 posthatching onwards, nests were checked twice a day, in the morning and in the late afternoon, to determine fledging date and the number of fledged nestlings.

We used a $2 \times 2 \times 2$ factorial design, that is 1) a "predation risk treatment" (PT), performed before egg laying, using either a sparrowhawk (*A. nisus*) to simulate an increased predation risk (PT+) or a song thrush (*Turdus philomelos*) as a control (PT−); 2) a "maternal parasite treatment" (MP), performed before egg laying in both predator-treated and control nests, by either infesting nests with hen fleas (MP+) or heat treating them to kill parasites (MP−); and 3) a "nestling parasite treatment" (NP) performed at the beginning of the incubation after the eggs were transferred between treatment plots and untreated ones (foster plots). This treatment also included 2 levels: nests exposed to parasites (NP+) and un-infested nests (NP−) (Figure 1).

Predation risk treatment

In order to increase the perceived predation risk, we simulated the presence of predators at the level of whole plots by exposing female great tits in plots assigned to PT+ (43 nests) to the calls of a diurnal avian predator, the sparrowhawk, played from portable loudspeakers (Fox-Pro NX3 game caller; FOXPRO Inc. Wildlife Equipment, Lewistown, PA) for 2h either in the morning or in the evening, alternated daily. Calls were accompanied by the presence of taxidermic sparrowhawk models perched alternately in 2 of 8 wooden poles planted in central locations in each plot. In the PT−, we used song thrush models and calls (35 nests). The sparrowhawk was chosen as a predator model since it is a frequent natural postfledging predator of great tits, while song thrushes are not considered either a threat or competition for great tits. Both species are naturally present in the area (personal observations).

The experimental treatment in a plot started when 5 of the nest boxes in the plot showed evidence of occupation with a layer of
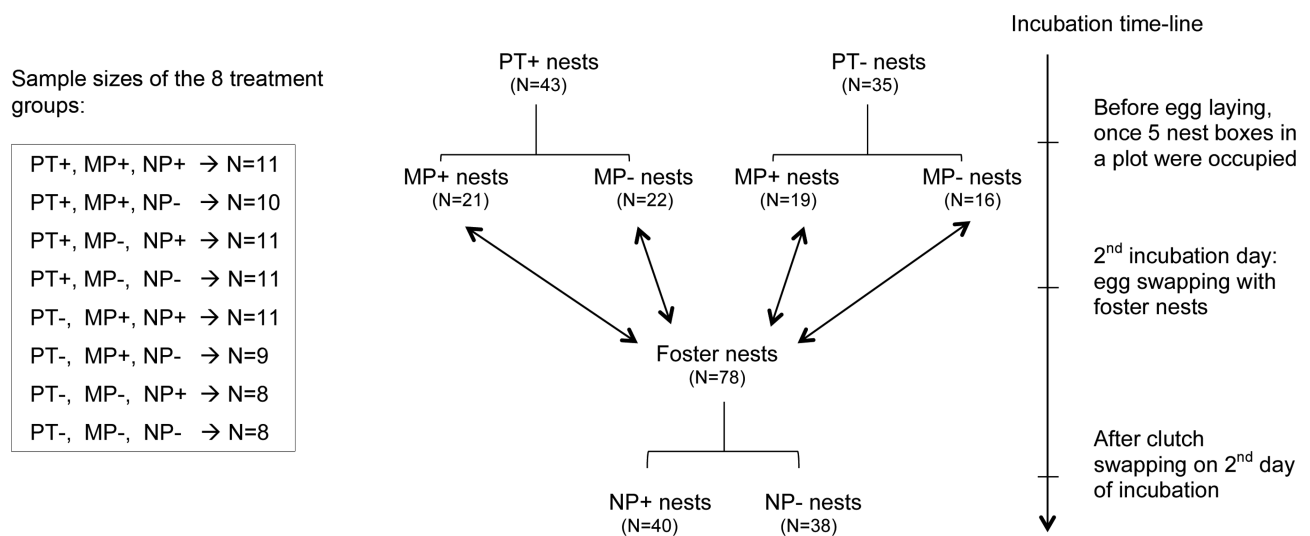

Figure 1
Graphical scheme of the experimental design and sample size. PT = predation risk treatment (PT+ = increased predation risk; PT− = control); MP = maternal parasite treatment (MP+ = infested maternal nests; MP− = parasite-free maternal nests); NP = nestling parasite treatment (NP+ = infested rearing nests; NP− = un-infested rearing nests).

fresh moss reaching 2–3 cm. Plots were sequentially allocated to the treatments according to their timing. To randomize the allocation, and to avoid differences in the timing of breeding between plots (Verhulst and Tinbergen 1991), we randomly created 5 blocks composed of 4 types of treatment groups, with 2 foster types in each block (necessary to allow a higher number of clutch exchanges). The first plot to have reached the predefined threshold was assigned to the first treatment in the first block, the second plot to the second treatment in the first block, etc. When the first 4 plots were assigned to the treatments in the first block, we assigned the following plots according to the second block and so on. In case that more than one plot reached the threshold on the same day, we rolled a dice to decide the order of assignment to one of the treatments. Overall, 5 plots were randomly assigned to the PT+, 5 to the PT−, and 10 to be foster plots. Every fifth day, the simulation was stopped in the plot for 1 day to prevent habituation. The use of vocalizations increases the detectability of the models and helps to avoid habituation (Ghalambor and Martin 2000). The remaining 10 plots (foster plots) remained untreated.

Maternal parasite treatment

Once a nest reached 2–3 cm in height, the nesting material was heat-treated in a microwave to eliminate any naturally occurring nest parasites, and the nest box was thoroughly cleaned using a hard brush to remove larvae and remaining parasites (Richner et al. 1993). This was done in all the nest boxes. Half the nests of each level of the predator treatment, PT+ and PT−, were subsequently infested with 15 male and 35 female adult hen fleas (*C. gallinae*) (MP+) (*n* = 40; 21 nests from the PT+ and 19 from the PT−). Hen fleas are naturally occurring ectoparasites in bird nests. Their prevalence in great tit nests can reach 80% (Oppliger et al. 1994). Fleas were collected in the same forest from old nesting material before the start of the breeding season and kept in a climatic chamber at 4 °C until the day they were used for the treatment. On the evening preceding the nest infestation, the 50-fleas groups were collected into plastic tubes with a small amount of humid moss and stored at 4 °C overnight. During transportation to the nest, fleas were kept on ice and were warmed up for a few minutes just before being released into the nest box. The other nests remained parasite-free (MP−) (*n* = 41).

Egg swapping

On the second day of full incubation, whole clutches of similar size (± 1 egg) and with a similar laying date (± 1 day) from the nests exposed to the PT and to the MP were swapped with clutches from foster nests. Swapping whole clutches allows disentangling the effects of the maternal treatments from posthatching effects. From the 2 maternal treatments (PT and MP), 81 pairs of nests were successfully swapped. During the exchange, the eggs from one nest were substituted with warm dummy eggs and carefully stored in small cotton-padded boxes and kept warm. Whole clutches were weighed to the nearest 0.1 g. Once all the suitable clutches in each plot were successfully swapped, the predator simulations were stopped.

Nestling parasite treatment

After clutch swapping, half of the nests in the foster plots (*n* = 41) were infested with 60 adult hen fleas (20 males and 40 females) (NP+), while the other half were left un-infested (NP−) (*n* = 40), following the same protocol described above. Fleas were removed from foster nests in the beginning of the breeding season, but not a second time before infestation.

Nestling growth

On the second day after hatching, all nestlings were weighed to the nearest 0.1 g and were individually marked by selectively removing tuft feathers from their heads, backs, and wings. This method allowed us to individually identify them until they were old enough to be permanently ringed (on day 8 posthatch). On days 8 and 15 after hatching, body mass (to 0.1 g), wing length (to 0.5 mm), and tarsus length (to 0.1 mm) were measured. On day 8, a small blood sample was collected from the nestling meta-tarsal vein for molecular sexing and to determine paternity. After collection, blood samples were transferred into 500 µl of absolute ethanol. Paternity was analyzed using 11 microsatellite loci (Saladin et al. 2003). Nestlings were considered extrapair if their genotype mismatched their putative social father's genotype at 2 or more loci. Nestlings were sexed using the sexing primers 2917/3088 (Ellegren 1996).

Statistical analysis

The statistical analysis was performed using R 2.15.1 (R Development Core Team). We used linear mixed effect models with restricted maximum likelihood to assess treatment effects on our primary outcomes—nestling morphological traits—as well as on variables such as clutch size, laying date, fledging age, hatching date, duration of the incubation, and mean egg mass per clutch. Generalized linear mixed models (GLMM; R package lme4, Bates et al. 2011) with binomial error structure were used to assess the percentage of male nestlings in a brood and the probability of extrapair paternity. For GLMMs, significance was tested via likelihood ratio tests (LRT) of nested models (Bolker et al. 2009). Plot of origin was included as random effect in all models except for fledging success, which showed considerable overdispersion. A GLMM was first used to assess the fledging success. Subsequently, and since the random effect plot of origin was not significant in a likelihood ratio test, fledging success was analyzed using a generalized linear model with a quasi-binomial error structure and no random effect. In models for nestling morphological traits, the random effects "nest of origin" nested within plot were included to control for genetic and environmental correlations between nestlings. Initial models, including all interactions between the treatments and nestling sex were tested first. Since morphological trait models include large numbers of covariates and interactions tested, our analyses may suffer an inflated risk of type I errors. As a precaution, we first compared the full model with a "null" model comprising all terms present in the full model other than treatments and their interactions using a likelihood ratio test (Forstmeier and Schielzeth 2011), before removing nonsignificant interactions from models. In these tests, significant results justify model simplification and removal of nonsignificant interactions with reduced risk of performing type I errors. Nonsignificant interactions (with *P* > 0.05) were removed to improve interpretability of main effects (Engqvist 2005). Nested models were compared by their Akaike Information Criterion (AIC) values when estimated with Maximum Likelihood.

To interpret significant interactions, models were stratified by treatment level. Main effects, including covariates and treatments, were always retained in the model. Depending on the response variable, we included other covariates, that is, hatching period, laying date, clutch, and brood size (for details of the specific models see Supplementary Table A1). Initial models are reported in the tables. In the text, the results of the best fitting models are reported.

Treatment effects on nestling mass, wing, and tarsus growth were examined with the difference in consecutive measurements as

response variable (day 15 – day 8; for mass also the difference day 8 – day 2). Fledging success was calculated as the proportion of hatchlings that fledged from each nest. Brood size was centered on its mean. Maternal body condition was estimated by the residuals of body mass regressed on tarsus length. Unless mentioned otherwise, we reported model estimates and standard errors. Parameter estimates from models with their standard errors are also shown in Figures 2–4.

As an alternative analysis, the growth of nestling morphological traits was analyzed using a repeated measurements approach.

Results of these analyses did not differ from the present report and are, thus, not reported here.

Nest desertion was categorized to no desertion, desertion in the early stages of the nest construction, desertion in late stages, and desertion after eggs were laid. We tested the association between our maternal treatments and nest desertion using Fisher's exact test.

After the egg exchange was performed, 5 out of the 162 exchanged nests had complete brood failure (3 foster nests and 2 control nests from PT− and MP−) and were removed from all analyses.

a

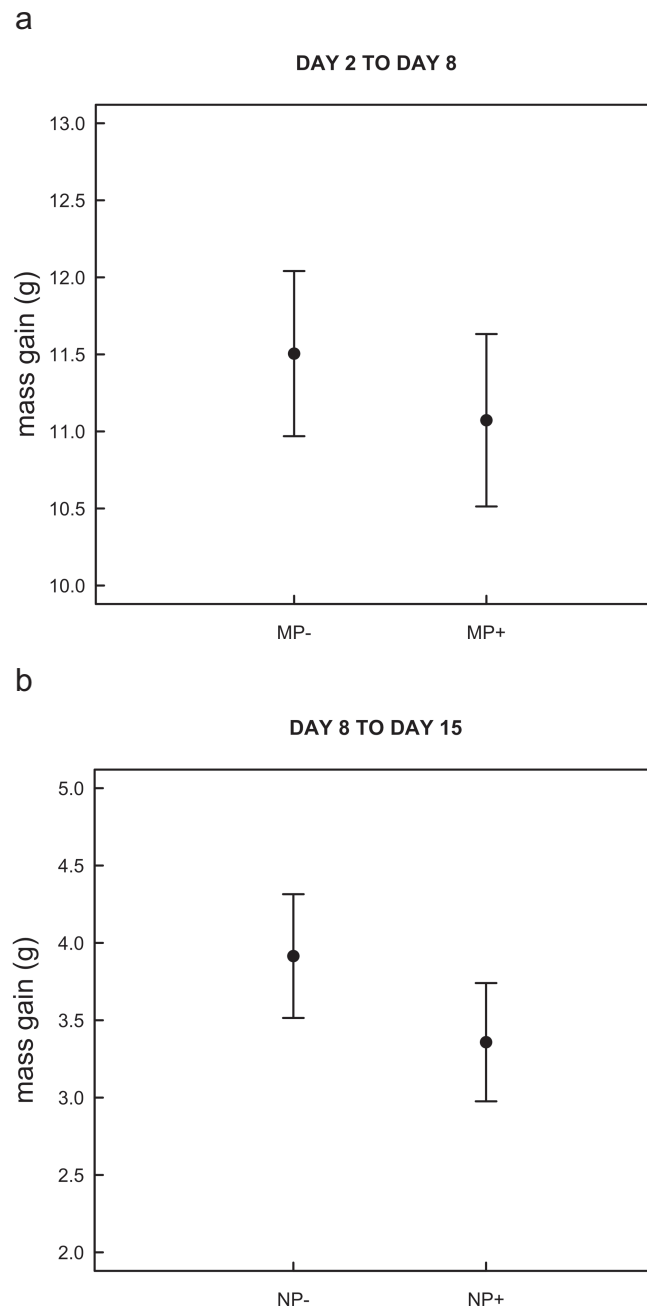**DAY 2 TO DAY 8**

b

DAY 8 TO DAY 15

Figure 2
(a) Mass gain from day 2 to day 8 (mean ± SE) of nestlings. Nestlings coming from MP+ gained less mass between day 2 and day 8 ($F_{1,63} = 3.876$, $P = 0.050$).
(b) Mass gain from day 8 to day 15 (mean ± SE) of nestlings. Nestlings between day 8 and day 15 showed a tendency for a lower mass gain in the NP+ group ($F_{1,57} = 3.275$, $P = 0.076$).
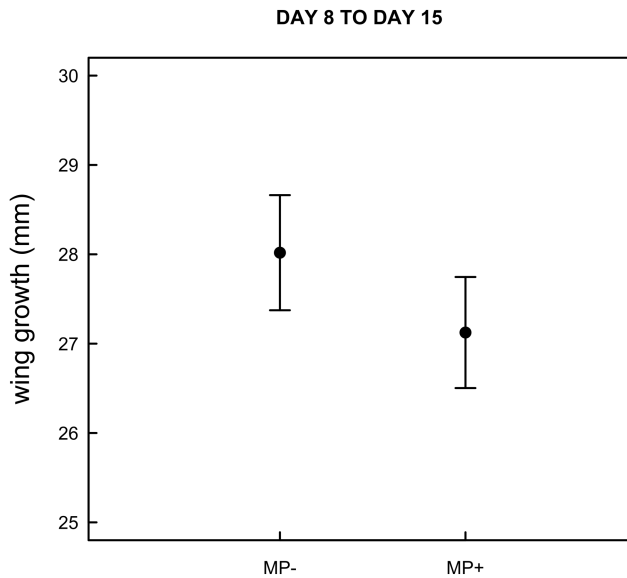
DAY 8 TO DAY 15

Figure 3
Wing growth (mean ± SE) of nestlings from MP+ and MP− from day 8 to day 15. Under MP+, nestlings showed a tendency for a lower wing growth compared to nestlings under MP− ($F_{1,56} = 3.460$, $P = 0.068$).
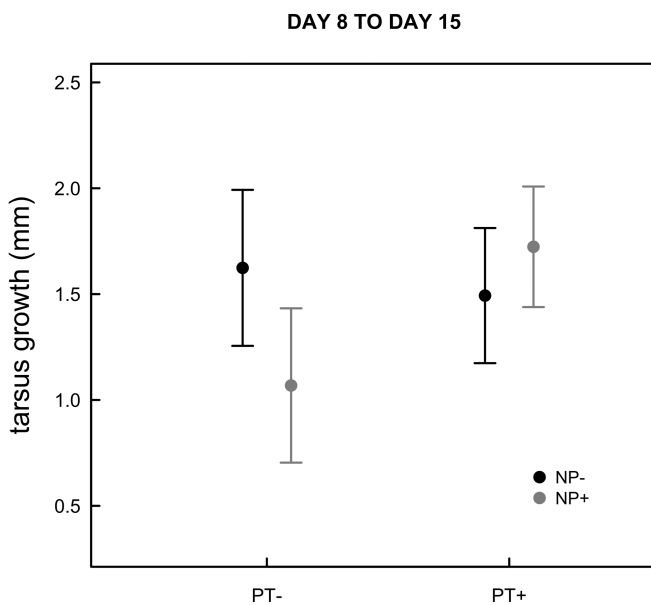
DAY 8 TO DAY 15

Figure 4
Tarsus growth (mean ± SE) of nestlings under PT− and PT+. Under PT−, tarsus growth did not significantly differ in the NP+ group compared to nestlings coming from NP− ($F_{1,18} = 2.385$, $P = 0.140$), and no significant difference between NP+ and NP− groups was found under PT+ ($F_{1,26} = 0.754$, $P = 0.393$).

Ethical note

This study was approved by the Ethical Committee of the Agricultural Office of the Canton Bern, Switzerland (experimentation permit BE 22/11 to AB). Bird catching and ringing were performed with permission of the Federal Agency for the Environment of the Canton of Bern, Switzerland (ringing permit 2992).

Steps were taken to minimize animal suffering. Bird measurements and egg manipulations never lasted more than 30 min. Our manipulation caused no apparent nest desertion. Nestlings and parents were always handled in the proximity of their nest boxes to minimize the disturbance to neighboring birds. Eggs were transferred from one nest to the other with particular attention, as previously done (Berthouly et al. 2008; Gallizzi et al. 2008). Nestlings were marked by selectively removing combinations of tuft feathers, which are naturally lost once the head feathers grow. Less than 5 µl (<1% of body mass) of blood was taken from nestlings for sex and paternity analyses. Hen fleas occur naturally in great tit nests; the numbers of fleas used to infest the parasite-treated nests resemble those found under natural conditions (Oppliger et al. 1994).

RESULTS

Nestling growth

Results of models for morphological growth are provided in Table 1 for body mass gain, Table 2 for wing growth, and Table 3 for tarsus growth.

The test comparing full model of mass gain and null model was not significant both between day 2 and day 8 and between day 8 and day 15 (day 2–8: AIC null model = 1599.064, df = 8; AIC full model = 1609.672, df = 18, LRT = 9.392; $P = 0.495$; day 8–15: AIC null model = 1492.019, df = 8; AIC full model = 1505.505, df = 18, LRT = 6.514; $P = 0.770$). Neither the 3-way interaction between the 3 treatments, nor the 2-way interaction between PT and each of the flea treatments, and between the 2 flea treatments significantly affected mass gain either between days 2 and 8, or between days 8 and 15 (Table 1). MP significantly affected mass gain between days 2 and 8, with nestlings gaining less mass in the MP+ group ($F_{1,63} = 3.876$, $P = 0.050$, Figure 2a). Nestlings between days 8 and 15 showed a tendency for a lower mass gain in the NP+ group ($F_{1,57} = 3.275$, $P = 0.076$, Figure 2b).

For wing length, the full model did not significantly differ from the null model without treatments and their interactions (AIC null model = 1924.829, df = 8; AIC full model = 1933.793, df = 18, LRT = 11.036; $P = 0.355$). Nestling originating from MP+ showed a tendency for a slower wing growth from day 8 to 15 ($F_{1,56} = 3.460$, $P = 0.068$, Figure 3). None of the other treatments or their interactions had a significant influence on wing growth (Table 2).

For tarsus length, the likelihood ratio test applied to compare full and null model was not significant (AIC null model = 1337.115, df = 7; AIC full model = 1333.532, df = 17, LRT = 16.417; $P = 0.088$). The 3-way interaction between the 3 treatments and the 2-way interactions between the 2 parasite treatments and between PT and MP were not significant in the model of tarsus growth (Table 3). The interaction between PT and NP was significant, showing differences in tarsus growth (interaction PT × NP: $F_{1,56} = 4.515$, $P = 0.038$). However, when stratifying the analysis by PT, we found no significant effect of the NP. Nestlings of PT+ had no differences in tarsus growth in the NP+ group compared to the NP− group ($F_{1,26} = 0.754$, $P = 0.393$; Figure 4), and tarsus growth did not significantly differ for nestlings of PT− according to NP treatment ($F_{1,18} = 2.385$, $P = 0.140$; Figure 4).

Incubation and eggs

For detailed tables of duration of incubation, laying date, clutch size, mean egg mass, and hatching date see Table 4. The duration of incubation in the nest of origin was not significantly associated

Table 1

Anova table of LMM for nestling mass gain from day 2 to day 8 and from day 8 to day 15, with relevant random effects nest of origin, nested in plot

Variable	Days 2–8				Days 8–15			
	Coefficient (SE)	df	F	P	Coefficient (SE)	df	F	P
(Intercept)	11.540 (0.544)	—	—	—	5.849 (0.711)	—	—	—
Brood size	**−0.282 (0.068)**	**1, 63**	**17.086**	**<0.001**	**−0.231 (0.088)**	**1, 57**	**6.875**	**0.040**
Hatching period	**−0.341 (0.237)**	**1, 63**	**2.077**	**0.154**	**−0.698 (0.333)**	**1, 57**	**4.402**	**0.040**
MP	**−0.439 (0.223)**	**1, 63**	**3.876**	**0.050**	**−0.193 (0.303)**	**1, 57**	**0.404**	**0.527**
NP	**0.102 (0.225)**	**1, 63**	**0.204**	**0.653**	**−0.565 (0.312)**	**1, 57**	**3.275**	**0.076**
PT	**−0.031 (0.224)**	**1, 63**	**0.020**	**0.888**	**−0.064 (0.306)**	**1, 57**	**0.044**	**0.834**
Sex	**0.478 (0.069)**	**1, 493**	**47.752**	**<0.001**	**0.133 (0.091)**	**1, 409**	**2.151**	**0.143**
MP × NP	0.482 (0.448)	1, 61	1.154	0.287	0.045 (0.622)	1, 55	0.005	0.942
PT × sex	0.030 (0.139)	1, 490	0.047	0.829	−0.059 (0.184)	1, 406	0.104	0.747
MP × sex	−0.103 (0.139)	1, 490	0.546	0.460	0.017 (0.184)	1, 406	0.008	0.927
NP × sex	0.179 (0.140)	1, 492	1.633	0.202	−0.237 (0.181)	1, 408	1.711	0.192
PT × NP	−0.549 (0.447)	1, 62	1.507	0.224	0.511 (0.610)	1, 56	0.701	0.406
PT × MP	−0.187 (1.027)	1, 60	<0.001	0.990	0.044 (0.642)	1, 54	0.005	0.945
PT × MP × NP	−0.661 (0.914)	1, 59	0.522	0.473	−0.209 (1.278)	1, 53	0.027	0.871

Variables in bold font were retained in the final model. Values for nonsignificant interactions represent values just before removal in backward elimination (significance level for interactions $P < 0.05$). The reference level for the coefficients is a female nestling under PT−, MP−, and NP−. Anova, analysis of variance; LMM, linear mixed effect model; SE, standard error.

Table 2

Anova table of LMM for nestling wing growth from day 8 to day 15, with relevant random effects nest of origin, nested in plot

Variable	Coefficient (SE)	df	F	P
(Intercept)	31.174 (1.143)			
Brood size	**−0.377 (0.141)**	**1, 56**	**7.118**	**0.010**
Hatching period	**−1.118 (0.536)**	**1, 56**	**4.351**	**0.041**
MP	**−0.907 (0.487)**	**1, 56**	**3.460**	**0.068**
NP	**−0.717 (0.507)**	**1, 56**	**2.003**	**0.162**
PT	**0.018 (0.491)**	**1, 56**	**0.001**	**0.971**
Sex	**0.678 (0.150)**	**1, 401**	**20.482**	**<0.001**
MP × NP	−0.326 (0.989)	1, 55	0.109	0.743
PT × Sex	0.144 (0.304)	1, 398	0.225	0.635
MP × Sex	−0.415 (0.300)	1, 399	1.911	0.168
NP × Sex	0.401 (0.299)	1, 400	1.791	0.182
PT × NP	−0.151 (1.000)	1, 53	0.023	0.880
PT × MP	−0.187 (1.027)	1, 54	0.033	0.856
PT × MP × NP	−2.293 (2.027)	1, 52	1.280	0.263

Variables in bold font were retained in the final model. Values for nonsignificant interactions represent values just before removal in backward elimination (significance level for interactions $P < 0.05$). The reference level for the coefficients is a female nestling under PT−, MP−, and NP−. Anova, analysis of variance; LMM, linear mixed effect model; SE, standard error.

with either PT or MP or their interaction. There was a significant negative association of incubation duration and laying date. Laying date did not differ significantly among maternal treatments (either PT or MP) or their interaction. Clutch size was not significantly influenced by PT but was larger under MP+.

Mean egg mass per clutch did not differ significantly among the treatments or among their interaction with laying date, but slightly increased when eggs were laid later in the season. Hatching date both in rearing nests and nests of origin was not related to the treatments or their interactions, or to clutch size.

The probability of a nest to hold at least one extrapair young was not significantly influenced by the maternal treatments (interaction PT × MP: $P = 0.346$; PT: $−0.846 \pm 0.633$, $P = 0.182$; MP: 1.026 ± 0.639, $P = 0.108$).

Fledging success and age

Fledging success was measured as the number of fledged nestlings divided by the number of hatched nestlings. The 3-way interaction

of PT × MP × NP was significant ($P = 0.050$). Fledging success of nestlings under PT+ was not significantly influenced by the interaction between MP and NP ($−0.597 \pm 1.288$, $P = 0.644$), nor by the MP ($−0.760 \pm 0.627$, $P = 0.218$) or NP ($−0.166 \pm 0.590$, $P = 0.778$). Under PT−, on the other hand, fledging success was significantly related to the interaction between the 2 flea treatments ($P = 0.024$). Indeed, under PT− and MP−, fledging success was reduced for nestlings under NP+ ($−2.792 \pm 1.438$, $P = 0.020$, Figure 5), while under PT− and MP+ fledging success was not significantly influenced by the NP (0.779 ± 0.941, $P = 0.400$, Figure 5). Fledging age was not significantly affected by the different treatments or by their interaction (Table 4).

Proportion of males

The proportion of male nestlings in a brood (8 days after hatching) was not significantly influenced by the PT and MP (PT: $−0.233 \pm 0.218$, $P = 0.286$; MP: $−0.165 \pm 0.211$, $P = 0.434$) or by their interaction (interaction PT × MP: $P = 0.102$). Also maternal

Table 3

Anova table of LMM for nestling tarsus growth from day 8 to day 15, with relevant random effects nest of origin, nested in plot

Variable	Coefficient (SE)	df	F	P
(Intercept)	2.313 (0.538)	—	—	—
Brood size	**−0.051 (0.065)**	**1, 56**	**0.624**	**0.433**
Hatching period	**0.979 (0.218)**	**1, 56**	**20.151**	**<0.001**
MP	**0.203 (0.216)**	**1, 56**	**0.883**	**0.351**
NP	**−0.611 (0.322)**	**1, 56**	**3.598**	**0.063**
PT	**−0.681 (0.305)**	**1, 56**	**4.988**	**0.029**
Sex	**0.048 (0.080)**	**1, 409**	**0.352**	**0.553**
MP × NP	0.534 (0.432)	1, 55	1.527	0.222
PT × Sex	−0.100 (0.162)	1, 406	0.378	0.539
MP × Sex	0.256 (0.161)	1, 406	2.519	0.113
NP × Sex	−0.243 (0.161)	1, 406	2.290	0.131
PT × NP	**0.915 (0.431)**	**1, 56**	**4.515**	**0.038**
PT × MP	−0.101 (0.437)	1, 54	0.053	0.819
PT × MP × NP	−1.172 (0.869)	1, 53	1.819	0.183

Variables in bold font were retained in the final model. Values for nonsignificant interactions represent values just before removal in backward elimination (significance level for interactions $P < 0.05$). The reference level for the coefficients is a female nestling under PT−, MP−, and NP−. Anova, analysis of variance; LMM, linear mixed effect model; SE, standard error.

body condition did not significantly influence the proportion of male nestlings ($−0.135 \pm 0.110$, $P = 0.219$).

Neither of the maternal treatments increased the probability of nest desertion (predator treatment: $\chi^2 = 7.141$, $P = 0.314$; flea treatment: $\chi^2 = 4.098$, $P = 0.696$).

DISCUSSION

The purpose of this experiment was to evaluate whether the combination of different sources of stress before incubation—increased perceived predation risk and the presence of parasites—may have interactive effects on reproductive investment and morphological traits in nestlings. Such interactions have been rarely investigated so far, partly due to the complexity of experimentally approaching this question. Here, we address this question using a robust experimental design in a natural setting, combined with a posthatching parasite treatment.

We found no strong evidence for interactions among the 3 treatments. Also the main effects of the treatments were not consistent. Our predictions of negative effects of parasite presence during the nestling phase (Richner et al. 1993; Christe et al. 1996), or possibly protective effect of early exposure of mothers to parasites (Heeb et al. 1998; Buechler et al. 2002), or of trade-offs between protection against parasites and the effect of maternal exposure to predators (Coslovsky and Richner 2012), were thus not confirmed.

Despite the robustness of our design, it is possible that the complexity and the required number of interactions, and the relatively small effects of each treatment alone, made it difficult to identify treatment effects even with a relatively large sample size. Despite the small effect sizes and the lack of statistical significance, we could detect some common trends in the direction of the applied treatments, regardless of the model or analysis used, at least for the main morphological traits.

The detrimental effects of parasites are visible in mass gain and wing growth. Nestlings whose mothers were exposed to parasites showed reduced mass gain in earlier phases of growth (day 2 to day 8). In contrast, the effects of parasites after hatching were visible only at later stages (day 8 to day 15) when nestlings exposed to parasites showed a tendency for slower growth. These observations are consistent with previous experiments demonstrating that

the presence of parasites affects offspring growth and development (Richner et al. 1993; Brown et al. 1995; Reed et al. 2012). Considering the direction of the estimates, the maternal effect induced by parasites was possibly stronger in the early growth phases and became nonsignificant later, even though the direction remained the same. The effect of parasites in the rearing nest was visible only later in nestling growth, where parasite load may have been larger due to a second generation of fleas born in the nest. Hen fleas need about 19 days to complete a reproductive cycle (Tripet and Richner 1999). There was thus sufficient time between infestation of rearing nest and the later nestling growth phase (days 8–15) for second-generation fleas to emerge and thus increase the effect on nestling growth during this period.

Following previous experiments, we predicted that the negative effects of the parasites on body growth would be reduced if mothers prepare their offspring to the presence of nest parasites (Gallizzi et al. 2008). However, contrary to our predictions, when mothers were exposed to parasites before egg laying, offspring in the early phases of growth did not seem able to cope better with this stressor.

Similar to the result of mass in the earlier phases, nestling wing growth showed a tendency to be slower when mothers were previously exposed to parasites, suggesting some long-term detrimental effects of parasite-induced maternal effects also after hatching (Gallizzi et al. 2008). Although not significant, the direction of the estimates related to the NP showed also in this case a negative influence of fleas on nestling wing growth.

Reduced allocation to growth is possibly determined by maternal effects that stimulate the young to increase the immune response (Soler et al. 2003; Brommer 2004). A study by Bize et al. (2003) showed a negative effect of parasites on wing development of alpine swift nestlings during the earlier phases of growth, giving evidence for the costs of parasitism to direct resources toward immune defense.

Although for an environment where the postfledging predation risk is increased, we expect higher investment in traits that could enhance the escaping abilities of fledglings, such as increased wing length at the expense of reduced body mass (Rigby and Jokela 2000; Saino et al. 2005; Hawlena and Schmitz 2010; Coslovsky and Richner 2011), neither wing growth nor mass gain were affected by the predator treatment. The effects of this treatment

Table 4

Anova table of LMM for incubation, egg measurements, and fledging age

Measurement	Variable	Coefficient (SE)	df	F	P
Duration of incubation	Intercept	14.756 (1.199)	—	—	—
	Laying date	**−0.065 (0.023)**	**1, 62**	**7.638**	**0.007**
	PT	**−0.199 (0.184)**	**1, 8**	**1.171**	**0.311**
	MP	**−0.097 (0.185)**	**1, 62**	**0.277**	**0.600**
	Clutch size	**−0.030 (0.068)**	**1, 62**	**0.191**	**0.664**
	PT X MP	0.441 (0.370)	1, 61	1.419	0.238
Laying date	Intercept	46.624 (1.147)	—	—	—
	PT	**−0.282 (1.420)**	**1, 8**	**0.039**	**0.848**
	MP	**−0.155 (0.953)**	**1, 66**	**0.027**	**0.871**
	PT × MP	−2.745 (1.907)	1, 65	2.070	0.155
Clutch size	Intercept	9.452 (1.684)	—	—	—
	Laying date	**−0.025 (0.035)**	**1, 65**	**0.503**	**0.480**
	PT	**0.227 (0.456)**	**1, 8**	**0.248**	**0.632**
	MP	**0.586 (0.287)**	**1, 65**	**4.162**	**0.045**
	PT × MP	0.924 (0.579)	1, 64	2.551	0.115
Mean egg mass	Intercept	1.237 (0.131)	—	—	—
	Laying date	**0.008 (0.003)**	**1, 65**	**7.785**	**0.007**
	PT	**−0.029 (0.024)**	**1, 8**	**1.391**	**0.272**
	MP	**−0.001 (0.024)**	**1, 65**	**0.002**	**0.961**
	PT × Laying date	0.006 (0.006)	1, 64	1.192	0.279
	MP × Laying date	−0.001 (0.006)	1, 63	0.020	0.887
Hatching date (rearing)	Intercept	63.418 (2.758)	—	—	—
	PT	**−0.287 (1.406)**	**1, 8**	**0.042**	**0.843**
	MP	**0.042 (0.763)**	**1, 64**	**0.003**	**0.956**
	NP	**−0.708 (0.770)**	**1, 65**	**0.845**	**0.362**
	Clutch size	**0.526 (0.313)**	**1, 66**	**2.822**	**0.098**
	PT × MP	−1.911 (1.534)	1, 63	1.552	0.217
	PT × NP	0.818 (1.563)	1, 61	0.274	0.602
	MP × NP	1.005 (1.563)	1, 62	0.643	0.522
	PT × MP × NP	−1.524 (3.095)	1, 60	0.242	0.624
Hatching date (origin)	Intercept	66.693 (2.951)	—	—	—
	PT	**−0.439 (1.466)**	**1, 8**	**0.090**	**0.772**
	MP	**0.194 (0.743)**	**1, 63**	**0.068**	**0.795**
	Clutch size	**0.131 (0.321)**	**1, 63**	**0.167**	**0.684**
	PT × MP	−1.096 (1.485)	1, 62	0.545	0.463
Fledging age	Intercept	16.945 (0.904)	—	—	—
	Brood size	**0.213 (0.115)**	**1, 57**	**3.447**	**0.068**
	PT	**0.334 (0.388)**	**1, 8**	**0.743**	**0.414**
	MP	**−0.120 (0.357)**	**1, 57**	**0.113**	**0.738**
	NP	**−0.489 (0.353)**	**1, 57**	**1.910**	**0.172**
	PT × MP	−0.580 (0.708)	1, 56	0.671	0.416
	PT × NP	0.255 (0.718)	1, 55	0.126	0.724
	MP × NP	−0.051 (0.729)	1, 54	0.005	0.945
	PT × MP × NP	−0.766 (1.504)	1, 53	0.259	0.613

Variables in bold font were retained in the final model. Values for nonsignificant interactions represent values just before removal in backward elimination (significance level for interactions $P < 0.05$). The reference levels for the coefficients are PT−, MP−, and NP−. Anova, analysis of variance; LMM, linear mixed effect model; SE, standard error.

may have been too small to be detected in our population or may have been concealed by the presence of parasites. Alternatively, the effects of parasite exposure on different physiological functions (Berthouly et al. 2008) may be stronger than the effects of the perceived predation risk, suggesting a hierarchy of environmental factors (Cushman and McGarigal 2004). This hierarchy likely depends on the conditions encountered during the specific breeding season, and it becomes especially relevant in case of a resource allocation trade-off.

The effect of the parasite treatments on tarsus growth was complex. The interaction between maternal PT and NP suggested that the effects of the parasite treatment on skeletal growth depended on the presence of predators. However, when stratifying the analysis on the maternal predator treatment, we found no significant differences between the parasite treatment groups, possibly due to the lower sample size in the stratified groups.

Besides the main morphological results, analyses on incubation parameters, fledging age, and fledging success showed an increase in clutch size when mothers were exposed to fleas. Ectoparasites are known to influence optimum clutch and brood sizes in hosts (Moss and Camin 1970; Heeb et al. 1998). When ectoparasites have a relatively long life cycle, parasite load per nestling will decrease with brood size via a dilution effect (Richner and Heeb 1995). Infestations during the nestling phase are often related to infestations before egg laying and females anticipating heavy parasite loads may increase clutch size to reduce the impact on each nestling.

Fledging success was influenced by the interaction of all 3 treatments. Nestling survival depended on exposure to parasites both pre-laying and during the nestling period, but only when mothers were not under increased pre-laying predation risk. As predicted, when mothers were exposed to fleas before laying, their offspring showed better resistance to the parasites' detrimental effects during growth,
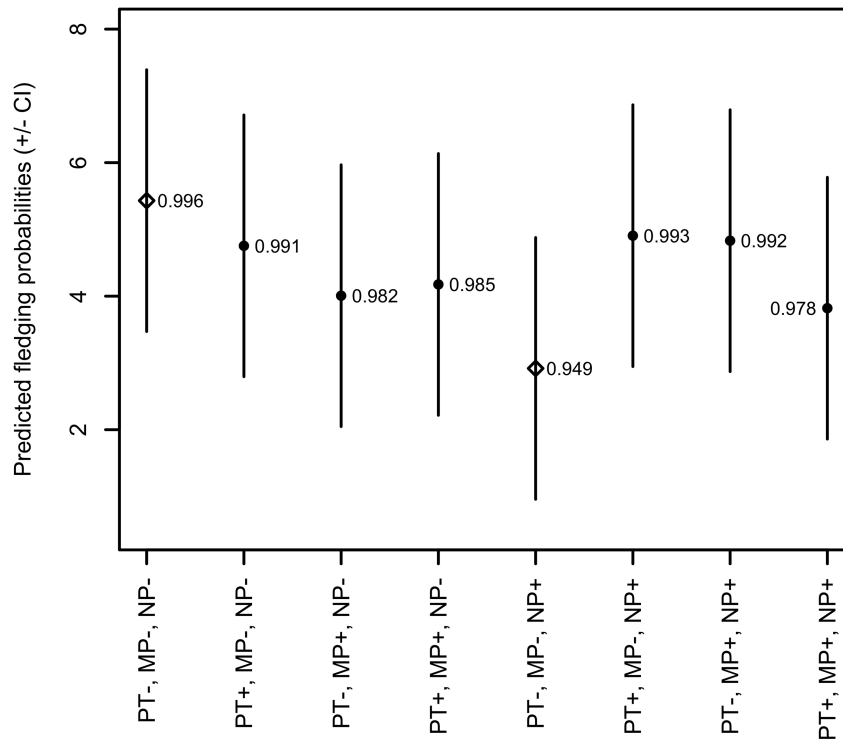
Figure 5
Predicted fledging probability (mean ± confidence interval) of nestlings from a GLM (generalized linear model) with quasi-binomial error structure. Under PT− and MP−, fledging success was reduced for nestlings under NP+ ($P = 0.020$). We provide values on the logit scale and labeled the mean with the back-transformed value for each treatment group.

as seen by similar nestling survival among infested and noninfested nests, while survival decreased for nestlings exposed to fleas during growth if their mothers had not previously been exposed to the parasite. This suggests a positive maternal effect for resisting fleas.

In summary, we found no strong interactive effects of a pre-laying parasite treatment, pre-laying predator treatment, and posthatching parasite treatment. However, the strength of these results may be limited due to the complexity of the design and its required sample sizes. Nevertheless, parasites seem to have negative effects on mass gain and wing growth both via maternal effects and via direct exposure of nestlings, as previously shown. The strength of these effects may differ with nestling age. Although we could not demonstrate that the 2 stressors interact, we cannot conclude either that these interactive effects do not exist. Since this interaction is strongly expected given that both risk of predation and exposure to parasites are common in nature, further studies with even larger sample sizes should be strongly encouraged.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

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