Manipulation of hunger levels affects great spotted cuckoo and magpie host nestlings differently

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Brood parasitic nestlings usually exhibit an exaggerated begging behaviour, which is mainly attributed to reduced inclusive fitness costs since they typically share the nest with unrelated individuals. However, energetic costs also constrain begging expression and accordingly a relation between food requirements and intensity of begging behaviour could also exist in brood parasites, just as in nesting bird species. Here, we tested this hypothesis in the great spotted cuckoo *Clamator glandarius* and its main host, the magpie *Pica pica*, by studying the effect of an appetite enhancer, cyproheptadine hydrochloride, on nestling provisioning and development (size, body mass and cell-mediated immune response). To study nestling provisioning, neck-collars were meticulously placed around nestling necks allowing normal respiration but avoiding the ingestion of food delivered by adult magpies during ca 2.5 h. Loss in body mass during neck-collar trials was used as a proxy for energetic begging costs, while the amount of food received during these trials and growth during the whole nestling period were used as variables reflecting short- and long-term effects of the experimental treatment. During neck-collar trials, we found that experimental nestlings of both species received more food than control nestlings. However, experimental magpies, but not cuckoos, lost more body mass than control nestlings. These results suggest a short-term beneficial effect of an escalated begging behaviour in both species that would be energetically cheaper for cuckoos than for magpies. We found positive long-term effects of the appetite enhancer only in magpies (in terms of tarsus and wing length at fledging, but not in terms of immune response and body mass): suggesting that exaggerated begging would be beneficial for hosts only. We discuss the possible effect of begging behaviour on the risk of predation and on inclusive fitness, but also the possibility that our results may be explained by some kind of limitation in the capability of food assimilation by parasitic species.

In species with parental care, parents use signals emitted by their offspring to regulate their effort and resource allocation (Kilner and Johnstone 1997, Mock and Parker 1997, Budden and Wright 2001). However, since parents and offspring are not genetically identical, a parent–offspring conflict on the investment of parental care arises (Trivers 1974). A set of theoretical models suggests that the resolution of this conflict, and hence the evolutionary stability of parent–offspring relationships, is achieved by a penalization cost of any exaggerated offspring behaviour (see Godfray and Johnstone 2000, for a review of resolutions of this conflict). This hypothesis is based on the prediction that any possible benefit of an increase in offspring solicitations (i.e. obtaining a greater amount of parental resources) should be counteracted by its associated costs. Accordingly, the intensity of begging signals would honestly reflect offspring food requirements caused either by the level of hunger, body condition or need (sensu Godfray 1991; see Mock et al. 2011, for a review about the possible information provided by offspring signals of solicitation).

In altricial birds, offspring solicitation is manifested as conspicuous calls and extravagant movements such as stretching of the neck, wing shivering and gaping (Redondo and Castro 1992a), but also by scramble competition between siblings (Van Heezik and Seddon 1996, Neuenschwander et al. 2003). Thus, an intensified offspring solicitation would imply additional physiological costs (Kilner 2001, Rodríguez-Gironés et al. 2001, Moreno-Rueda 2010, Noguera et al. 2010) or a greater risk of predation (Redondo and Castro 1992b, Haskell 1994, 1999, Leech and Leonard 1997, Briskie et al. 1999, Dearborn 1999, Ibáñez-Álamo et al. 2011). Moreover, an exaggerated begging behaviour would also imply a reduction in inclusive fitness by depriving food from relatives, either nest mates or future unborn siblings (Briskie et al. 1994). In the case of obligate avian brood parasites such as cuckoos and cowbirds, offspring
solicitation signals are free of inclusive fitness costs because other species (hosts) take care of the parasitic offspring (Payne 1977, Rothstein 1990, Davies 2000). Consequently begging behaviour is predicted to be cheaper for brood parasites than for nesting species (Davies and Brooke 1988, Lotem 1998, Soler et al. 1999b, Lichtenstein and Dearborn 2004) and nestlings of parasitic species could even afford an elevated begging intensity independently of their food requirements (Redondo and Zúñiga 2002, Rivers 2007, but see Soler et al. 2012). These hypotheses explain why brood parasitic nestlings typically display more exaggerated begging behaviours than those of their hosts (Davies et al. 1998, Dearborn 1998, Lichtenstein and Sealy 1998, Kilner and Davies 1999, Soler et al. 1999b, Rodriguez-Gironés et al. 2002, Hauber and Ramsey 2003). In addition, the exaggeration of the begging signals by parasite nestlings would facilitate an adequate provisioning rate in the case of evictor parasites (Kilner et al. 1999, Grim and Honza 2001, Butchart et al. 2003), and could confer advantages when competing for food with host nestlings in the case of non-evictor parasites (Soler et al. 1995, Lichtenstein and Sealy 1998).

The well founded assumption that brood parasites exaggerate their begging signals further than host nestlings does not however preclude the possibility that their begging behaviour could still be constrained by the costs associated to the production of those signals, i.e. begging behaviour by brood parasites could still be both energetically expensive to produce (Soler et al. 1999b) and attractive to predators (Arcese et al. 1996, Dearborn 1999). Thus, the intensity of begging signals in nestlings of brood parasites could still honestly reflect their food requirements (Kilner and Davies 1999, Soler and Soler 1999, Hauber 2003, Lichtenstein and Dearborn 2004, Tanaka and Ueda 2005, Soler et al. 2012). Then, although there is a robust theoretical and empirical background supporting the prediction that parasite nestlings are able to beg at superior levels than their hosts, it is still unclear how begging behaviour of brood parasitic nestlings varies according to their food requirements (Redondo and Zúñiga 2002, Lichtenstein and Dearborn 2004, Rivers 2007).

Here, we study possible interspecific differences in the costs and benefits associated to an experimental increase in the level of hunger and begging signals in nestlings of a brood parasite, the great spotted cuckoo Clamator glandarius, and those of its main host in Europe, the magpie Pica pica. Great spotted cuckoo nestlings do not evict eggs or hatchlings and as a consequence parasitized magpie nests usually harbour more than one cuckoo egg (average 2.4 eggs, Soler and Soler 1991, Soler and Soler 2000), either from the same or different females (Martínez et al. 1998). We used a new pharmacological approach consisting in the administration of an appetite stimulant, cyproheptadine hydrochloride, to increase the hunger sensation of nestlings (Martín-Gámez et al. 2011). Cyproheptadine is a type 2 serotonin receptor antagonist, which acts directly on the hunger centre sited on the hypothalamus (Stone et al. 1961, Chakrabarty et al. 1967). Cyproheptadine is broadly used in humans, including children, as a safe and effective appetite stimulant (Chinuck et al. 2007, Couluris et al. 2008, Mahachoklertwattana et al. 2009).

It is also often used in other animals, mainly in cats Felis catus (Plumb 1999), and its property as appetite stimulant has been manifested in two bird species, the domestic fowl Gallus gallus (Injidi and Forbes 1987, Muralidharan et al. 1998, but see Rao and Varadaraju 1979) and the domestic pigeon Columba livia (Gunturkun et al. 1989). In a previous study on magpie nestlings, cyproheptadine has been shown to increase the begging frequency and food provisioning of experimental nestlings compared to their control nest mates (Martín-Gámez et al. 2011). Cyproheptadine is also used as antihistaminic because it acts blocking H1 histamine receptors (Stone et al. 1961) and, like other antihistaminic substances, cyproheptadine has minimal side effects, consisting mainly on transient drowsiness (Homnick et al. 2004, and references therein) and a possible reduction of the immune response. However, the last effect may be disregarded in magpies, since we have previously found a positive effect of cyproheptadine on the immune response of magpies (Martín-Gámez et al. 2011).

Furthermore, the use of this methodology allows manipulating begging and hunger level while nestlings are raised in their natural environment. It has the advantage of taking into account several important predicted costs affecting the outcome of signalling dishonest level of hunger that cannot be controlled in laboratory conditions such as the competition between siblings (Johnstone 1999, Rodriguez-Gironés 1999, Kilner 2001, Royle et al. 2002, Neuenschwander et al. 2003), vigilance for parent arrival (Roulin 2001) or those costs related to sibling negotiation during the absence of parents (Johnstone and Roulin 2003). In consequence, and conversely to traditional methodologies based on food deprivation to increase begging signals, this experimental approach allows us to estimate variables (e.g. size, body mass or immune state at fledging) related to the net balance between benefits and costs associated with the exaggerated begging signals in natural conditions.

Briefly, our design consisted of experimental broods of two great-spotted cuckoo and two magpie nestlings of similar weight, where one nestling of each species was provided with cyproheptadine every two days along the nestling period. The other two nestlings were provided with mineral water and used as controls. Afterwards, we used neck-collars (i.e. ‘ligature method’, Johnson et al. 1980, Soler et al. 1995) during ca 2.5 h to quantify the amount of food received by each nestling and the individual change in body mass (used as an index of physiological costs of begging, Kilner 2001). Finally, at the end of the nestling period, we estimated the effect of the experimental treatment on two variables related to recruitment probability: cell-mediated immune response (Møller and Saino 2004, Cichon and Dubiec 2005, Moreno et al. 2005), and body size and mass (Magrath 1991, Gebhart-Henrich and Richner 1998, Schwagmeyer and Mock 2008). We have previously found a positive effect of cyproheptadine on these measurements at fledging in non-parasitized magpie broods. Experimental nestlings were heavier, larger and showed a better immune state than their control nestmates (Martín-Gámez et al. 2011). However, a negative effect of the cyproheptadine treatment on nestling development
can also be expected if physiological costs associated with an increased level of hunger undo the benefits of enhanced feeding by parents.

Hence, if great spotted cuckoo nestlings demand food according to their food requirements (hypothesis 1), we predict that the administration of the appetite-enhancer should have a similar effect in the nestlings of both species (prediction 1). On the other hand, if great spotted cuckoo nestlings demand food independently of their requirements (hypothesis 2), the effect of the experimental treatment should be detected only in magpie nestlings (prediction 2).

**Methods**

**Study species**

The magpie is a territorial, sedentary, and relatively long-lived passerine bird with a well-studied biology (Birkhead 1991). Magpies occur throughout large parts of the Holarctic region, where they are very abundant in some regions. They lay a single clutch between March and May with 5–7 eggs (rank 3–10). Females start to incubate before clutch completion (usually once the fourth egg is laid), which results in broods that hatch asynchronously where usually some of the smallest nestlings starve soon after hatching (Birkhead 1991, Cramp 1994).

The great spotted cuckoo is an obligate brood parasite, which in Europe mainly parasitizes magpie nests (in our study area 36.40% (n = 272) and 36.14% (n = 324) of magpie nests were parasitized during the breeding seasons of 2002 and 2003, respectively). Unlike common cuckoos, great spotted cuckoo nestlings do not evict eggs or host hatchlings (Soler and Soler 1991, Soler and Soler 2000), but some characteristics confer great spotted cuckoo nestlings with crucial advantages in the competition with host nestlings, which usually results in the starvation of host chicks in parasitized nests (Soler et al. 1996). These cuckoo characteristics are: a shorter incubation period than that of magpies (12–14 vs 21–22 d, Cramp 1985, 1994), a faster growth rate than host chicks (Soler and Soler 1991), the presence of a gape with well-developed palatal papillae (Soler et al. 1995), and an exaggerated begging behaviour compared to that of host nestlings (Soler et al. 1999b). For a more detailed review of the peculiarities of this system, see Soler and Soler (2000).

**General field procedures**

Fieldwork was carried out during the springs of 2002 and 2003 in a magpie population breeding in the Hoya of Guadix (37°18’N, 3°11’W, southern Spain). This place is a high-altitude plateau (approx. 1000 m a.s.l.) with small cereal crops (specially barley) alternated with dispersed plots of almond groves (mainly in the proximity of villages) or holm oak trees, which are both preferentially used by magpies to build their nests (Soler 1990). At the beginning of the breeding season we searched for new nests and, once a nest was finished, we visited it regularly to determine laying date. During the laying period, nests were visited at least twice a week to determine clutch size and the occurrence of brood parasitism.

**Experimental broods**

In order to reduce the probability of starvation of host nestlings due to competition with cuckoo nestlings (see above), we created experimental broods containing two great spotted cuckoo nestlings and two magpie nestlings of similar weight. We set up the experimental broods when hatchlings were 2–4 d old. Great spotted cuckoo nestlings usually hatch some days before magpie nestlings do (see above). Thus to obtain experimental broods with four nestlings of similar size, great spotted cuckoo and magpie nestlings had to come from at least two different magpie nests. The use of two, three or four different magpie nests to create experimental broods was done completely at random. Of the 97 experimental broods that we created, 21 broods held nestlings from 2 different magpie pairs, 69 from 3 different pairs, and 7 from 4 different magpie pairs. Original broods were both naturally parasitized and non-parasitized by great spotted cuckoos. Since adult magpies were not individually marked but are generally faithful to their territory (Birkhead 1991, Molina-Morales et al. 2012), we tried to avoid using the same magpie pairs to create the experimental broods during the two consecutive study years by not using the same nor adjacent magpie territories in both years.

Once the experimental brood was created, nestlings were weighed and marked by colouring one of their tarsi with non-toxic and waterproof ink (Uni Posca, PC-8k). In order to randomize our treatment assignments, nestlings of each species were ranked according to their weights and the four possible combinations of treatment assignments were alternated among nests (i.e. 1) the heaviest magpie and heaviest cuckoo received cyproheptadine; 2) the heaviest magpie and heaviest cuckoo received water; 3) the heaviest magpie and lightest cuckoo received cyproheptadine; and 4) the heaviest magpie and lightest cuckoo received water). Treatments consisted in the oral administration (by a plastic – 1 ml syringe) every two days of 0.1 mg of cyproheptadine hydrochloride (Acofarma, Barcelona, Spain) diluted in 0.25 ml mineral water to control nestlings. We calculated the dose based on the one recommended for children (0.4 mg kg⁻¹ ml⁻¹) and extrapolated this to one nestling of 100 g. Doses and drug concentration were also similar to those previously used in domestic chickens *Gallus gallus* (for more details about the use of cyproheptadine in nestlings, see Martín-Gálvez et al. 2011). After the first dose, we revisited experimental broods every two days in order to re-colour the tarsi, weigh nestlings and supply them with new doses (six times along the nesting period).

**Neck-collars trials**

We used neck-collars (Johnson et al. 1980, Soler et al. 1995) to determine the possible short-term benefits (i.e. food acquisition) and physiological costs (i.e. costs related to begging effort) associated with the experimental increase of a nestling's sensation of hunger. We applied neck-collars to all nestlings within each brood at the age of 10–12 d. Since application of neck-collars is a laborious and time-consuming process, we can also expect that physiological costs associated with an increased level of hunger undo the benefits of enhanced feeding by parents.
consuming task, neck collar trials could be performed in only a subset of experimental broods (n = 49). Neck-collars consisted of pieces (ca 80 mm) of fine wire (ø 1 mm) lined with plastic, which were meticulously placed around nestling necks allowing normal respiration but avoiding the ingestion of food delivered by adult magpies. After ca two and a half hours (141.76 ± 14.60 min (mean ± SE)), we revisited the nests to remove collars and collect food samples from nestlings’ throats. Food samples were stored in absolute ethanol until their desiccation in the laboratory (24 h at 60°C). The weight of dry samples (in mg; precision scale Adam equipment 120/0.0001g) was used as an estimation of the biomass received by each nestling. Nestlings were weighed before and after the neck-collar trial in order to determine body-mass loss (in grams). This was used as an indicator of physiological costs associated to the begging effort (Kilner 2001). Body-mass loss and the biomass of food received were standardized dividing them by the duration (in hours) of the neck-collar trial. Since collars should prevent nestlings from swallowing any food, nestlings that increased their body mass during the neck-collar period were not used for the statistical analyses. Nests in which regurgitated boluses of food were detected in the nest cup that could not be assigned to any nestlings were not included in the analyses. Only those nests with reliable food samples and body-mass loss from at least two nestlings were included in the analyses (n = 42 broods in both cases).

No nestling died or resulted injured during neck-collars trials. Moreover, we did not find any long-term negative effects on nestling development as a consequence of neck-collar trials, since all measured variables of nestlings close to fledging did not differ significantly between nests that were used for neck-collar trials (n = 49) and those that were not (n = 36; all p > 0.36; GLMMs with year of study, nestling species and collar treatment as between fixed effects; and nest identity (nested in year) as a random factor. We also included body mass before the experiment as a covariate because adult magpies preferentially feed nestlings of larger size (Soler et al. 1995) and nestling body mass could also affect weight loss and/or faecal production during the time of neck-collars (Martin-Gálvez et al. 2011). All two-way interactions were included in the initial statistical model. Final models were obtained by removing non-significant fixed effects using a sequential step-down procedure (Zuur et al. 2009). However, the interaction between treatment and nest identity, the interaction between species and nest identity and their associated main effects were always maintained in the statistical model irrespective of their significance. In this way, broods were allowed to differ in the slopes of their responses (i.e. random slope model, see for more details Schielzeth and Forstmeier 2009) and the model was controlled for random effects of nest identity (i.e. within-nests mean values were equalled to be zero, while maintaining original within-nest variance). Moreover, because we were interested in testing if the treatment affected magpie and great spotted cuckoo nestlings similarly (prediction 1) or differentially (prediction 2), we also maintained in the model the interaction between cyproheptadine treatment and species irrespective to its statistical significance. We used post-hoc LSD tests to analyse the effect of the experimental treatment within each species. Error terms were calculated by the Satterthwaite’s method, and we used the type-III sums of squares and the over-parameterized model to code the two categorical factors.

Measurements of magpie and cuckoo nestlings soon before fledging

We estimated the effect of our treatment on body size and mass and immune state of nestlings at the end of the nestling period. Because great spotted cuckoo nestlings leave the nest a few days before magpie nestlings do (Soler and Soler 1991) they were measured two days after the last dose (ca 14–16 d old) and magpie nestlings two days later (ca 16–18 d old). We weighed nestlings (Pesola 300 ± 1 g) and measured tarsi (with a digital calliper, Mitutoyo, accuracy ± 0.01 mm), wing, and tail length (with a ruler, ± 0.1 cm). At this age, we also estimated the in vivo cell-mediated immune response by measuring values of skin swelling elicited by the injection of the mitogen phytohemagglutinin (PHA-P, reference number: L8754, Sigma Chemical) (hereafter immune response). This is a commonly used measurement in evolutionary ecology to estimate cell mediated immunity (Tella et al. 2008), although it also reflects other components of the immune system such as MHC molecules (Goto et al. 1978, Martin et al. 2006). We injected fledglings subcutaneously in the right wing web with 0.5 mg of PHA dissolved in 0.1 ml of physiological saline solution (Bausch and Lomb). As a control, the left wing web was injected with 0.1 ml of physiological saline solution. Before and 24 h after the injection, we measured the thickness of each wing web at the injection site with a digital pressure-sensitive micrometer (Mitutoyo, models 547-301, ± 0.01 mm). The immune response was then estimated as the change in thickness of the right minus that of the left wing web (Lochmiller et al. 1993). We repeated measurements of each wing web three times, and used the mean values in the analyses.

After square root transformations of tarsus and tail lengths, the distribution frequencies for all measures obtained at the end of the nestling period did not significantly differ from normal distribution (Kolmogorov–Smirnov test for continuous variables, p > 0.2). Also, variances of established groups were homogeneous (Levene’s test of homogeneity of variances, p > 0.05) and therefore we used parametric statistical analyses. The effect of cyproheptadine treatment on the received biomass and body-mass loss was estimated by means of a general linear mixed model (GLMM) with year, treatment and species as fixed factors; and nest identity (nested in year and in collar treatment) considered as random effect.
body mass and immune response) was explored by using general linear models with a paired design. Nestlings were matched according to the experimental broods and measurements from these nestlings across conditions (experimental vs control or magpie vs cuckoo) were treated like repeated measures. Univariate and multivariate approaches were employed by including separately or jointly all dependent variables associated to nestling development (i.e. Wilks lambda test). This statistical approach requires a balanced design, which implied a reduction of our sample sizes. In order to use the largest possible sample size we performed two sets of statistical analyses. First, we tested for each species separately the effect of the treatment by using 68 broods and 70 broods where respectively the two magpie nestlings and the two cuckoo nestlings survived. For these analyses we included experimental treatment as a within factor. The study year (between factor) and its interaction with treatment were included in the initial model, but were removed from final models if both did not reach statistical significance. Secondly, we used the subset of experimental broods where all nestling survived (n = 61) to test if the experimental treatment did or did not affect differently both species (i.e. the interaction between species and the experimental treatment). This model had two within factors, each one of two levels: ‘species’ (great spotted cuckoo and magpie) and ‘treatment’ (experimental and control); and year of study and interactions with species and treatment as between factors. Non-significant between-factors were removed from the final model. For these analyses we included experimental treatment as a within factor. The study year (between factor) and its interaction with treatment were included in the initial model, but were removed from final models if both did not reach statistical significance. Secondly, we used the subset of experimental broods where all nestling survived (n = 61) to test if the experimental treatment did or did not affect differently both species (i.e. the interaction between species and the experimental treatment). This model had two within factors, each one of two levels: ‘species’ (great spotted cuckoo and magpie) and ‘treatment’ (experimental and control); and year of study and interactions with species and treatment as between factors. Non-significant between-factors were removed from the final model. This design allowed us to determine whether the treatment effect was similar (prediction 1) or different (prediction 2) between both species (interaction of within factors). Similarly as in the models for each species, year of study and their interactions with treatment and/or species were removed from the model only if all of these factors were statistically not significant.

All analyses were performed with STATISTICA 8.0 software (StatSoft 2008) and a sigma-restricted model was used to code categorical factors and the type VI error (the effective hypothesis method) to obtain the sums of squares.

Results

Effect of cyproheptadine during neck-collars trials

The cyproheptadine treatment resulted in an increase in the amount of food received by nestlings, and this effect did not differ between great spotted cuckoo and magpie nestlings (see treatment effect and interaction term between species and treatment in Table 1A; Fig. 1A). On the other hand, the effect of the treatment on body-mass loss was different between species (see interaction term between treatment and nestling species in Table 1B, Fig. 1B). Only experimental magpie nestlings tended to lose more body-mass than their control conspecifics during neck-collar trials.

Finally, the effect of the treatment did not differ between broods (see non-significant interaction terms between nest and treatment in Table 1A, B). Although the biomass that the nestlings received during neck-collars trials differed between years, the effect of the experiment on this variable was similar in both years (see non-significant interaction between year and treatment in Table 1A).

Effect of cyproheptadine on nestling development (body size and mass and immune response)

We did not find a global statistically significant effect of the cyproheptadine treatment on the whole suite of nestling traits in neither of the species (multivariate model, Wilks λ tests, treatment effect, Table 2). Nonetheless, when looking at nestling traits separately, experimental magpie nestlings had longer tarsi (49.28 mm ± 0.35 vs 48.44 mm ± 0.35, n = 68 broods) and longer wings (87.10 mm ± 0.91 vs 85.02 mm ± 1.15, n = 68 broods) than control ones (Table 2), but this was not the case for great spotted cuckoo nestlings (rursus length: 30.41 mm ± 0.25 vs 30.62 mm ± 0.25, n = 75 broods, wing length: 84.04 mm ± 1.19 vs 84.09 mm ± 1.36, n = 75 broods, Table 2). For both species, no effect of the treatment was found on immune response or body mass (Table 2).

Table 1. Results from GLMMs analysing the effect of cyproheptadine during neck-collars trials on the square root of biomass received per hour (A) and the square root of body-mass loss per hour (B).

<table>
<thead>
<tr>
<th>Factors</th>
<th>Effects</th>
<th>MS</th>
<th>Error MS</th>
<th>DF</th>
<th>F</th>
<th>p</th>
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<tr>
<td>A) Biomass received</td>
<td></td>
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</table>

Crosses indicate the interaction between factors.
compared to great spotted cuckoo nestlings (1.49 ± 0.05 vs 1.34 ± 0.045; F\textsubscript{1,54} = 4.90, p = 0.031).

Most of the nestling traits differed between years, except for wing length and body mass in magpie nestlings and body mass in cuckoo nestlings (Table 2). However, statistical interactions between year and treatment were not significant in most cases, except for tail length in magpie nestlings (Table 2).

Discussion

In this study, we experimentally tested whether there were different benefits and costs associated with intensified

To analyse if the treatment had different effects on nestling traits between species, we used the subset of experimental broods where all nestlings survived (n = 61 broods). The interaction between cyproheptadine and species reached significance for tarsus length (F\textsubscript{1,59} = 4.05, p = 0.049), but not for wing length (F\textsubscript{1,60} = 1.13, p = 0.29). With this subset of data we also checked for differences in nestling traits between species at the end of the nestling period. The magpie is a larger bird species than the great spotted cuckoo, and this size difference was already apparent a few days before fledging. Magpie nestlings were larger (longer tarsi, wing and tail length) and heavier compared to cuckoo nestlings (all p-values < 0.038). In addition, magpie nestlings also showed a better immune response compared to great spotted cuckoo nestlings (1.49 ± 0.05 vs 1.34 ± 0.045; F\textsubscript{1,54} = 4.90, p = 0.031).

Most of the nestling traits differed between years, except for wing length and body mass in magpie nestlings and body mass in cuckoo nestlings (Table 2). However, statistical interactions between year and treatment were not significant in most cases, except for tail length in magpie nestlings (Table 2).

Table 2. Univariable and multivariable analyses of the cyproheptadine effect on measurements associated to nestling development.

<table>
<thead>
<tr>
<th></th>
<th>Magpie nestlings</th>
<th></th>
<th></th>
<th>Cuckoo nestlings</th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MS (error)</td>
<td>DF</td>
<td>F p</td>
<td>MS (error)</td>
<td>DF</td>
<td>F p</td>
</tr>
<tr>
<td>Immune response</td>
<td>Year</td>
<td>4.77 (0.31)</td>
<td>1,66 15.48</td>
<td>&lt; 0.001</td>
<td>6.56 (0.19)</td>
<td>1,68 33.80</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>0.04 (0.15)</td>
<td>1,66 0.27</td>
<td>0.61 0.61</td>
<td>0.19 (0.21)</td>
<td>1,68 0.94</td>
</tr>
<tr>
<td></td>
<td>Year × treatment</td>
<td>0.40 (0.15)</td>
<td>1,66 0.26</td>
<td>0.61 0.61</td>
<td>0.12 (0.21)</td>
<td>1,68 0.57</td>
</tr>
<tr>
<td>(Tarsus length)\textsuperscript{1/2}</td>
<td>Year</td>
<td>0.39 (0.06)</td>
<td>1,66 6.38</td>
<td>0.014 0.014</td>
<td>0.33 (0.05)</td>
<td>1,73 6.67</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>0.12 (0.02)</td>
<td>1,66 5.43</td>
<td>0.023 0.023</td>
<td>0.01 (0.03)</td>
<td>1,73 0.42</td>
</tr>
<tr>
<td></td>
<td>Year × treatment</td>
<td>0.01</td>
<td>1,66 0.27</td>
<td>0.61 0.61</td>
<td>0.04 (0.03)</td>
<td>1,73 1.33</td>
</tr>
<tr>
<td>Wing length</td>
<td>Year</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>766 (164)</td>
<td>1,73 4.66</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>147 (31)</td>
<td>1,67 4.73</td>
<td>0.033 0.033</td>
<td>0 (79)</td>
<td>1,73 0.01</td>
</tr>
<tr>
<td></td>
<td>Year × treatment</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>146 (79)</td>
<td>1,73 1.84</td>
</tr>
<tr>
<td>(Tail length)\textsuperscript{1/2}</td>
<td>Year</td>
<td>3.28 (0.80)</td>
<td>1,65 4.10</td>
<td>0.05 0.05</td>
<td>8.23 (0.96)</td>
<td>1,73 8.54</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>0.20 (0.14)</td>
<td>1,65 1.43</td>
<td>0.24 0.24</td>
<td>0.40 (0.49)</td>
<td>1,73 0.80</td>
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<tr>
<td></td>
<td>Year × treatment</td>
<td>0.68 (0.14)</td>
<td>1,65 4.87</td>
<td>0.031 0.031</td>
<td>0.13 (0.49)</td>
<td>1,73 0.27</td>
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<tr>
<td>Body mass</td>
<td>Treatment</td>
<td>257 (167)</td>
<td>1,66 1.53</td>
<td>0.22 0.22</td>
<td>10 (247)</td>
<td>1,74 0.04</td>
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<tr>
<td></td>
<td>Wilks λ tests</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>All variables</td>
<td>Year</td>
<td>0.67</td>
<td>5,58 5.82</td>
<td>&lt; 0.001</td>
<td>0.58</td>
<td>5,64 9.40</td>
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<tr>
<td></td>
<td>Treatment</td>
<td>0.93</td>
<td>5,58 0.85</td>
<td>0.52 0.52</td>
<td>0.94</td>
<td>5,64 0.87</td>
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<tr>
<td></td>
<td>Year × treatment</td>
<td>0.91</td>
<td>5,58 1.16</td>
<td>0.34 0.34</td>
<td>0.96</td>
<td>5,64 0.61</td>
</tr>
</tbody>
</table>
solicitation signals in brood parasite and host nestlings. This was done by exploring the short and long-term effects of an appetite stimulant (cyproheptadine) in magpie and great spotted cuckoo nestlings. We found that, independently of the species, nestlings treated with cyproheptadine, and thus with an increased sensation of hunger, obtained a greater amount of food than controls. This is in accordance with previous results from magpie nestlings in non-parasitized nests (Martín-Gálvez et al. 2011), and indicates that cyproheptadine, probably through an increase of begging signals, affected food allocation by parents (Fig. 1). In fact, video-recordings in non-parasitized magpie nests confirmed an intensified begging activity in nestlings supplemented with cyproheptadine (Martín-Gálvez et al. 2011). In accordance with prediction 1, our results suggest that the intensity of begging behaviour varies according to nestling requirements of food (level of hunger), not only in the case of magpies, but also of the parasitic great spotted cuckoo.

Although there generally exists good evidence that brood parasitic nestlings do beg in relation to the time of food deprivation (i.e. hunger level, Kilner and Davies 1999, Hauber 2003, Lichtenstein and Dearborn 2004), opposite findings have been reported by two studies. The first found that begging intensity of great spotted cuckoo chicks did not vary in relation to the duration (0.5, 1 and 2.5 h) of food deprivation, while that of magpie nestlings did (Redondo and Zúñiga 2002). However, this study did not take into account the statistical interaction between species and the time of food deprivation (i.e. a differential effect of food deprivation on begging intensity depending on nestling species). In another experiment where nestlings were trained to beg for food at different rates, cuckoo nestlings consistently did increase their begging intensity depending on the duration of food deprivation (0.5, 1 and 2.5 h) (Rodríguez-Gironés pers. comm.), thereby signalling their nutritional state. Furthermore, Soler et al. (1999b) found that hand-reared great spotted cuckoo and magpie nestlings both consistently stopped begging when they had been satiated, suggesting that begging behaviour in both species could be influenced, at least partially, by the food ingested. Moreover, in a recent study, Soler et al. (2012) showed that when food is restricted, both magpie and great spotted cuckoo nestlings significantly begged more than when fed ad libitum. In addition, the begging intensity of both species increased as the food deprivation period advanced. Our results are in agreement with these latter studies, since cyproheptadine, by increasing hunger level also increased the amount of food received by experimental nestlings of both species. The other study claiming that begging behaviour of brood parasitic species does not depend on the nutritional state of nestlings was based on the finding that the effects of food deprivation on several parameters of begging behaviour of cowbird and host nestlings did not differ (Rivers et al. 2007). This was interpreted as evidence that begging intensity of parasitic offspring does not reflect their level of hunger. However, these results can also be interpreted as host and parasitic nestlings reacting in a similar way to the experiment of food deprivation and, consequently, if we assume that host nestlings beg for food honestly, these results should be considered as an evidence of honest begging behaviour of brood parasites.

Furthermore, our results indicate non-preferential feeding of cuckoos by adult magpies (species effect, Table 1A), which contradicts a previous paper where preferential allocation was reported (Soler et al. 1995). These earlier results were obtained in both natural and experimentally parasitized nests in which great spotted cuckoo nestlings were three days older than magpie nestlings (the usual situation in naturally parasitized nests) and parasitic nestlings were therefore of larger size than host nestlings. This size advantage is crucial for cuckoo nestlings to be preferentially fed by magpie adults (Soler et al. 1995). In order to increase the survival probability of all nestlings in the experimental broods, we here deliberately eliminated this initial advantage in size of cuckoos by creating experimentally nests holding magpie and cuckoo nestlings of similar weight, which would greatly reduce the preferential feeding of cuckoo nestlings by magpie adults (Soler et al. 1995).

We also estimated the short-term physiological costs associated to the experimentally increased level of hunger, which we calculated as the loss of body mass experienced by nestlings during the neck-collar trials. The significant interaction between treatment and species identity showed that only experimental magpie nestlings, but not experimental cuckoo nestlings, lost body mass at a higher rate than their conspecific controls (Fig. 1B). This result suggests that the costs associated to an escalated level of hunger differ between species. Great spotted cuckoo nestlings, by exhibiting intensified begging, would obtain more food, but pay differentially less additional physiological costs than magpie nestlings. Perhaps, these differences in begging costs between species could be due to differences in their usual begging position: magpie nestlings tend to extend their tarsi to beg for food, while great spotted cuckoo nestlings usually maintain crouched during begging and seldom rise on their tarsi (unpubl.).

Then, others factors than physiological costs should explain why great spotted cuckoo nestlings do not escalate even more their begging signals despite the advantage it confers. One possible explanation would be the increase in predation risk. For instance, Dearborn (1999) found that nests of indigo buntings Passerina cyanea with nestlings of the brood parasitic brown headed cowbirds Molothrus ater were more frequently predated than non-parasitized broods, and concluded that such difference was partially caused by the noisy begging calls of cowbird nestlings. In relation to the magpie-great spotted cuckoo system, both no differences in predation rates between parasitized and non-parasitized nests (Redondo and Zúñiga 2002) and greater predation rates for non-parasitized nests have been found (Soler et al. 1998). Also, another study previously showed that the noisiest non-parasitized magpie nests suffered predation more frequently (Redondo and Castro 1992b). The fact that great spotted cuckoo nestlings outcompete nestmates more often than cowbird nestlings (Davies 2000) result in parasitized magpie nests generally containing smaller brood sizes than non-parasitized nests (Soler and Soler 2000), which may result in nests with similar or smaller detectability to predators. However,
experimental parasitism with a great spotted cuckoo nestling of common blackbird *Turdus merula* broods (a bird species non-parasitized by this cuckoo species), caused a significant increase in predation rate (Ibáñez-Álamo et al. 2011). Then, it cannot be ruled out that great spotted cuckoos may suffer predation costs associated to their begging behaviour, which therefore might constrain the escalation begging signals. Another associated cost to exaggerated begging could be related to the loss of inclusive fitness. Great spotted cuckoo females may lay more than one egg per host nest (Martínez et al. 1998) and thus, the escalated begging behaviour of one cuckoo nestling might deprive food from relatives in the same nest (Rodríguez-Gironés et al. 2002). In any case, cuckoo nestlings could increase begging intensity without paying direct physiological costs, and this could help them to out-compete host nestlings.

The ligature or neck-collar method may however have some caveats because the difference in body mass loss between experimental and control nestlings could be due to a side effect of our drug. However, the main side effect of cyproheptadine is drowsiness, (see above) from which the expected influence on weight loss is just the opposite to the one detected (i.e. weight gain, Stiel et al. 1970). Another alternative explanation for this body mass loss could be that experimental nestlings could have produced more faecal sacs than control ones as a consequence of differences in the amount of food received before the experiment. Although adult magpies remove all faecal sacs after feeding and, thus, we cannot completely discard that possibility, a previous study under laboratory conditions showed that the weight of faeces produced by experimental and control nestlings did not differ (Martín-Gálvez et al. 2011). Furthermore, only experimental magpies lost more body mass than their control siblings (Results), even though experimental nestlings of both species were fed preferentially. Then, because these results do not suggest a link between the received biomass and body-mass loss, we believe that body-mass loss during neck-collar trials was an adequate indicator of energetic expenditure.

Finally, we also estimated the net balance between benefits and costs associated to an escalated level of hunger at the end of the nestling period of great spotted cuckoo and magpie nestlings. We previously reported a global and positive effect of cyproheptadine on magpie nestlings in non-parasitized broods soon before leaving the nest (i.e. experimental nestlings were larger tarsi, wing and tail lengths, heavier and had a better immune response than their control nestmates, Martín-Gálvez et al. 2011). In the present article, we did not detect a global effect of the cyproheptadine treatment on such measurements in neither species. Although we still found a positive significant effect of the appetite-enhancer on tarsus and wing length in magpie nestlings, these effects were not detected in cuckoos (Table 2). The reduced effect of the cyproheptadine treatment on magpie development compared to the effect in magpies of non-parasitized nests (Martín-Gálvez et al. 2011) could be related to a higher level of competition for food in the experimentally created parasitized nests of the present study. Still, the detected positive effects on tarsus and wing length suggest that the effect of cyproheptadine on magpie development was in the same direction than the one previously reported in non-parasitized broods. The fact that the cyproheptadine treatment had no effect on phenotypic variables in cuckoo nestlings close to fledging is quite interesting, because it suggests that cuckoos do not get any fitness benefits by showing an exaggerated begging behaviour. Experimental cuckoo nestlings tended to receive more food (neck-collar trials), but this advantage was not manifested in the measured nestling traits at the end of the nestling period. This indicates that cuckoos may be not as efficient as magpies allocating the extra food into growth. A physiological limitation in the processing and/or assimilation of nutrients might explain this result, but it is also possible that cuckoo nestlings invested the extra food in other not measured physiological traits. On the other hand, we found that magpie nestlings showed a better cell-mediated immune response compared to great spotted cuckoo nestlings, which is contrary to results of a previous study (Soler et al. 1999a). This result was probably also the consequence of the initial size advantage of cuckoo nestlings in the previous study, which was removed here by using cuckoo and host nestlings of similar weight (see above).

Summarizing, our results show that great spotted cuckoo and magpie nestlings received more food when treated with an appetite enhancer, which suggests that begging signals varied according to the nutritional state in the nestlings of both species. The appetite enhancer, however, increased physiological costs in magpies only, indicating that the display of an escalated hunger level may be more expensive for magpies than for cuckoos. Finally, experimental magpies, but not cuckoos, had longer tarsi and wings than their control conspecifics close to fledging. Experimental cuckoos may have been unable to translate the extra food into phenotypic advantages or may have allocated the extra food to other non-measured phenotypic traits.

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**References**


