Pied flycatcher nestlings incur immunological but not growth begging costs

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2 Short title: Nestling begging costs

3 **LAY SUMMARY**

4 Nestling begging signals should be costly in order to limit the benefits of selfish
decision to parents. Exactly which cost begging entails has been hotly debated in the
past. Here we show that begging impairs the immune system of pied flycatcher
nestlings. It also reduced nestling growth but nestlings were able to recover while
resting at night. Since most of the controversy was about growth costs, this study may
help to settle the debate.
ABSTRACT

Many theoretical models on the evolution of nestling begging assume this behavior is costly, so that only nestlings in real need of food would profit from giving intensive signals to parents. However, evidence accumulated for the last two decades is either contradictory (growth costs) or scant (immunological cost). Here, we experimentally test the existence of both costs in pied flycatcher (*Ficedula hypoleuca*) nestlings, a species in which parents appropriately respond to honest begging signals. Nestlings were paired by nest of origin and similar body mass. In each pair, a nestling was forced to beg for 51 s/meal, while the other begged for only 3.4 s/meal, both receiving the same amount of food. Simultaneously, the nestling immune response to an antigen (phytohaemagglutinin) was measured. Experimental nestlings showed reduced immunocompetence compared to control chicks, which in this species could be regarded as a genuine direct cost. High-begging nestlings also gained less mass during the daylight activity hours. However, they lost less mass while resting at night, resulting in similar mass gains for both groups across the whole daily cycle. This suggests that negative effects of excess begging upon mass gain can be compensated for by nestlings, thus avoiding the negative fitness consequences (i.e. cost) of a retarded growth. Mixed results found in previous studies may reflect interspecific differences in compensatory changes in mass gain. But if such differences do not map into fitness consequences, they may be of little help to answer the question of whether begging entails direct growth costs.
INTRODUCTION

Compared with other vertebrates, altricial nestlings show an extremely fast postnatal development (Erickson et al. 2001) and parents must sustain high rates of food provisioning in order to fulfill offspring demands (Starck and Ricklefs 1998). Food transfer from parents to nestlings is largely mediated by begging signals, a complex array of postures, vocalizations and colorful mouth structures affecting both the amount of food delivered and how it is allocated among the brood (Kilner and Johnstone 1997; Budden and Wright 2001; Kilner 2002). Suboptimal growth may have long-lasting negative consequences upon fitness (Lindström 1999; Metcalfe and Monaghan 2001). Therefore, there is an evolutionary incentive for nestlings to secure food at the expenses of their nestmates, especially when food becomes insufficient or unpredictable, as it is often the case (Leech & Leonard 1996). Nestlings may be selected to obtain a disproportionate share of the food delivered, or coerce parents into bringing food at rates that benefit them, but are harmful to parental fitness (Royle et al. 2002; Hoover & Reetz 2006). For these reasons, there is wide consensus among behavioral biologists that begging signals have evolved within this evolutionary scenario of conflicting interests among family members (Trivers 1974; Kilner and Hinde 2008).

Parents are known to rely on begging signals to make decisions about how much food deliver to the nest and how distribute it among the brood (Budden and Wright 2001, Searcy and Nowicki 2005). On the other hand, there is evidence that begging signals reliably covary with nestling hunger in a finely-graded, informative fashion (Kilner and Johnstone 1997; Searcy and Nowicki 2005). Moreover, nestlings usually beg below their maximum capacity (Chappell and Bachman 2002), despite being able of escalating begging intensity in response to factors other than nutritional need (e.g. nestmate size or
begging, Rodríguez-Gironés et al. 2002). Such evidence seems at odds with the reasonable expectation that nestlings are selected to overplay signals in order to secure more food, despite the potential harm caused to other family members (review in Mock et al. 2011).

Theoretical models have found two plausible evolutionary routes that may lead to a stable resolution of this conflict, depending on the behavioural mechanism underlying parental feeding decisions. First, nestlings may engage in a scramble competition of signals and parents passively allocate food to the offspring presenting the greatest stimulus (Macnair and Parker 1979). Second, nestlings may display ‘honest’ signals and parents actively monitor offspring begging because it conveys information about their nutritional need or hunger (which is cryptic to parents), then distributing food according to their assessment of the signals of different chicks (Godfray 1991). Both mechanisms of parental allocation may be relevant to parent-offspring communication in multiple broods (Royle et al. 2004; Andrews and Smiseth 2013). Moreover, both mechanisms may lead to a stable evolutionary equilibrium where parents give more food to nestlings that beg at higher levels and nestlings display reliable signals of need, provided that more intense begging entails a cost that reduces nestling fitness in direct proportion to the degree of signal escalation (Parker et al. 2002; Royle et al. 2002). In this scenario, begging cost is an increasing function of signal intensity (Godfray 1991; Parker et al. 2002) and offspring differing in nutritional need have different optimal signaling levels at equilibrium because of the differential marginal benefits accrued from obtaining a given amount of extra food via more intensive (costly) begging (Maynard Smith and Harper 2003; Royle et al. 2004). Honest begging by nestlings is also predicted by theoretical signaling models in which nestlings in different nutritional condition would
differ in the cost incurred by begging escalation, for example if satiated nestlings that
pretend to be hungry, begging at a higher (out-of-equilibrium) level for the same
amount of food, pay a special cost (Hurd 1995; Számadó 1999; Lachmann et al. 2001).
A final possibility is that begging is totally cost-free and an honest equilibrium is
reached because parents and offspring share overlapping interests that allow a partially
informative communicative exchange (Maynard Smith 1994; Bergstrom and Lachmann
1998; Lachmann et al. 2001). The last possibility has not yet been explored in detail by
empirical studies (Számadó 2011) but, although theoretically feasible, it may not fully
apply to the problem of how finely graded, informative begging signals may evolve
under manifest sibling competition (Brilot and Johnstone 2003; Maynard Smith and
Harper 2003; but see Lachmann et al. 2001).
Two such putative begging costs have so far been explored in some detail. First, noisy
begging may attract eavesdropping predators to the nest (McDonald et al. 2009; Haff
and Magrath 2011). This cost would be shared by all members of the brood, as long as
predators would not be selective on those nestlings begging louder, but usually kill the
entire brood. Second, nestlings begging more intensively might incur direct individual
costs, such as a higher metabolic expenditure, increased attentiveness, reduced time to
sleep or lower digestive efficiency (Moreno-Rueda 2007; Grodzinski et al. 2009). Some
models predict different results depending on whether the cost of begging is shared or
individual (e.g. Macnair and Parker 1979; Harper 1986; Motro 1989; Godfray and
Parker 1992), and it is not entirely clear whether predation costs could guarantee signal
honesty (Godfray 1995; but see Parker et al. 2002).
The most obvious way in which begging could directly reduce nestling fitness is
increasing metabolic expenditure. Begging signals involve buoyant physical
performance (vigorous posturing and calling) that must be metabolically sustained. Increased metabolic expenditure may have negative fitness consequences because it can be both energetically demanding (Chapell and Bachman 2002) and cause oxidative stress (Costantini 2014).

Considerable effort has been devoted to solve the question of whether begging is sufficiently costly in terms of energetic expenditure, but results are still inconclusive. First, several studies found that metabolic rate measured as oxygen consumption increased ca. 27% above resting metabolic rate during begging but this comprised a tiny fraction (<0.25%) of the total daily energy budget which could be easily compensated for by a few extra feedings (Chappell and Bachman 2002). Such energetic demands might, however, be of biological importance considering the limited metabolic scope of developing nestlings, and could still impact growth negatively (Verhulst and Wiersma 1997), especially under conditions of suboptimal food abundance (Leech and Leonard 1996). This idea stimulated a second wave of empirical studies covering different bird species (five passerines and a dove) aimed at finding whether actively growing nestlings that were forced to beg at high rates incurred a growth cost, but again with mixed, inconclusive results. Some studies (Kilner 2001; Rodríguez-Gironés et al. 2001) found a negative effect of begging upon nestling growth, while others (Rodríguez-Gironés et al. 2001; Leonard et al. 2003) did not. Sometimes, different studies on the same species arrived at opposite conclusions (Kedar et al. 2000; Moreno-Rueda 2010; Moreno-Rueda and Redondo 2011, 2012; Soler et al. 2014).

Several explanations have been advanced to account for this confusing mixture of empirical results. Species may differ in begging effort (Kilner 2001), growth rates and peak energy demands (Rodríguez-Gironés et al. 2001; Leonard et al. 2003), alternative
non-begging ways of sibling competition (e.g. jostling, Chappell and Bachman 2002; Moreno-Rueda 2007), or allocation to different parts (e.g. growth versus immunity) of the energy budget (Moreno-Rueda 2010). For example, in small insectivorous birds, nestlings may be less likely to incur growth costs due to their short begging bouts and tight growth constraints (Kilner 2001). Also, hole-nesting species, being less constrained by predation costs, may have evolved more flamboyant begging signals (Briskie et al. 1999) and also rely more on alternative ways of sibling competition (e.g. physical interference for favorable positions close to the nest entrance), and thus may better afford the cost of an experimentally induced begging effort. Finally, discrepancies between studies may result from differences in experimental setups, e.g. diet (Moreno-Rueda and Redondo 2012), or the time scale over which measurements of costs are made (Soler et al. 2014).

More recently, some studies have explored the possibility that begging affects metabolically demanding processes other than growth, such as immune function (Buchanan et al. 2007), in house sparrows *Passer domesticus* (Moreno-Rueda 2010; Soler et al. 2014), southern shrikes *Lanius meridionalis* (Moreno-Rueda and Redondo 2011, 2012) and magpies *Pica pica* (Moreno-Rueda et al. 2012). All these studies found that intense begging reduces immune function, providing less controversial evidence than studies on growth costs but the number of species tested is still small. Clearly, there is a need to increase the diversity of the data set before any firm conclusion can be reached.

In this study, we contribute experimental evidence supporting the existence of begging costs in nestlings of the pied flycatcher (*Ficedula hypoleuca*), a small hole-nesting, insectivorous passerine. Begging by nestling pied flycatchers reliably covaries with
nutritional need (Wright et al. 2010) and affects allocation of parental feedings (Gottlander 1987). Hence, begging can be regarded as a stable, honest signaling system in this species. Body mass at fledging is a powerful predictor of subsequent survival until reproduction (Potti et al. 2002), so there is an incentive for signal overplay too.

In this study, experimental nestlings were forced to beg for longer than their control nestmates for the same amount of food. The rationale for this experimental design lies on the following assumptions:

1) Nestlings are free to choose their optimal (equilibrium) begging level which is determined by the differential benefits of food according to need and a cost which depends on begging effort, but not need (i.e. we assume a differential benefit (Godfray 1991; Johnstone 1997), rather than a differential cost signaling model (Szamado 1999; Lachmann et al. 2001; Szamado 2011). By manipulating begging effort while holding need constant (equal food amounts given to similar-size nestlings on an identical time schedule) we expect to find measurable variations in begging cost.

2) By focusing on growth and immune costs, we assume that all nestlings, irrespective of their need, are constrained to pay similar marginal costs (Getty 2006) for a given deviation in begging effort (Szamado 2011). Cost is an intrinsic property of the signal caused by the unavoidable physical activity required to produce it, and says nothing about whether such cost is either an ‘efficacy’ or a ‘strategic’ cost (Maynard Smith & Harper 2003). In other words, even if we assume that begging may be costly at equilibrium (because needy offspring have to expend more in muscular activity), such cost should be regarded as an investment (Getty 2006) rather than a ‘handicap’ (Grafen 1990). Therefore, we expect a difference (Bergstrom & Lachmann 1998) in cost between experimental treatments which represents the cumulative marginal costs of
deviating from an equilibrial, freely chosen begging effort by control nestlings to a
higher, further away from equilibrium, enforced begging effort by experimental ones.

3) The only meaningful way to test that signal costs are balancing signal overplay at
equilibrium (assumption 1) above) is to force nestlings to beg outside their natural
signaling range (Moreno-Rueda 2007; Lachmann et al. 2001; Számadó 2011). However,
precisely which signal components should be experimentally altered, and to what
extent, are open empirical questions, nonetheless because too large experimental
alterations outside the natural range of options that nestlings can choose may provide
significant, but biologically meaningless results. In response to variations in parental
feeding rates, begging rates by pied flycatcher nestlings may vary by orders of
magnitude within a given period of time (say, hours) but begging bouts by individual
nestlings in a given feeding visit are much less variable and, by definition, are equal or
shorter than the duration of the begging bout of the whole brood. We assume that the
key component of begging effort is the duration of begging bouts, rather than the rate of
begging bouts per hour, because nestlings have the opportunity to rest and be fed in the
interval between two successive feeding visits (which may alter the benefit/cost balance
between successive bouts) and parents can more easily compare the effort of different
nestlings begging simultaneously during the same bout. Therefore, we forced
experimental nestlings to beg for much longer begging bouts while summing hourly
rates within the range of natural broods.

Results in this study showed that experimental nestlings experienced a reduced T-cell
mediated immune response. Begging also affected nestling mass gain, but only in the
short term. This finding may shed light on the complexity of growth costs and help
putting into perspective the apparently contradictory results found in previous studies.
MATERIALS AND METHODS

The study was carried out during the spring of 2013 in an extensively studied pied flycatcher population at La Hiruela (central Spain; details in Potti and Montalvo 1990; Camacho et al. 2015). The study area is an old oak (*Quercus pyrenaica*) forest provided with nest boxes. Nests were inspected regularly to determine the exact date of hatching (day 1).

During the previous year, we had obtained samples of parent and nestling behavior by placing miniature cameras (Sony Go-Pro) inside nest boxes at eleven 5 or 6-chick broods when nestlings were 7 days old. A decoy camera of identical external appearance was placed during the preceding 24 h to ensure parental habituation. From these video recordings, we measured rates of parental visits and begging bouts by the whole brood by using JWatcher 1.0 software (Blumstein and Daniel 2007). This dataset served to ensure that our experimental setup induced excess begging in experimental nestlings in a way similar to other studies (Kilner 2001; Kedar et al. 2000; Leonard et al. 2003; Moreno-Rueda 2010; Moreno-Rueda and Redondo 2011, 2012; Soler et al. 2014) but within the range of natural begging rates in wild broods.

The experiment was performed with 40 chicks from 20 nests, starting when nestlings were 7 days old, at their peak of daily mass gain. In the afternoon of the day before the experiment, we took one pair of nestlings of similar body mass from the nests, leaving at least three nestlings to prevent parental desertion. Nestlings were placed in a warm chamber and carried to a nearby laboratory. Transportation lasted about 20 min. On that afternoon, nestlings were conditioned to a begging stimulus (a playback of a parental feeding call recorded from the same population) while fed *ad libitum.*
We randomly assigned one nestling of each pair of nestmates to either a high begging (HB) or a low begging (LB) treatment. Nestlings were maintained isolated in small (5 cm diameter) cups lined with a cotton fabric. While resting, nestlings were covered with a duster, simulating brooding by the mother. This procedure precluded nestlings from begging between trials. Heating was provided by bulb lamps whose distance to artificial nests could be regulated in order to maintain a temperature close to 35 ºC within nest cups. Temperature was monitored with a probe digital thermometer. Nestlings were grouped by treatment at opposite sides of the laboratory to minimize interference caused by spontaneous begging. Both sides were chosen randomly (but not swapped between treatments) and were at equal distances from sources of environmental noise and vibrations, such as the laboratory entrance and the testing chamber (see below). Both groups of nestlings were kept under identical conditions of illumination, temperature and humidity. All this ensured that both groups of nestlings were similarly impacted by environmental stress factors which could potentially affect immune response (Romero 2004). The whole begging session started at 08:00 (local hour) and ended at 20:45. During the night, artificial nests were covered with a cardboard opaque to dim light. Previously, nestlings were weighed with a digital balance (Sartorius®; accuracy 0.01 g). We estimated the food to be ingested by nestlings according to their mass during the experimental day, following the allometric relationship calculated by Weathers (1996):

\[
\text{daily food to be consumed} = 0.98 \times M^{0.814}, \text{ where } M \text{ is nestling body mass in grams.}
\]

Daily food intake was divided into 18 equal portions corresponding to the 18 begging trials, which were performed every 45 min during a 12:45 h begging session. Any deviations from expected food intake during a trial were compensated for in subsequent
trials. Food consisted in the alternation of dipteran larvae and tiny omelette chunks that were weighed individually.

During each feeding trial, nestlings were carried to an adjacent room and stimulated to beg by using the same stimulus as before (feeding call playback). However, while LB nestlings were fed immediately after gaping, HB nestlings were repeatedly stimulated to beg for 1 min before being fed. Five begging trials, starting at 09:30 and evenly distributed every 135 minutes were recorded with a digital video camera Handycam HDR-XR155E (Sony®). A trained observer transcribed the video recordings into continuous numeric sequences of ordinal begging ranks using JWatcher 1.0 software (Blumstein and Daniel 2007). Postural begging ranks were categorized in ascending order of vigor and muscular activity: 1 (gaping, tarsi flexed), 2 (gaping, neck extended, tarsi flexed), 3 (gaping, neck extended, body up) and 4 (gaping fully stretched on extended tarsi, sometimes including wing flapping) (modified from Redondo and Castro 1992). Time spent begging (at any postural intensity) and mean ordinal rank of postural intensity were computed for each nestling at each recorded begging trial. We then computed average begging time and average postural rank for each nestling as individual measures of begging effort. Some nestlings failed to beg at all during some trials, particularly during the central hours of the day. To measure individual repeatability across trials we selected four recorded trials (excluding the central one at 14:00 h) where all nestlings begged except three LB nestlings at three different trials. Begging failures were computed as zero for estimating average time begging and postural intensity but were omitted for repeatability analyses.

Body mass of nestlings was measured three times, at 8:00 h at the start of the begging session (mass 1), at 21:00 at the end of begging trials (mass 2), and the next day, at
08:00 h, exactly 24 hours after the first measurement (mass 3). Nestlings were weighed only after they had defecated. When a nestling produced a fecal sac within the next 15 minutes after it was weighed, it was discounted. Diurnal mass gain during the whole begging session was estimated as mass 2 minus mass 1. Nocturnal (negative) mass gain was estimated as mass 3 minus mass 2. Total daily mass gain was estimated as mass 3 minus mass 1. We computed mass lost attributable to metabolic expenditure ($M_{EE}$) during begging as $M_{EE} = M_I - M_G - M_F$, where $M_I$ is food mass ingested, $M_G$ is mass gained, and $M_F$ is the mass of feces. Then, we calculated the difference $D_{EE} = M_{EE}(HB) - M_{EE}(LB)$ between pairs of nestmates as an estimate of the marginal metabolic cost of begging (Kilner 2001). Two nestlings (one HB and one LB) vomited between two consecutive trials and were excluded from $M_{EE}$ analyses. Fecal sacs were not weighed individually but collected in closed containers and kept refrigerated until weighed. Two measures of cumulative fecal mass were taken, at the end (21:00 h) and ca. the middle (13:00 h) of the whole begging session.

We also measured how the experimental treatment affected cell-mediated immune response. Immediately before the onset of the experiment (07:30 h), we injected into the left patagium of each chick 0.2 mg of phytohaemagglutinin (PHA-P, L-8754, Sigma Aldrich) diluted in 0.04 ml of isotonic phosphate buffer (following Moreno et al. 2005). PHA-P is an innocuous protein which induces an immune response in birds causing a swelling of the patagium skin that is positively correlated with the strength of the T-cell mediated immune response (Kennedy and Nager 2006), although other components of the immune system are also involved (Martin et al. 2006). Previously, we had measured (three times) the patagium thickness with a pressure-sensitive micrometer (Mitutoyo®; accuracy: 0.01 mm). At the end of the day (21:00 h) and at the end of the experiment
(24 h later), we again measured the patagium thickness, calculating the T-cell mediated
immune response as the difference between measurements. Patagium measurements
were performed by a trained person blind with regard to nestling treatment.

The day after the experiment, nestlings were fed *ad libitum* again, marked with non-
toxic waterproof ink and returned back to their nests during the morning. On the
following days, we regularly checked nests to monitor the fate of chicks used in the
experiment. With one exception, all nestlings looked well until they were ringed when
13 days old. One nestling died of starvation some days after the experiment had
concluded.

For statistical analyses, we performed Linear Mixed Effects Models of Restricted
Maximum Likelihood (REML-LMM; Zuur et al. 2009), by using the package "nlme"
(Pinheiro et al. 2012) in R (R Development Core Team 2012). In each model, nest of
origin was introduced as a random factor to control for variance among nests, thus
avoiding problems of statistical independence (Hurlbert 1984). In some models, initial
body mass and food consumed were introduced as predictors to examine possible
effects on the dependent variables. For every model, we checked for homoscedasticity
(residuals vs. fitted plots), and log-transformed the variable "time begging" in order to
fulfill homoscedasticity requirements. We also checked visually for normality of
residuals (normal quantile plots), which never deviated from a normal distribution
according to Kolmogorov-Smirnov tests (Quinn and Keough 2002). Means are given
with one standard error (SE). The complete dataset can be found in Supplementary
Material.

The experimental procedure was approved by the CSIC Ethical Committee (ref.
CGL2011-29694) and the Andalusian Committee of Animal Experimentation (ref.
to comply with Spanish and European legislation on the protection of animals used for scientific purposes.

RESULTS

The experimental treatment succeeded at making HB nestlings beg for much longer bouts (51.2 s) than LB nestlings (3.4 s), and also at higher postural intensities (Table 1). Most of the variation in begging time and intensity was explained by treatment (Table 2). Individual repeatabilities within treatment across four begging trials were low, which comes at no surprise considering that treatment involved the experimenter either enforcing (HB) or preventing (LB) begging by nestlings. Video recordings from nests showed that pied flycatcher broods in the wild begged with short begging bouts similar to LB nestlings (4.8 s ± 1.23 SE, total range 0.1-21.5, range of mean values per brood 3.1-8.5). Therefore, begging effort per bout of HB nestlings substantially exceeded the natural range in this species (Fig. 1). Since experimental nestlings were stimulated to beg once every 45 min, hourly begging rates of HB nestlings (68 s/h) were within the range of wild broods (135 s/h ± 29.4 SE, range 21-344).

The effect of begging on cell-mediated immune response

Chicks begging for longer and with higher intensity had a smaller diurnal immune response than LB nestlings (Table 1). Food consumed or initial body mass did not affect initial immune response ($F_{1, 19} = 2.48, P = 0.13; F_{1, 19} = 2.85, P = 0.11$, respectively), and their inclusion in the model did not remove the significant effect of treatment on initial immune response ($\chi^2 = 27.80, P < 0.001$). Final immune response remained significantly smaller in HB nestlings than in LB nestlings (Fig. 2). Again, food consumed and initial body mass did not affect final immune response (respectively, $F_{1,}$
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$19 = 2.51, P = 0.13; F_{1, 19} = 0.26, P = 0.62$), and their inclusion in the model did not qualitatively affect the results ($\chi^2 = 10.05, P = 0.0015$). Immune response decreased during the night (paired t-test, $t = -2.36, P = 0.023$), irrespective of treatment (Table 1).

Food consumed and initial body mass did not affect the change in immune response during the night (respectively, $F_{1, 19} < 0.01, P = 0.95; F_{1, 19} = 0.85, P = 0.37$), and their inclusion in the model did not qualitatively alter the results, although rendered them marginally non-significant ($\chi^2 = 2.97, P = 0.08$).

**The effect of begging on mass gain and metabolic expenditure**

There were no differences in initial body mass or food consumed between high begging (HB) and low begging (LB) nestlings (Table 1). Nestlings in the HB treatment gained significantly less mass than LB nestlings during the diurnal phase of active begging when begging trials took place (diurnal mass gain; Table 1). Diurnal mass gain was not significantly affected by the amount of food consumed ($F_{1, 19} = 0.46, P = 0.51$) or initial body mass ($F_{1, 19} = 0.24, P = 0.63$). Therefore, the effect of treatment on diurnal mass gain remained significant when the amount of food consumed and initial body mass were included in the model ($\chi^2 = 7.82, P = 0.005$). However, total mass gain during the 24 h period did not significantly differ between treatments (Table 1). Neither was total mass gain during 24 h affected by food consumed ($F_{1, 19} = 0.02, P = 0.89$) or initial body mass ($F_{1, 19} = 1.06, P = 0.32$), and their inclusion in the model did not change the results ($\chi^2 = 0.20, P = 0.65$). The reason for the discrepancy between diurnal and total mass gain was that HB nestlings lost significantly less mass during the night resting phase than LB nestlings (Table 1). In this case, initial body mass negatively influenced nocturnal mass loss ($\beta = -0.39, F_{1, 19} = 6.82, P = 0.017$). There was no effect of food consumed ($F_{1, 19} = 2.19, P = 0.15$). Nonetheless, the effect of treatment on nocturnal
mass loss remained significant even after controlling for initial body mass and consumed food ($\chi^2 = 4.84, P = 0.028$). Summarizing, HB nestlings grew less during the daylight, active begging time but also lost less mass during the night, resulting in similar growth rates for both treatments during a 24 h period (Fig. 3).

There were no differences in metabolic expenditure $M_{EE}$ according to treatment (Table 1). $M_{EE}$ comprised a larger fraction of diurnal mass gain in HB than in LB nestlings (40.0 % ± 4.04 and 32.5 % ± 5.08, respectively) but differences were not significant ($F_{1, 17} = 1.16, P = 0.29$). The mean marginal metabolic cost of begging ($D_{EE}$) was 0.09 g (± 0.087 SE, N = 18) of mass lost by HB nestlings attributable to begging. $D_{EE}$ was neither correlated with differences between nestlings in a pair (HB-LB) in time begging ($r = -0.10$, NS) nor differences in postural intensity ($r = 0.05$, NS).

The effect of begging on fecal mass production

Experimental treatment had no effect on total fecal mass excreted at the end of the begging session. However, HB nestlings excreted a marginally ($P = 0.061$) larger fraction of cumulative fecal mass during the first half of the begging session than LB nestlings (Table 1). Among HB nestlings, the amount of time spent begging had a positive, marginally significant effect on the amount of feces excreted during the first half of the begging session, after controlling for food intake ($\beta = 0.39, F_{1, 19} = 3.98, P = 0.062$) but this trend was not evident in the control LB group ($\beta = -0.04, F_{1, 19} = 0.02, P = 0.88$). This suggests that begging had a mild, short-term effect upon the digestive dynamics of nestlings.

DISCUSSION
Compared with their LB nestmates in the control group, experimental HB nestlings invested a great deal in begging effort. Begging bouts of LB nestlings (3.4 s) were similar in duration to begging bouts at wild nests (5 s). Sustained begging bouts of HB nestlings were 15 times longer (51 s) and well above the maximum duration of begging bouts recorded in the wild (21.5 s). It is reasonable to assume that variation in the duration of begging bouts recorded at natural broods may in part reflect variations in nestling nutritional need, with well-fed chicks begging at shorter durations. Nestlings in this study received ca. 0.5 g of food per g of body mass over a 24 h period (Table 1). This food amount is above the average estimated for a 7-days old, 9 g pied flycatcher nestling in the wild (0.44 g per g of body mass) and close to the highest food intake (0.67 g/g) established in a previous experimental study testing the effects of nutritional need upon begging and digestion (Wright et al. 2010). Therefore, as both LB and HB nestlings could be considered as belonging to a well-fed category of signalers, the long begging bouts performed by HB nestlings, above the range recorded at natural broods, can be considered as out-of-equilibrium signals (Számadó 2011).

Hourly begging rates of experimental nestlings were within the range observed at wild broods. Some previous studies on growth costs also reported keeping hourly begging rates of enforced HB nestlings within the natural range of wild broods (Kilner 2001; Leonard et al. 2003; Moreno-Rueda et al. 2012). Others did not report natural begging rates but adjusted their testing schedule to approach natural rates of feeding visits by parents (Kedar et al. 2000; Moreno-Rueda 2010; Moreno-Rueda and Redondo 2011, 2012). Comparatively, the HB/LB ratio of begging duration in this study (15) was higher (6, Kilner 2001; 3.5 and 5, Rodriguez-Gironés et al. 2001; 6, Leonard et al. 2003; 6.7, Moreno-Rueda et al. 2012) or similar (16.7 Moreno-Rueda and Redondo 2011; 14.1
Moreno-Rueda and Redondo 2012) to those reported by previous studies. Only in two
of them did the HB/LB time ratio exceeded that in this study (24, Moreno-Rueda 2010;
36, Soler et al. 2014). In summary, experimental HB nestlings in this study were forced
to beg at least as hard as in studies that did show a growth cost (Kilner 2001;
Rodríguez-Gironés et al. 2001; Moreno-Rueda and Redondo 2011; Moreno-Rueda et al.
2012). Mass gain measured during 24 h in the laboratory (0.86 g) was lower than in
the field at the same age (1.36 g), which is common for hand-reared wild birds and can
be attributed to general stress caused by husbandry practices (Flammer and Clubb 1994)
even if fed with high-quality diets, as in this study.

Excess begging experimentally induced had a two-fold impact upon nestling
physiology, reducing both the mass gain during activity hours and immune response.
Such effects can hardly be explained in terms of metabolic expenditure (measured as
mass lost during the begging session). Metabolic expenditure was similar between HB
and LB nestlings, either in absolute terms or as a fraction of diurnal mass gain and
differences in begging effort between pairs of nestlings had no effect upon mass lost
attributable to begging. The small, non-significant effect of excess begging upon
metabolic expenditure is consistent with previous respirometry studies (Chappell and
Bachman 2002). As an alternative to energetic expenditure, vigorous physical
exercising associated to intensive begging may cause oxidative stress (Noguera et al.
2010; Boncoraglio et al. 2012; Costantini 2014). Both growth and immune function
contribute to the production of free radicals (Alonso-Álvarez et al. 2007; Costantini and
Møller 2009; Sorci and Faivre 2009), and nestlings sustaining a high begging effort may
be able to momentarily downregulate other oxidative processes in order to keep up with
oxidative balance (Moreno-Rueda et al. 2012). A final possibility is that it is not the
physiological processes leading nestlings to beg with greater effort (Buchanan et al. 2007). Some studies have found that endogenous levels of testosterone and glucocorticoids may simultaneously promote more intense begging and reduce both growth and immunocompetence in pied flycatchers (Goodship and Buchanan 2006, 2007) and other birds (Quillfeldt et al. 2006; Buchanan et al. 2007; Loiseau et al. 2008).

The fact that experimental nestlings in this study begged not only for longer, but also at higher postural intensities, is consistent with this last possibility. Effects of endogenous hormones on begging, growth and immune response may vary among different species (Smiseth et al. 2011) and this variation could provide an additional explanation for the different results obtained in previous studies about the impact of begging on growth.

T-cell mediated immune response was highly impacted by experimental treatment at the end of the begging session (Cohen’s d = 1.15) and also, but with a milder effect, 24 hours later (Cohen’s d = 0.64). This change is expected in phytohaemagglutinin assays (Navarro et al. 2003) as a result of a rapid (3-12 h) transient infiltration of heterophils and lymphocytes in the injected tissue, later followed by macrophage infiltration (ca. 24 h), the swelling typically remitting after 48 h (Smits et al. 1999; Martin et al. 2006).

Experimentally induced excess begging has been shown to impair T-cell mediated immune response in each and every species tested so far: house sparrows (Moreno-Rueda 2010; Soler et al. 2014), magpies (Moreno-Rueda et al. 2012), southern shrikes (Moreno-Rueda and Redondo 2011, 2012), and pied flycatchers (this study). By contrast, Romano et al. (2011) found that female barn swallow (Hirundo rustica) nestlings injected with an antigen (LPS, a bacterial lipopolysaccharide) increased their begging intensity. However, their study was not specifically designed to test the effect
of enforced begging on immune response and chicks were allowed to beg freely. This
detail is of critical importance, as long as marginal begging costs predicted by signaling
models can be evaluated only by manipulating nestlings into begging above their
preferred (out-of-equilibrium) levels (Kilner 2001; Számadó 2011). Furthermore,
Romano et al. (2011) measured the begging response of nestlings two days after the
inoculation, when nutritional condition of nestlings (body mass, feather quality and
gape coloration) had already deteriorated as a result of the immune challenge.
Therefore, begging response in that study might be affected by both immune challenge
and nestling condition (Jacob et al. 2011). One potential drawback of studies showing
an impact of enforced begging upon immune response is that, to make experimental HB
nestlings beg with greater effort, they were stimulated more than their LB nestmates
(Moreno-Rueda 2010; Moreno-Rueda and Redondo 2011, 2012; Moreno-Rueda et al.
2012; Soler et al. 2014; this study). More stimulation may cause stress, which may
elevate glucocorticoid levels and depress immune response (Saino et al. 2003). Some
studies on growth costs (Kedar et al. 2000; Kilner 2001) also suffer from the same
drawback but not others (Rodriguez-Gironés et al. 2001; Leonard et al. 2003). There is
not an obvious relationship in these studies between stimulation schedule and whether
they found a significant impact on growth (Kilner 2001; Rodríguez-Gironés et al. 2001)
or not (Kedar et al. 2000; Leonard et al. 2003). Glucocorticoids may affect both growth
and immune response simultaneously (Saino et al. 2003), which suggests that
differential stimulation may not be the only cause of reduced immune response in HB
nestlings. However, the possibility that experimenter-induced stress may affect results
in studies of immunological costs of begging should be addressed in future studies.
If, as suggested by this and previous studies, there is a generalized immunological impact of excess begging, this effect could be qualified as a genuine direct begging cost of the type hypothesized by begging models. First, cost should affect the nestling fitness function by reducing viability (Számadó 2011). Downregulating immune function may reduce nestling survival prospects in case of a parasitic infection. Pied flycatcher nestlings from Iberian populations suffer from a high (above 20%) prevalence of haematozoan and arthropod nest-dwelling ectoparasites (Merino and Potti 1995, 1996). Nestlings showing a reduced immune response are worse at coping with an eventual parasitic infection (Tschirren et al. 2007; Pitala et al. 2010), and indeed have a reduced viability in the long term (Cichoń and Dubiec 2005; Bowers et al. 2014). A second assumption of signaling models is that marginal begging costs should not be offset by marginal benefits (i.e. extra food), thus rendering fitness returns negative for nestlings begging above the honest equilibrium level (Számadó 2011). T-cell immune response at fledging is a better predictor of survival to first reproduction than body mass or condition in pied flycatchers (Moreno et al. 2005). This implies that gaining mass by begging intensively may be of little use if immune function is impaired as a result of signal overplay. Furthermore, it is not immediately obvious whether obtaining extra food would compensate for an impaired immune response. In an experimental study in which we simultaneously manipulated marginal benefits and costs of extra begging, we found that southern shrike nestlings receiving 30% extra food were able to compensate for the impact of excess begging on growth, but immune response was affected independently of the amount of food ingested (Moreno-Rueda and Redondo 2012). We therefore conclude that excess begging is costly for pied flycatcher nestlings as a result of impaired immunocompetence.
The impact of excess begging on mass gain was large at the end of the begging session (Cohen’s $d = 0.65$) but had virtually vanished the morning after (Cohen’s $d = 0.06$). Nestlings that had begged intensively and gained less mass during the daylight hours were capable of a compensatory regulation of mass loss while resting later at night. At least two possible mechanisms might be involved in the compensatory phase of nocturnal mass loss. First, a large fraction (ca. 3/4) of a nestling energy budget is allocated to dissipative processes such as maintenance and thermoregulation (Verhulst and Wiersma 1997). During the night, HB nestlings may have reduced basal metabolism or heat production in favor of growth (Moe et al. 2004; Vézina et al. 2009; Killpack and Karasov 2012). Second, experimental treatment may have affected digestive dynamics, allowing HB nestlings to delay food assimilation. For example, pigeons are able to postpone digestion at low temperatures in order to use the extra heat from digestion-related thermogenesis (Laurila et al. 2003). Unfortunately, we did not keep a continuous record of the daily changes in fecal production throughout the begging session neither we measured fecal production the morning after. But we found a marginally significant trend for HB nestlings to excrete a lower fraction of the total fecal mass on the second half of the begging session. In canaries *Serinus canaria*, enforced begging during a relatively short (6 h) trial also increased fecal sac production (Kilner 2001). Alternatively, the lower diurnal mass gain of HB nestlings may have been a consequence of a lower digestive efficiency (Kilner 2001; Budden & Wright 2008; Grodzinski et al. 2009; Wright et al 2010) that would have been compensated for during the night, when begging ceased. Does excess begging have an impact on pied flycatcher nestling mass gain? The answer to this question will depend on the time scale of measurement. It did after 13 h of
begging, but not 11 h afterwards. Other studies have also found begging effects upon mass gain to be dependent on time scale or age. For example, canaries showed an impact of begging on M_{EE} at 8 days, but not at 6 or 10 days, and HB-LB differences in mass gain during a 24 h period also varied with age (Fig. 2 in Kilner 2001). Magpies sustaining high begging rates for three consecutive days also showed an impact on mass gain at 24 h, but not at 48-72 h (Moreno-Rueda et al. 2012). In shrikes, the impact of begging on mass gain over 24 h depended on the amount and quality of ingested food (Moreno-Rueda & Redondo 2011, 2012). And house sparrows showed an impact after 60 h of sustained begging effort but not at 6, 12, 72, 84 and 108 h (Kedar et al. 2000; Moreno-Rueda 2010; Soler et al. 2014). Two of these studies found an impact of excess begging upon body condition measured as residuals of a regression of body mass against wing length at 72 h (Kedar et al. 2000) and between 48 and 108 h (Soler et al. 2014). However, this last finding may prove difficult to interpret in biologically meaningful terms. First, body condition measured as Model I regression residuals may suffer from several statistical hindrances, such as slope overestimation and lack of allometric linearity, especially for growing nestlings (Green 2001). Second, variations in nestling body mass relative to structural size may reflect patterns of mass allocation to different body parts in response to a variety of environmental conditions (Potti 1999, 2000; Szép and Møller 2000).

The possibility exists that mixed results found in previous studies on the effect of begging on nestling growth are not simply the outcome of differences in experimental setups, but actually reflect the ability of nestlings to show flexible variations in mass gain in response to excess begging according to different circumstances. But if begging impact on mass gain can be compensated for under many situations then it fails to
qualify as a genuine direct cost (Számadó 2011). This raises the interesting question of why growth in some species is more easily affected by begging than in others. However, begging-induced growth variations may not offer a universal explanation for the cost predicted by signaling models. Immunological costs seem a promising avenue for future studies, particularly those aimed at testing differential benefit signaling models.
REFERENCES


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FIGURE LEGENDS

Figure 1. Duration of sustained begging bouts per trial by High-Begging experimental nestlings (HB), Low-Begging control nestlings (LB) and broods in the wild. Shown are medians, interquartile range (boxes) and 10th-90th percentiles (error bars).

Figure 2. Changes in T-cell mediated immune response (patagium swelling) to an antigen (PHA) of High-Begging (HB) and Low-Begging (LB) nestlings after 13 and 24 h of inoculation. Error bars are SE around means.

Figure 3. Changes in body mass of High-Begging (black dots) and Low-Begging (open dots) at the onset of the experiment (0 h), after 13 h of active begging (diurnal phase) and 24 h afterwards. Asterisks indicate significant differences \((P = 0.005)\) between groups. Error bars are SE around means.
TABLES

Table 1

Mean ± SE for each variable measured in the study and the effect of Treatment (fixed), controlling for Nest (random).

<table>
<thead>
<tr>
<th></th>
<th>LB (n = 20)</th>
<th>HB (n = 20)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body mass (g)</td>
<td>7.79 ± 0.21</td>
<td>7.76 ± 0.22</td>
<td>0.05&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Consumed food (g)</td>
<td>3.93 ± 0.08</td>
<td>3.96 ± 0.07</td>
<td>0.60&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Feces mass (g)</td>
<td>1.25 ± 0.08</td>
<td>1.32 ± 0.10</td>
<td>0.32&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Feces mass at 13:00 h (%)</td>
<td>24.1 ± 2.85</td>
<td>30.6 ± 1.91</td>
<td>3.96&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Time begging (s/trial)</td>
<td>3.43 ± 0.32</td>
<td>51.18 ± 2.57</td>
<td>662.7&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Begging postural intensity</td>
<td>1.78 ± 0.07</td>
<td>2.14 ± 0.04</td>
<td>21.70&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diurnal mass gain (g)</td>
<td>2.02 ± 0.06</td>
<td>1.85 ± 0.06</td>
<td>7.46&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nocturnal mass gain (g)</td>
<td>-1.16 ± 0.05</td>
<td>-1.00 ± 0.04</td>
<td>5.21&lt;sub&gt;*&lt;/sub&gt;</td>
</tr>
<tr>
<td>Total mass gain (g)</td>
<td>0.87 ± 0.09</td>
<td>0.85 ± 0.07</td>
<td>0.02&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Metabolic expenditure (g)</td>
<td>1.78 ± 0.11</td>
<td>1.73 ± 0.08</td>
<td>0.24&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Diurnal immune response (mm)</td>
<td>4.21 ± 0.19</td>
<td>3.41 ± 0.19</td>
<td>28.30&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nocturnal immune response (mm)</td>
<td>-0.43 ± 0.21</td>
<td>-0.15 ± 0.12</td>
<td>1.90&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td>Final immune response (mm)</td>
<td>3.78 ± 0.22</td>
<td>3.25 ± 0.14</td>
<td>11.31&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>F</sup> values from Restricted Maximum Likelihood Estimation Linear Mixed Models (REML-LMM). Degrees of freedom df = 1,19, except for Metabolic Expenditure (df = 1,17). Time Begging was log transformed prior to analyses.

<sup>P</sup>-values: * <i>P < 0.05</i>, **<i>P < 0.01</i>, ***<i>P < 0.001</i>, ns for not significant.
Table 2

ANOVA estimation of variance components due to Treatment (fixed) and Nestling (random) of Time Begging and Postural Intensity.

<table>
<thead>
<tr>
<th></th>
<th>Time Begging</th>
<th></th>
<th>Postural Intensity</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>df</td>
<td>MS</td>
<td>F</td>
<td>MS</td>
</tr>
<tr>
<td>Effect</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>2</td>
<td>6989.65</td>
<td>7642.64***</td>
<td>315.28</td>
</tr>
<tr>
<td>Nestling(Treatment)</td>
<td>38</td>
<td>0.92</td>
<td>3.64***</td>
<td>0.26</td>
</tr>
<tr>
<td>Error</td>
<td>117</td>
<td>0.25</td>
<td></td>
<td>0.11</td>
</tr>
</tbody>
</table>

Repeatability (intraclass correlation coefficient)

<table>
<thead>
<tr>
<th></th>
<th>Time Begging</th>
<th>Postural Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB</td>
<td>0.45***</td>
<td>0.52***</td>
</tr>
<tr>
<td>LB</td>
<td>0.22*</td>
<td>0.33***</td>
</tr>
</tbody>
</table>

Repeatability of begging variables across four begging trials for each treatment level (HB and LB) indicates the fraction of total variance explained by Nestling (random).

Time begging was log transformed prior to analyses.

P-values: * P < 0.05, ***P < 0.001.
Figure 1

![Box plot showing begging bout durations for High, Low, and Wild broods]

- **Begging bout duration (s)**
  - **High**
  - **Low**
  - **Wild broods**

The box plot displays the distribution of begging bout durations for three different groups: High, Low, and Wild broods. The box plot includes the median, quartiles, and outliers for each group.
Figure 2

![Bar chart showing patagium thickness (mm) at different times after PHA injection. The chart compares HB and LB groups, with error bars indicating variability.]
Figure 3

![Graph showing the relationship between Nestling mass (g) and Time (h).](image)

- **Time (h)**: 0, 12, 24
- **Nestling mass (g)**: 7.5, 8.0, 8.5, 9.0, 9.5, 10.0

**Note:** The graph indicates a significant difference at the 0.01 level (marked with **).
LAY SUMMARY

Nestling begging signals should be costly in order to limit the benefits of selfish
deception to parents. Exactly which cost begging entails has been hotly debated in the
past. Here we show that begging impairs the immune system of pied flycatcher
nestlings. It also reduced nestling growth but nestlings were able to recover while
resting at night. Since most of the controversy was about growth costs, this study may
help to settle the debate.