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Pied flycatcher nestlings incur immunological but not growth begging costs

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1   **Pied flycatcher nestlings incur immunological but not growth begging costs**

2   Short title: Nestling begging costs

3   **LAY SUMMARY**

4   Nestling begging signals should be costly in order to limit the benefits of selfish

5   deception to parents. Exactly which cost begging entails has been hotly debated in the

6   past. Here we show that begging impairs the immune system of pied flycatcher

7   nestlings. It also reduced nestling growth but nestlings were able to recover while

8   resting at night. Since most of the controversy was about growth costs, this study may

9   help to settle the debate.

10   **ABSTRACT**

11   Many theoretical models on the evolution of nestling begging assume this behavior is

12   costly, so that only nestlings in real need of food would profit from giving intensive

13   signals to parents. However, evidence accumulated for the last two decades is either

14   contradictory (growth costs) or scant (immunological cost). Here, we experimentally

15   test the existence of both costs in pied flycatcher (*Ficedula hypoleuca*) nestlings, a

16   species in which parents appropriately respond to honest begging signals. Nestlings

17   were paired by nest of origin and similar body mass. In each pair, a nestling was forced

18   to beg for 51 s/meal, while the other begged for only 3.4 s/meal, both receiving the

19   same amount of food. Simultaneously, the nestling immune response to an antigen

20   (phytohaemagglutinin) was measured. Experimental nestlings showed reduced

21   immunocompetence compared to control chicks, which in this species could be regarded

22   as a genuine direct cost. High-begging nestlings also gained less mass during the

23   daylight activity hours. However, they lost less mass while resting at night, resulting in

24   similar mass gains for both groups across the whole daily cycle. This suggests that

25   negative effects of excess begging upon mass gain can be compensated for by nestlings,

26   thus avoiding the negative fitness consequences (i.e. cost) of a retarded growth. Mixed

27   results found in previous studies may reflect interspecific differences in compensatory

28   changes in mass gain. But if such differences do not map into fitness consequences,

29   they may be of little help to answer the question of whether begging entails direct

30   growth costs.

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33 **INTRODUCTION**

34 Compared with other vertebrates, altricial nestlings show an extremely fast postnatal

35 development (Erickson et al. 2001) and parents must sustain high rates of food

36 provisioning in order to fulfill offspring demands (Starck and Ricklefs 1998). Food

37 transfer from parents to nestlings is largely mediated by begging signals, a complex

38 array of postures, vocalizations and colorful mouth structures affecting both the amount

39 of food delivered and how it is allocated among the brood (Kilner and Johnstone 1997;

40 Budden and Wright 2001; Kilner 2002). Suboptimal growth may have long-lasting

41 negative consequences upon fitness (Lindström 1999; Metcalfe and Monaghan 2001).

42 Therefore, there is an evolutionary incentive for nestlings to secure food at the expenses

43 of their nestmates, especially when food becomes insufficient or unpredictable, as it is

44 often the case (Leech & Leonard 1996). Nestlings may be selected to obtain a

45 disproportionate share of the food delivered, or coerce parents into bringing food at

46 rates that benefit them, but are harmful to parental fitness (Royle et al. 2002; Hoover &

47 Reetz 2006). For these reasons, there is wide consensus among behavioral biologists

48 that begging signals have evolved within this evolutionary scenario of conflicting

49 interests among family members (Trivers 1974; Kilner and Hinde 2008).

50 Parents are known to rely on begging signals to make decisions about how much food

51 deliver to the nest and how distribute it among the brood (Budden and Wright 2001,

52 Searcy and Nowicki 2005). On the other hand, there is evidence that begging signals

53 reliably covary with nestling hunger in a finely-graded, informative fashion (Kilner and

54 Johnstone 1997; Searcy and Nowicki 2005). Moreover, nestlings usually beg below

55 their maximum capacity (Chappell and Bachman 2002), despite being able of escalating

56 begging intensity in response to factors other than nutritional need (e.g. nestmate size or

57 begging, Rodríguez-Gironés et al. 2002). Such evidence seems at odds with the

58 reasonable expectation that nestlings are selected to overplay signals in order to secure

59 more food, despite the potential harm caused to other family members (review in Mock

60 et al. 2011).

61 Theoretical models have found two plausible evolutionary routes that may lead to a

62 stable resolution of this conflict, depending on the behavioural mechanism underlying

63 parental feeding decisions. First, nestlings may engage in a scramble competition of

64 signals and parents passively allocate food to the offspring presenting the greatest

65 stimulus (Macnair and Parker 1979). Second, nestlings may display 'honest' signals and

66 parents actively monitor offspring begging because it conveys information about their

67 nutritional need or hunger (which is cryptic to parents), then distributing food according

68 to their assessment of the signals of different chicks (Godfray 1991). Both mechanisms

69 of parental allocation may be relevant to parent-offspring communication in multiple

70 broods (Royle et al. 2004; Andrews and Smiseth 2013). Moreover, both mechanisms

71 may lead to a stable evolutionary equilibrium where parents give more food to nestlings

72 that beg at higher levels and nestlings display reliable signals of need, provided that

73 more intense begging entails a cost that reduces nestling fitness in direct proportion to

74 the degree of signal escalation (Parker et al. 2002; Royle et al. 2002). In this scenario,

75 begging cost is an increasing function of signal intensity (Godfray 1991; Parker et al.

76 2002) and offspring differing in nutritional need have different optimal signaling levels

77 at equilibrium because of the differential marginal benefits accrued from obtaining a

78 given amount of extra food via more intensive (costly) begging (Maynard Smith and

79 Harper 2003; Royle et al. 2004). Honest begging by nestlings is also predicted by

80 theoretical signaling models in which nestlings in different nutritional condition would

81   differ in the cost incurred by begging scalation, for example if satiated nestlings that

82   pretend to be hungry, begging at a higher (out-of-equilibrium) level for the same

83   amount of food, pay a special cost (Hurd 1995; Számadó 1999; Lachmann et al. 2001).

84   A final possibility is that begging is totally cost-free and an honest equilibrium is

85   reached because parents and offspring share overlapping interests that allow a partially

86   informative communicative exchange (Maynard Smith 1994; Bergstrom and Lachmann

87   1998; Lachmann et al. 2001). The last possibility has not yet been explored in detail by

88   empirical studies (Számadó 2011) but, although theoretically feasible, it may not fully

89   apply to the problem of how finely graded, informative begging signals may evolve

90   under manifest sibling competition (Brilot and Johnstone 2003; Maynard Smith and

91   Harper 2003; but see Lachmann et al. 2001).

92   Two such putative begging costs have so far been explored in some detail. First, noisy

93   begging may attract eavesdropping predators to the nest (McDonald et al. 2009; Haff

94   and Magrath 2011). This cost would be shared by all members of the brood, as long as

95   predators would not be selective on those nestlings begging louder, but usually kill the

96   entire brood. Second, nestlings begging more intensively might incur direct individual

97   costs, such as a higher metabolic expenditure, increased attentiveness, reduced time to

98   sleep or lower digestive efficiency (Moreno-Rueda 2007; Grodzinski et al. 2009). Some

99   models predict different results depending on whether the cost of begging is shared or

100   individual (e.g. Macnair and Parker 1979; Harper 1986; Motro 1989; Godfray and

101   Parker 1992), and it is not entirely clear whether predation costs could guarantee signal

102   honesty (Godfray 1995; but see Parker et al. 2002).

103   The most obvious way in which begging could directly reduce nestling fitness is

104   increasing metabolic expenditure. Begging signals involve buoyant physical

105   performance (vigorous posturing and calling) that must be metabolically sustained.

106   Increased metabolic expenditure may have negative fitness consequences because it can

107   be both energetically demanding (Chapell and Bachman 2002) and cause oxidative

108   stress (Costantini 2014)

109   Considerable effort has been devoted to solve the question of whether begging is

110   sufficiently costly in terms of energetic expenditure, but results are still inconclusive.

111   First, several studies found that metabolic rate measured as oxygen consumption

112   increased ca. 27% above resting metabolic rate during begging but this comprised a tiny

113   fraction (<0.25%) of the total daily energy budget which could be easily compensated

114   for by a few extra feedings (Chappell and Bachman 2002). Such energetic demands

115   might, however, be of biological importance considering the limited metabolic scope of

116   developing nestlings, and could still impact growth negatively (Verhulst and Wiersma

117   1997), especially under conditions of suboptimal food abundance (Leech and Leonard

118   1996). This idea stimulated a second wave of empirical studies covering different bird

119   species (five passerines and a dove) aimed at finding whether actively growing nestlings

120   that were forced to beg at high rates incurred a growth cost, but again with mixed,

121   inconclusive results. Some studies (Kilner 2001; Rodríguez-Gironés et al. 2001) found a

122   negative effect of begging upon nestling growth, while others (Rodríguez-Gironés et al.

123   2001; Leonard et al. 2003) did not. Sometimes, different studies on the same species

124   arrived at opposite conclusions (Kedar et al. 2000; Moreno-Rueda 2010; Moreno-Rueda

125   and Redondo 2011, 2012; Soler et al. 2014).

126   Several explanations have been advanced to account for this confusing mixture of

127   empirical results. Species may differ in begging effort (Kilner 2001), growth rates and

128   peak energy demands (Rodríguez-Gironés et al. 2001; Leonard et al. 2003), alternative

129   non-begging ways of sibling competition (e.g. jostling, Chappell and Bachman 2002;

130   Moreno-Rueda 2007), or allocation to different parts (e.g. growth versus immunity) of

131   the energy budget (Moreno-Rueda 2010). For example, in small insectivorous birds,

132   nestlings may be less likely to incur growth costs due to their short begging bouts and

133   tight growth constraints (Kilner 2001). Also, hole-nesting species, being less

134   constrained by predation costs, may have evolved more flamboyant begging signals

135   (Briskie et al. 1999) and also rely more on alternative ways of sibling competition (e.g.

136   physical interference for favorable positions close to the nest entrance), and thus may

137   better afford the cost of an experimentally induced begging effort. Finally, discrepancies

138   between studies may result from differences in experimental setups, e.g. diet (Moreno-

139   Rueda and Redondo 2012), or the time scale over which measurements of costs are

140   made (Soler et al. 2014).

141   More recently, some studies have explored the possibility that begging affects

142   metabolically demanding processes other than growth, such as immune function

143   (Buchanan et al. 2007), in house sparrows *Passer domesticus* (Moreno-Rueda 2010;

144   Soler et. al 2014), southern shrikes *Lanius meridionalis* (Moreno-Rueda and Redondo

145   2011, 2012) and magpies *Pica pica* (Moreno-Rueda et al. 2012). All these studies found

146   that intense begging reduces immune function, providing less controversial evidence

147   than studies on growth costs but the number of species tested is still small. Clearly,

148   there is a need to increase the diversity of the data set before any firm conclusion can be

149   reached.

150   In this study, we contribute experimental evidence supporting the existence of begging

151   costs in nestlings of the pied flycatcher (*Ficedula hypoleuca*), a small hole-nesting,

152   insectivorous passerine. Begging by nestling pied flycatchers reliably covaries with

153   nutritional need (Wright et al. 2010) and affects allocation of parental feedings

154   (Gottlander 1987). Hence, begging can be regarded as a stable, honest signaling system

155   in this species. Body mass at fledging is a powerful predictor of subsequent survival

156   until reproduction (Potti et al. 2002), so there is an incentive for signal overplay too.

157   In this study, experimental nestlings were forced to beg for longer than their control

158   nestmates for the same amount of food. The rationale for this experimental design lies

159   on the following assumptions:

160   1) Nestlings are free to choose their optimal (equilibrium) begging level which is

161   determined by the differential benefits of food according to need and a cost which

162   depends on begging effort, but not need (i.e. we assume a differential benefit (Godfray

163   1991; Johnstone 1997), rather than a differential cost signaling model (Számadó 1999;

164   Lachmann et al. 2001; Számadó 2011). By manipulating begging effort while holding

165   need constant (equal food amounts given to similar-size nestlings on an identical time

166   schedule) we expect to find measurable variations in begging cost.

167   2) By focusing on growth and immune costs, we assume that all nestlings, irrespective

168   of their need, are constrained to pay similar marginal costs (Getty 2006) for a given

169   deviation in begging effort (Számadó 2011). Cost is an intrinsic property of the signal

170   caused by the unavoidable physical activity required to produce it, and says nothing

171   about whether such cost is either an 'efficacy' or a 'strategic' cost (Maynard Smith &

172   Harper 2003). In other words, even if we assume that begging may be costly at

173   equilibrium (because needy offspring have to expend more in muscular activity), such

174   cost should be regarded as an investment (Getty 2006) rather than a 'handicap' (Grafen

175   1990). Therefore, we expect a *difference* (Bergstrom & Lachmann 1998) in cost

176   between experimental treatments which represents the cumulative marginal costs of

177   deviating from an equilibrial, freely chosen begging effort by control nestlings to a

178   higher, further away from equilibrium, enforced begging effort by experimental ones.

179   3) The only meaningful way to test that signal costs are balancing signal overplay at

180   equilibrium (assumption 1) above) is to force nestlings to beg outside their natural

181   signaling range (Moreno-Rueda 2007; Lachmann et al. 2001; Számadó 2011). However,

182   precisely which signal components should be experimentally altered, and to what

183   extent, are open empirical questions, nonetheless because too large experimental

184   alterations outside the natural range of options that nestlings can choose may provide

185   significant, but biologically meaningless results. In response to variations in parental

186   feeding rates, begging rates by pied flycatcher nestlings may vary by orders of

187   magnitude within a given period of time (say, hours) but begging bouts by individual

188   nestlings in a given feeding visit are much less variable and, by definition, are equal or

189   shorter than the duration of the begging bout of the whole brood. We assume that the

190   key component of begging effort is the duration of begging bouts, rather than the rate of

191   begging bouts per hour, because nestlings have the opportunity to rest and be fed in the

192   interval between two successive feeding visits (which may alter the benefit/cost balance

193   between successive bouts) and parents can more easily compare the effort of different

194   nestlings begging simultaneously during the same bout. Therefore, we forced

195   experimental nestlings to beg for much longer begging bouts while summing hourly

196   rates within the range of natural broods.

197   Results in this study showed that experimental nestlings experienced a reduced T-cell

198   mediated immune response. Begging also affected nestling mass gain, but only in the

199   short term. This finding may shed light on the complexity of growth costs and help

200   putting into perspective the apparently contradictory results found in previous studies.

MATERIALS AND METHODS

201

202   The study was carried out during the spring of 2013 in an extensively studied pied

203   flycatcher population at La Hiruela (central Spain; details in Potti and Montalvo 1990;

204   Camacho et al. 2015). The study area is an old oak (*Quercus pyrenaica*) forest provided

205   with nest boxes. Nests were inspected regularly to determine the exact date of hatching

206   (day 1).

207   During the previous year, we had obtained samples of parent and nestling behavior by

208   placing miniature cameras (Sony Go-Pro) inside nest boxes at eleven 5 or 6-chick

209   broods when nestlings were 7 days old. A decoy camera of identical external

210   appearance was placed during the preceding 24 h to ensure parental habituation. From

211   these video recordings, we measured rates of parental visits and begging bouts by the

212   whole brood by using JWatcher 1.0 software (Blumstein and Daniel 2007). This dataset

213   served to ensure that our experimental setup induced excess begging in experimental

214   nestlings in a way similar to other studies (Kilner 2001; Kedar et al. 2000; Leonard et

215   al. 2003; Moreno-Rueda 2010; Moreno-Rueda and Redondo 2011, 2012; Soler et al.

216   2014) but within the range of natural begging rates in wild broods.

217   The experiment was performed with 40 chicks from 20 nests, starting when nestlings

218   were 7 days old, at their peak of daily mass gain. In the afternoon of the day before the

219   experiment, we took one pair of nestlings of similar body mass from the nests, leaving

220   at least three nestlings to prevent parental desertion. Nestlings were placed in a warm

221   chamber and carried to a nearby laboratory. Transportation lasted about 20 min. On that

222   afternoon, nestlings were conditioned to a begging stimulus (a playback of a parental

223   feeding call recorded from the same population) while fed *ad libitum*.

224   We randomly assigned one nestling of each pair of nestmates to either a high begging

225   (HB) or a low begging (LB) treatment. Nestlings were maintained isolated in small (5

226   cm diameter) cups lined with a cotton fabric. While resting, nestlings were covered with

227   a duster, simulating brooding by the mother. This procedure precluded nestlings from

228   begging between trials. Heating was provided by bulb lamps whose distance to artificial

229   nests could be regulated in order to maintain a temperature close to 35 ºC within nest

230   cups. Temperature was monitored with a probe digital thermometer. Nestlings were

231   grouped by treatment at opposite sides of the laboratory to minimize interference caused

232   by spontaneous begging. Both sides were chosen randomly (but not swapped between

233   treatments) and were at equal distances from sources of environmental noise and

234   vibrations, such as the laboratory entrance and the testing chamber (see below). Both

235   groups of nestlings were kept under identical conditions of illumination, temperature

236   and humidity. All this ensured that both groups of nestlings were similarly impacted by

237   environmental stress factors which could potentially affect immune response (Romero

238   2004). The whole begging session started at 08:00 (local hour) and ended at 20:45.

239   During the night, artificial nests were covered with a cardboard opaque to dim light.

240   Previously, nestlings were weighed with a digital balance (Sartorius®; accuracy 0.01 g).

241   We estimated the food to be ingested by nestlings according to their mass during the

242   experimental day, following the allometric relationship calculated by Weathers (1996):

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

244   Daily food intake was divided into 18 equal portions corresponding to the 18 begging

245   trials, which were performed every 45 min during a 12:45 h begging session. Any

246   deviations from expected food intake during a trial were compensated for in subsequent

247   trials. Food consisted in the alternation of dipteran larvae and tiny omelette chunks that

248   were weighed individually.

249   During each feeding trial, nestlings were carried to an adjacent room and stimulated to

250   beg by using the same stimulus as before (feeding call playback). However, while LB

251   nestlings were fed immediately after gaping, HB nestlings were repeatedly stimulated to

252   beg for 1 min before being fed. Five begging trials, starting at 09:30 and evenly

253   distributed every 135 minutes were recorded with a digital video camera Handycam

254   HDR-XR155E (Sony®). A trained observer transcribed the video recordings into

255   continuous numeric sequences of ordinal begging ranks using JWatcher 1.0 software

256   (Blumstein and Daniel 2007). Postural begging ranks were categorized in ascending

257   order of vigor and muscular activity: 1 (gaping, tarsi flexed), 2 (gaping, neck extended,

258   tarsi flexed), 3 (gaping, neck extended, body up) and 4 (gaping fully stretched on

259   extended tarsi, sometimes including wing flapping) (modified from Redondo and Castro

260   1992). Time spent begging (at any postural intensity) and mean ordinal rank of postural

261   intensity were computed for each nestling at each recorded begging trial. We then

262   computed average begging time and average postural rank for each nestling as

263   individual measures of begging effort. Some nestlings failed to beg at all during some

264   trials, particularly during the central hours of the day. To measure individual

265   repeatability across trials we selected four recorded trials (excluding the central one at

266   14:00 h) where all nestlings begged except three LB nestlings at three different trials.

267   Begging failures were computed as zero for estimating average time begging and

268   postural intensity but were omitted for repeatability analyses.

269   Body mass of nestlings was measured three times, at 8:00 h at the start of the begging

270   session (mass 1), at 21:00 at the end of begging trials (mass 2), and the next day, at

271 08:00 h, exactly 24 hours after the first measurement (mass 3). Nestlings were weighed

272 only after they had defecated. When a nestling produced a fecal sac within the next 15

273 minutes after it was weighed, it was discounted. Diurnal mass gain during the whole

274 begging session was estimated as mass 2 minus mass 1. Nocturnal (negative) mass gain

275 was estimated as mass 3 minus mass 2. Total daily mass gain was estimated as mass 3

276 minus mass 1. We computed mass lost attributable to metabolic expenditure (M_{EE})

277 during begging as $M_{EE} = M_I - M_G - M_F$, where M_I is food mass ingested, M_G is mass

278 gained, and M_F is the mass of feces. Then, we calculated the difference $D_{EE} = M_{EE}(HB)$

279 - $M_{EE}(LB)$ between pairs of nestmates as an estimate of the marginal metabolic cost of

280 begging (Kilner 2001). Two nestlings (one HB and one LB) vomited between two

281 consecutive trials and were excluded from M_{EE} analyses. Fecal sacs were not weighed

282 individually but collected in closed containers and kept refrigerated until weighed. Two

283 measures of cumulative fecal mass were taken, at the end (21:00 h) and ca. the middle

284 (13:00 h) of the whole begging session.

285 We also measured how the experimental treatment affected cell-mediated immune

286 response. Immediately before the onset of the experiment (07:30 h), we injected into the

287 left patagium of each chick 0.2 mg of phytohaemagglutinin (PHA-P, L-8754, Sigma

288 Aldrich) diluted in 0.04 ml of isotonic phosphate buffer (following Moreno et al. 2005).

289 PHA-P is an innocuous protein which induces an immune response in birds causing a

290 swelling of the patagium skin that is positively correlated with the strength of the T-cell

291 mediated immune response (Kennedy and Nager 2006), although other components of

292 the immune system are also involved (Martin et al. 2006). Previously, we had measured

293 (three times) the patagium thickness with a pressure-sensitive micrometer (Mitutoyo®;

294 accuracy: 0.01 mm). At the end of the day (21:00 h) and at the end of the experiment

295   (24 h later), we again measured the patagium thickness, calculating the T-cell mediated

296   immune response as the difference between measurements. Patagium measurements

297   were performed by a trained person blind with regard to nestling treatment.

298   The day after the experiment, nestlings were fed *ad libitum* again, marked with non-

299   toxic waterproof ink and returned back to their nests during the morning. On the

300   following days, we regularly checked nests to monitor the fate of chicks used in the

301   experiment. With one exception, all nestlings looked well until they were ringed when

302   13 days old. One nestling died of starvation some days after the experiment had

303   concluded.

304   For statistical analyses, we performed Linear Mixed Effects Models of Restricted

305   Maximum Likelihood (REML-LMM; Zuur et al. 2009), by using the package "nlme"

306   (Pinheiro et al. 2012) in R (R Development Core Team 2012). In each model, nest of

307   origin was introduced as a random factor to control for variance among nests, thus

308   avoiding problems of statistical independence (Hurlbert 1984). In some models, initial

309   body mass and food consumed were introduced as predictors to examine possible

310   effects on the dependent variables. For every model, we checked for homoscedasticity

311   (residuals vs. fitted plots), and log-transformed the variable "time begging" in order to

312   fulfill homoscedasticity requirements. We also checked visually for normality of

313   residuals (normal quantile plots), which never deviated from a normal distribution

314   according to Kolmogorov-Smirnov tests (Quinn and Keough 2002). Means are given

315   with one standard error (SE). The complete dataset can be found in Supplementary

316   Material.

317   The experimental procedure was approved by the CSIC Ethical Committee (ref.

318   CGL2011-29694) and the Andalusian Committee of Animal Experimentation (ref.

319   2011_03Potti) to comply with Spanish and European legislation on the protection of

320   animals used for scientific purposes.

321   **RESULTS**

322   The experimental treatment succeeded at making HB nestlings beg for much longer

323   bouts (51.2 s) than LB nestlings (3.4 s), and also at higher postural intensities (Table 1).

324   Most of the variation in begging time and intensity was explained by treatment (Table

325   2). Individual repeatabilities within treatment across four begging trials were low, which

326   comes at no surprise considering that treatment involved the experimenter either

327   enforcing (HB) or preventing (LB) begging by nestlings. Video recordings from nests

328   showed that pied flycatcher broods in the wild begged with short begging bouts similar

329   to LB nestlings (4.8 s $\pm$ 1.23 SE, total range 0.1-21.5, range of mean values per brood

330   3.1-8.5). Therefore, begging effort per bout of HB nestlings substantially exceeded the

331   natural range in this species (Fig. 1). Since experimental nestlings were stimulated to

332   beg once every 45 min, hourly begging rates of HB nestlings (68 s/h) were within the

333   range of wild broods (135 s/h $\pm$ 29.4 SE, range 21-344).

334   **The effect of begging on cell-mediated immune response**

335   Chicks begging for longer and with higher intensity had a smaller diurnal immune

336   response than LB nestlings (Table 1). Food consumed or initial body mass did not affect

337   initial immune response ($F_{1, 19} = 2.48$, $P = 0.13$; $F_{1, 19} = 2.85$, $P = 0.11$, respectively),

338   and their inclusion in the model did not remove the significant effect of treatment on

339   initial immune response ($\chi^2 = 27.80$, $P < 0.001$). Final immune response remained

340   significantly smaller in HB nestlings than in LB nestlings (Fig. 2). Again, food

341   consumed and initial body mass did not affect final immune response (respectively, $F_{1,}$

342    $_{19} = 2.51$, $P = 0.13$; $F_{1, 19} = 0.26$, $P = 0.62$), and their inclusion in the model did not

343   qualitatively affect the results ($\chi^2 = 10.05$, $P = 0.0015$). Immune response decreased

344   during the night (paired t-test, t = -2.36, P = 0.023), irrespective of treatment (Table 1).

345   Food consumed and initial body mass did not affect the change in immune response

346   during the night (respectively, $F_{1, 19} < 0.01$, $P = 0.95$; $F_{1, 19} = 0.85$, $P = 0.37$), and their

347   inclusion in the model did not qualitatively alter the results, although rendered them

348   marginally non-significant ($\chi^2 = 2.97$, $P = 0.08$).

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

350   There were no differences in initial body mass or food consumed between high begging

351   (HB) and low begging (LB) nestlings (Table 1). Nestlings in the HB treatment gained

352   significantly less mass than LB nestlings during the diurnal phase of active begging

353   when begging trials took place (diurnal mass gain; Table 1). Diurnal mass gain was not

354   significantly affected by the amount of food consumed ($F_{1, 19} = 0.46$, $P = 0.51$) or initial

355   body mass ($F_{1, 19} = 0.24$, $P = 0.63$). Therefore, the effect of treatment on diurnal mass

356   gain remained significant when the amount of food consumed and initial body mass

357   were included in the model ($\chi^2 = 7.82$, $P = 0.005$). However, total mass gain during the

358   24 h period did not significantly differ between treatments (Table 1). Neither was total

359   mass gain during 24 h affected by food consumed ($F_{1, 19} = 0.02$, $P = 0.89$) or initial

360   body mass ($F_{1, 19} = 1.06$, $P = 0.32$), and their inclusion in the model did not change the

361   results ($\chi^2 = 0.20$, $P = 0.65$). The reason for the discrepancy between diurnal and total

362   mass gain was that HB nestlings lost significantly less mass during the night resting

363   phase than LB nestlings (Table 1). In this case, initial body mass negatively influenced

364   nocturnal mass loss ($\beta = -0.39$, $F_{1, 19} = 6.82$, $P = 0.017$). There was no effect of food

365   consumed ($F_{1, 19} = 2.19$, $P = 0.15$). Nonetheless, the effect of treatment on nocturnal

366   mass loss remained significant even after controlling for initial body mass and

367   consumed food ($\chi^2 = 4.84$, $P = 0.028$). Summarizing, HB nestlings grew less during the

368   daylight, active begging time but also lost less mass during the night, resulting in

369   similar growth rates for both treatments during a 24 h period (Fig. 3).

370   There were no differences in metabolic expenditure M_{EE} according to treatment (Table

371   1). M_{EE} comprised a larger fraction of diurnal mass gain in HB than in LB nestlings

372   (40.0 % ± 4.04 and 32.5 % ± 5.08, respectively) but differences were not significant ($F_{1,}$

373    $_{17} = 1.16$, $P = 0.29$). The mean marginal metabolic cost of begging (D_{EE}) was 0.09 g (±

374   0.087 SE, N = 18) of mass lost by HB nestlings attributable to begging. D_{EE} was neither

375   correlated with differences between nestlings in a pair (HB-LB) in time begging (r = -

376   0.10, NS) nor differences in postural intensity (r = 0.05, NS).

377   **The effect of begging on fecal mass production**

378   Experimental treatment had no effect on total fecal mass excreted at the end of the

379   begging session. However, HB nestlings excreted a marginally ($P = 0.061$) larger

380   fraction of cumulative fecal mass during the first half of the begging session than LB

381   nestlings (Table 1). Among HB nestlings, the amount of time spent begging had a

382   positive, marginally significant effect on the amount of feces excreted during the first

383   half of the begging session, after controlling for food intake ($\beta = 0.39$, $F_{1, 19} = 3.98$, $P =$

384   0.062) but this trend was not evident in the control LB group ($\beta = -0.04$, $F_{1, 19} = 0.02$, P

385   = 0.88). This suggests that begging had a mild, short-term effect upon the digestive

386   dynamics of nestlings.

387   **DISCUSSION**

388   Compared with their LB nestmates in the control group, experimental HB nestlings

389   invested a great deal in begging effort. Begging bouts of LB nestlings (3.4 s) were

390   similar in duration to begging bouts at wild nests (5 s). Sustained begging bouts of HB

391   nestlings were 15 times longer (51 s) and well above the maximum duration of begging

392   bouts recorded in the wild (21.5 s). It is reasonable to assume that variation in the

393   duration of begging bouts recorded at natural broods may in part reflect variations in

394   nestling nutritional need, with well-fed chicks begging at shorter durations. Nestlings in

395   this study received ca. 0.5 g of food per g of body mass over a 24 h period (Table 1).

396   This food amount is above the average estimated for a 7-days old, 9 g pied flycatcher

397   nestling in the wild (0.44 g per g of body mass) and close to the highest food intake

398   (0.67 g/g) established in a previous experimental study testing the effects of nutritional

399   need upon begging and digestion (Wright et al. 2010). Therefore, as both LB and HB

400   nestlings could be considered as belonging to a well-fed category of signalers, the long

401   begging bouts performed by HB nestlings, above the range recorded at natural broods,

402   can be considered as out-of-equilibrium signals (Számadó 2011).

403   Hourly begging rates of experimental nestlings were within the range observed at wild

404   broods. Some previous studies on growth costs also reported keeping hourly begging

405   rates of enforced HB nestlings within the natural range of wild broods (Kilner 2001;

406   Leonard et al. 2003; Moreno-Rueda et al. 2012). Others did not report natural begging

407   rates but adjusted their testing schedule to approach natural rates of feeding visits by

408   parents (Kedar et al. 2000; Moreno-Rueda 2010; Moreno-Rueda and Redondo 2011,

409   2012). Comparatively, the HB/LB ratio of begging duration in this study (15) was

410   higher (6, Kilner 2001; 3.5 and 5, Rodríguez-Gironés et al. 2001; 6, Leonard et al. 2003;

411   6.7, Moreno-Rueda et al. 2012) or similar (16.7 Moreno-Rueda and Redondo 2011; 14.1

412 Moreno-Rueda and Redondo 2012) to those reported by previous studies. Only in two

413 of them did the HB/LB time ratio exceeded that in this study (24, Moreno-Rueda 2010;

414 36, Soler et al. 2014). In summary, experimental HB nestlings in this study were forced

415 to beg at least as hard as in studies that did show a growth cost (Kilner 2001;

416 Rodríguez-Gironés et al. 2001; Moreno-Rueda and Redondo 2011; Moreno-Rueda et al.

417 2012). Mass gain measured during 24 h in the laboratory (0.86 g) was lower than in

418 the field at the same age (1.36 g), which is common for hand-reared wild birds and can

419 be attributed to general stress caused by husbandry practices (Flammer and Clubb 1994)

420 even if fed with high-quality diets, as in this study.

421 Excess begging experimentally induced had a two-fold impact upon nestling

422 physiology, reducing both the mass gain during activity hours and immune response.

423 Such effects can hardly be explained in terms of metabolic expenditure (measured as

424 mass lost during the begging session). Metabolic expenditure was similar between HB

425 and LB nestlings, either in absolute terms or as a fraction of diurnal mass gain and

426 differences in begging effort between pairs of nestlings had no effect upon mass lost

427 attributable to begging. The small, non-significant effect of excess begging upon

428 metabolic expenditure is consistent with previous respirometry studies (Chappell and

429 Bachman 2002). As an alternative to energetic expenditure, vigorous physical

430 exercising associated to intensive begging may cause oxidative stress (Noguera et al.

431 2010; Boncoraglio et al. 2012; Costantini 2014). Both growth and immune function

432 contribute to the production of free radicals (Alonso-Álvarez et al. 2007; Costantini and

433 Møller 2009; Sorci and Faivre 2009), and nestlings sustaining a high begging effort may

434 be able to momentarily downregulate other oxidative processes in order to keep up with

435 oxidative balance (Moreno-Rueda et al. 2012). A final possibility is that it is not the

436   physical effort of begging *per se* what is immunosuppressive , but rather the

437   physiological processes leading nestlings to beg with greater effort (Buchanan et al.

438   2007). Some studies have found that endogenous levels of testosterone and

439   glucocorticoids may simultaneously promote more intense begging and reduce both

440   growth and immunocompetence in pied flycatchers (Goodship and Buchanan 2006,

441   2007) and other birds (Quillfeldt et al. 2006; Buchanan et al. 2007; Loiseau et al. 2008).

442   The fact that experimental nestlings in this study begged not only for longer, but also at

443   higher postural intensities, is consistent with this last possibility. Effects of endogenous

444   hormones on begging, growth and immune response may vary among different species

445   (Smiseth et al. 2011) and this variation could provide an additional explanation for the

446   different results obtained in previous studies about the impact of begging on growth.

447   T-cell mediated immune response was highly impacted by experimental treatment at the

448   end of the begging session (Cohen's $d = 1.15$) and also, but with a milder effect, 24

449   hours later (Cohen`s $d = 0.64$). This change is expected in phytohaemagglutinin assays

450   (Navarro et al. 2003) as a result of a rapid (3-12 h) transient infiltration of heterophyls

451   and lymphocites in the injected tissue, later followed by macrophage infiltration (ca. 24

452   h), the swelling typically remitting after 48 h (Smits et al. 1999; Martin et al. 2006).

453   Experimentally induced excess begging has been shown to impair T-cell mediated

454   immune response in each and every species tested so far: house sparrows (Moreno-

455   Rueda 2010; Soler et al. 2014), magpies (Moreno-Rueda et al. 2012), southern shrikes

456   (Moreno-Rueda and Redondo 2011, 2012), and pied flycatchers (this study). By

457   contrast, Romano et al. (2011) found that female barn swallow (*Hirundo rustica*)

458   nestlings injected with an antigen (LPS, a bacterial lipopolysaccharide) increased their

459   begging intensity. However, their study was not specifically designed to test the effect

460 of enforced begging on immune response and chicks were allowed to beg freely. This

461 detail is of critical importance, as long as marginal begging costs predicted by signaling

462 models can be evaluated only by manipulating nestlings into begging above their

463 preferred (out-of-equilibrium) levels (Kilner 2001; Számadó 2011). Furthermore,

464 Romano et al. (2011) measured the begging response of nestlings two days after the

465 inoculation, when nutritional condition of nestlings (body mass, feather quality and

466 gape coloration) had already deteriorated as a result of the immune challenge.

467 Therefore, begging response in that study might be affected by both immune challenge

468 and nestling condition (Jacob et al. 2011). One potential drawback of studies showing

469 an impact of enforced begging upon immune response is that, to make experimental HB

470 nestlings beg with greater effort, they were stimulated more than their LB nestmates

471 (Moreno-Rueda 2010; Moreno-Rueda and Redondo 2011, 2012; Moreno-Rueda et al.

472 2012; Soler et al. 2014; this study). More stimulation may cause stress, which may

473 elevate glucocorticoid levels and depress immune response (Saino et al. 2003). Some

474 studies on growth costs (Kedar et al. 2000; Kilner 2001) also suffer from the same

475 drawback but not others (Rodriguez-Gironés et al. 2001; Leonard et al. 2003). There is

476 not an obvious relationship in these studies between stimulation schedule and whether

477 they found a significant impact on growth (Kilner 2001; Rodríguez-Gironés et al. 2001)

478 or not (Kedar et al. 2000; Leonard et al. 2003). Glucocorticoids may affect both growth

479 and immune response simultaneously (Saino et al. 2003), which suggests that

480 differential stimulation may not be the only cause of reduced immune response in HB

481 nestlings. However, the possibility that experimenter-induced stress may affect results

482 in studies of immunological costs of begging should be addressed in future studies.

483 If, as suggested by this and previous studies, there is a generalized immunological

484 impact of excess begging, this effect could be qualified as a genuine direct begging cost

485 of the type hypothesized by begging models. First, cost should affect the nestling fitness

486 function by reducing viability (Számadó 2011). Downregulating immune function may

487 reduce nestling survival prospects in case of a parasitic infection. Pied flycatcher

488 nestlings from Iberian populations suffer from a high (above 20 %) prevalence of

489 haematozoan and arthropod nest-dwelling ectoparasites (Merino and Potti 1995, 1996).

490 Nestlings showing a reduced immune response are worse at coping with an eventual

491 parasitic infection (Tschirren et al. 2007; Pitala et al. 2010), and indeed have a reduced

492 viability in the long term (Cichoń and Dubiec 2005; Bowers et al. 2014). A second

493 assumption of signaling models is that marginal begging costs should not be offset by

494 marginal benefits (i.e. extra food), thus rendering fitness returns negative for nestlings

495 begging above the honest equilibrium level (Számadó 2011). T-cell immune response at

496 fledging is a better predictor of survival to first reproduction than body mass or

497 condition in pied flycatchers (Moreno et al. 2005). This implies that gaining mass by

498 begging intensively may be of little use if immune function is impaired as a result of

499 signal overplay. Furthermore, it is not immediately obvious whether obtaining extra

500 food would compensate for an impaired immune response. In an experimental study in

501 which we simultaneously manipulated marginal benefits and costs of extra begging, we

502 found that southern shrike nestlings receiving 30% extra food were able to compensate

503 for the impact of excess begging on growth, but immune response was affected

504 independently of the amount of food ingested (Moreno-Rueda and Redondo 2012). We

505 therefore conclude that excess begging is costly for pied flycatcher nestlings as a result

506 of impaired immunocompetence.

507   The impact of excess begging on mass gain was large at the end of the begging session

508   (Cohen's d = 0.65) but had virtually vanished the morning after (Cohen's d = 0.06).

509   Nestlings that had begged intensively and gained less mass during the daylight hours

510   were capable of a compensatory regulation of mass loss while resting later at night. At

511   least two possible mechanisms might be involved in the compensatory phase of

512   nocturnal mass loss. First, a large fraction (ca. 3/4) of a nestling energy budget is

513   allocated to dissipative processes such as maintenance and thermoregulation (Verhulst

514   and Wiersma 1997). During the night, HB nestlings may have reduced basal

515   metabolism or heat production in favor of growth (Moe et al. 2004; Vézina et al. 2009;

516   Killpack and Karasov 2012). Second, experimental treatment may have affected

517   digestive dynamics, allowing HB nestlings to delay food assimilation. For example,

518   pigeons are able to postpone digestion at low temperatures in order to use the extra heat

519   from digestion-related thermogenesis (Laurila et al. 2003). Unfortunately, we did not

520   keep a continuous record of the daily changes in fecal production throughout the

521   begging session neither we measured fecal production the morning after. But we found

522   a marginally significant trend for HB nestlings to excrete a lower fraction of the total

523   fecal mass on the second half of the begging session. In canaries *Serinus canaria*,

524   enforced begging during a relatively short (6 h) trial also increased fecal sac production

525   (Kilner 2001). Alternatively, the lower diurnal mass gain of HB nestlings may have

526   been a consequence of a lower digestive efficiency (Kilner 2001; Budden & Wright

527   2008; Grodzinski et al. 2009; Wright et al 2010) that would have been compensated for

528   during the night, when begging ceased.

529   Does excess begging have an impact on pied flycatcher nestling mass gain? The answer

530   to this question will depend on the time scale of measurement. It did after 13 h of

531   begging, but not 11 h afterwards. Other studies have also found begging effects upon

532   mass gain to be dependent on time scale or age. For example, canaries showed an

533   impact of begging on M_{EE} at 8 days, but not at 6 or 10 days, and HB-LB differences in

534   mass gain during a 24 h period also varied with age (Fig. 2 in Kilner 2001). Magpies

535   sustaining high begging rates for three consecutive days also showed an impact on mass

536   gain at 24 h, but not at 48-72 h (Moreno-Rueda et al. 2012). In shrikes, the impact of

537   begging on mass gain over 24 h depended on the amount and quality of ingested food

538   (Moreno-Rueda & Redondo 2011, 2012). And house sparrows showed an impact after

539   60 h of sustained begging effort but not at 6, 12, 72, 84 and 108 h (Kedar et al. 2000;

540   Moreno-Rueda 2010; Soler et al. 2014). Two of these studies found an impact of excess

541   begging upon body condition measured as residuals of a regression of body mass

542   against wing length at 72 h (Kedar et al. 2000) and between 48 and 108 h (Soler et al.

543   2014). However, this last finding may prove difficult to interpret in biologically

544   meaningful terms. First, body condition measured as Model I regression residuals may

545   suffer from several statistical hindrances, such as slope overestimation and lack of

546   allometric linearity, especially for growing nestlings (Green 2001). Second, variations

547   in nestling body mass relative to structural size may reflect patterns of mass allocation

548   to different body parts in response to a variety of environmental conditions (Potti 1999,

549   2000; Szép and Møller 2000).

550   The possibility exists that mixed results found in previous studies on the effect of

551   begging on nestling growth are not simply the outcome of differences in experimental

552   setups, but actually reflect the ability of nestlings to show flexible variations in mass

553   gain in response to excess begging according to different circumstances. But if begging

554   impact on mass gain can be compensated for under many situations then it fails to

25

555   qualify as a genuine direct cost (Számadó 2011). This raises the interesting question of

556   why growth in some species is more easily affected by begging than in others.

557   However, begging-induced growth variations may not offer a universal explanation for

558   the cost predicted by signaling models. Immunological costs seem a promising avenue

559   for future studies, particularly those aimed at testing differential benefit signaling

560   models.

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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).

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

F 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.

P-values: * $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns for not significant.

Table 2

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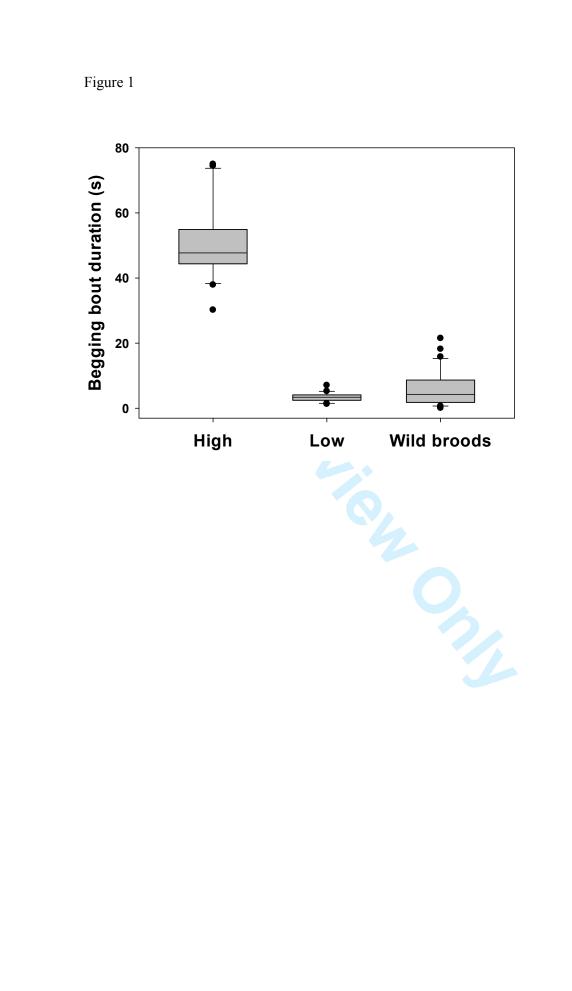
(random) of Time Begging and Postural Intensity.

		Time Begging		Postural Intensity	
	df	MS	F	MS	F
Effect					
Treatment	2	6989.65	7642.64***	315.28	1223.29***
Nestling(Treatment)	38	0.92	3.64***	0.26	2.54***
Error	117	0.25		0.11	
Repeatability (intraclass correlation coefficient)					
HB			0.45***		0.52***
LB			0.22*		0.33***

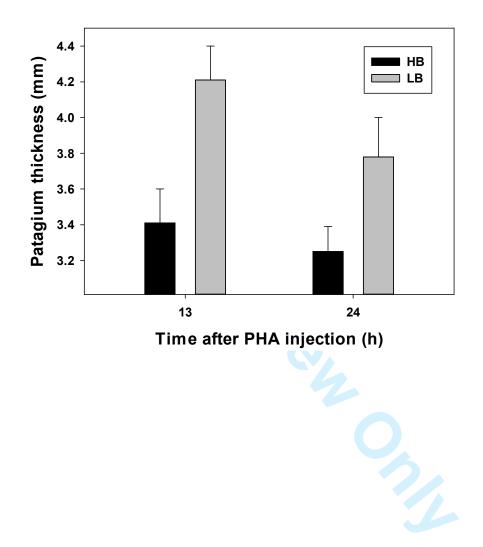Repeatability of begging variables across four begging trials for each treatment level

(HB and LB) indicates the fraction of total variance explained by Nestling (random).
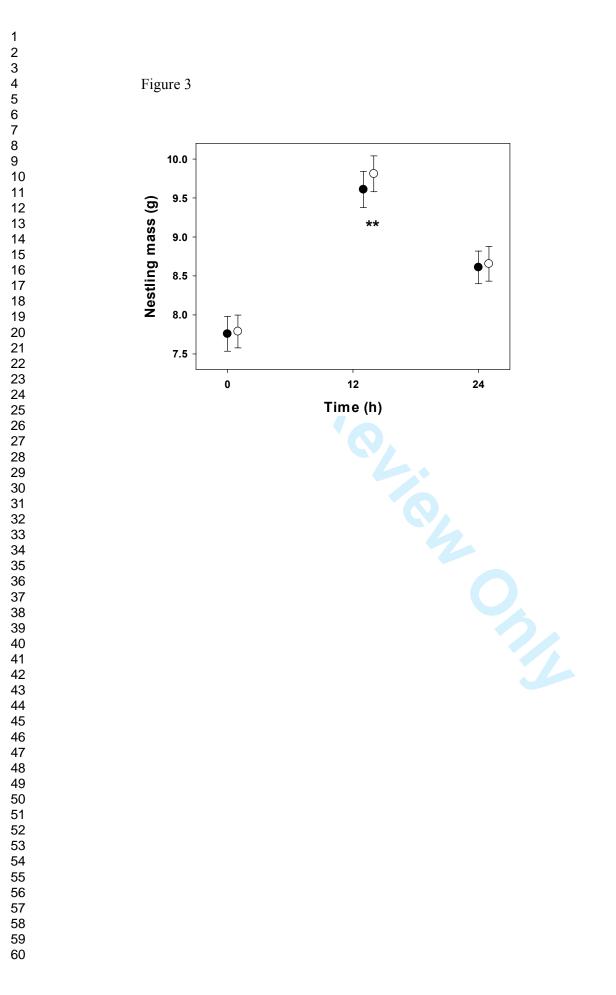
Time begging was log transformed prior to analyses.

P-values: * $P < 0.05$, ***$P < 0.001$.

Figure 1

Figure 2

Figure 3

1

1   **LAY SUMMARY**

2   Nestling begging signals should be costly in order to limit the benefits of selfish

3   deception to parents. Exactly which cost begging entails has been hotly debated in the

4   past. Here we show that begging impairs the immune system of pied flycatcher

5   nestlings. It also reduced nestling growth but nestlings were able to recover while

6   resting at night. Since most of the controversy was about growth costs, this study may

7   help to settle the debate.

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