Decoding colouration of begging traits by the experimental addition of the appetite enhancer cyproheptadine hydrochloride in magpie (*Pica pica*) nestlings

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The colouration of some traits in nestlings of altricial birds may influence parental food allocation as it may reflect physical condition or hunger. There is increasing evidence of the relationship between colouration of begging traits and nestling performance. However, evidence of the influence of hunger level on nestling colouration is scarce, mainly because of difficulty of distinguishing between the effects of physical condition and hunger levels. Here, we used the appetite stimulant cyproheptadine hydrochloride to increase the sensation of hunger of magpie (*Pica pica*) nestlings for eight days and assessed the effect on the colouration of rictal flanges, mouth and body skin. We found that nestlings administered with cyproheptadine had flanges more conspicuous (chromatic visual contrast), more UV coloured and less yellow coloured than their control nestmates. Conversely, mouths of experimental nestlings were more yellow coloured and less UV coloured than controls. Our pharmacological experiment affected the strength of the relationship between body mass and some colour components of body skin (chromatic and achromatic visual contrasts, UV–chroma and Yellow–chroma) and of rictal flanges (chromatic visual contrasts, UV–chroma and yellow–chroma), but not for mouth colouration. These results taken together suggest that the effect of the cyproheptadine on nestling colourations is probably mediated by an increase in hunger levels of nestlings for rictal flanges and body skin colourations, and by an increase in physical condition in the case of mouth coloration.

Keywords: Begging behaviour, cyproheptadine, honest signalling, nestling colouration, parent-offspring conflict, *Pica pica*, visual cues, visual contrasts.

Offspring of species with parental care are selected to demand a greater share of resources than the parents are selected to provide (Trivers 1974). This parent-offspring conflict, together with sibling competition for parental care, provokes coevolutionary processes selecting conspicuous morphological and behavioural traits that affect parental investment (Godfray 1995, Mock and Parker 1997). This is because parents might adaptively adjust their feeding effort to maximize their own fitness payoff (e.g. Smith et al. 1988, Ottosson et al. 1997, Kilner and Johnstone 1997, Burford et al. 1998, but see Clark and Lee 1998) by actively assessing the requirements or the quality of their offspring through solicitation signals (Redondo and Castro 1992, Price et al. 1996, Iacovides and Evans 1998, Sacchi et al. 2002), or by passively feeding the offspring that emerges victorious from scramble competition among siblings (Rodríguez-Gironés et al. 2001, Parker et al. 2002).

Studies of parent-offspring communication have been mainly carried out on altricial birds and focused on acoustic signals and postures (e.g. Wright and Leonard 2002). In these species, offspring solicitation is manifested as conspicuous calls and extravagant movements such as stretching of the neck, wing shivering and gaping (Redondo and Castro 1992). Visual cues, for instance the colouration of some nestling traits, such as rictal flange, gape and body skin (hereafter begging-related traits), may also influence parental decisions concerning the allocation of food among siblings. For instance, the efficacy of visual cues of nestlings attracting parental attention would depend on light conditions, and parents may use the conspicuous gapes and rictal flanges to locate nestlings within dimly lit nests (Heeb et al. 2003, Kilner and Davies 1998, Avilés et al. 2008, Wiebe and Slagsvold 2009). However, visual cues may signal inherent characteristics of nestlings linked to their fitness prospects. Indeed, mouth colouration of nestlings of several bird species reflects their body condition (de Ayala et al. 2007, Ewen et al. 2008, Parejo et al. 2010, Dugas and McGraw 2011, Jacob et al. 2011, Wisner 2011, Dugas 2012), immune state (Saino et al. 2000, 2003, Jourdie et al. 2004, Romano et al. 2011) or ectoparasite density (Dugas and Doumas 2014). Furthermore, the colouration of body skin may also inform parents of their phenotypic quality because for some species it is associated with fitness-related variables (Jourdie et al. 2004, Bize et al. 2006, Soler et al. 2007, Avilés et al. 2011).

On the other hand, nestling gapes of canaries (*Serinus canaria*, Kilner 1997) and other seed-regurgitating finches (Kilner and Davies 1998) experience a 'red flush' at the start of begging that become more intense with increasing food deprivation, which would inform the parents about the recent feeding history of their nestlings. Similarly, Jacob and Heeb (2013) have found that colouration of rictal flanges changed in response to food deprivation in nestlings of European starling (*Sturnus vulgaris*).

The main signalling functions of colouration of begging-related traits of nestlings would therefore seem to reliably reflect the phenotypic quality, the level of food deprivation (i.e. the level of hunger), or both. Nonetheless, evidence of a relationship between nestling colouration and the extent of food deprivation does not appear in all studied species (see e.g. Kilner and Davies 1998, Clotfelter et al. 2003, Saino et al. 2003, de Ayala et al. 2007, Wegrzyn 2013). This lack of support in some species could be due to interspecies variation, either in physiological mechanisms governing nutritional stress (i.e. with or without mouth 'red flush'), or in the duration of experimental deprivation needed to detect changes of colouration. However, it is also possible that the colouration of begging-related traits has not evolved in all species as a hunger signal. Moreover, the extent of food deprivation could affect nestling condition and *vice versa*. For instance, nestlings that experience long periods of food deprivation could develop poorer body condition, while those in better body condition would on average experience a lower level of hunger (see e.g. Clark 2002, for a detailed review of the concept and the factors affecting hunger in the short and long-term). Consequently, it is possible that the commonly detected relationship between colouration of begging-related traits and condition was partially mediated by the effects of food deprivation on colouration of such traits. Disentangling the effects of hunger and body condition would be important for identifying the prime cause of variation in solicitation signals of nestlings in general (Mock et al. 2011, Boncoraglio et al. 2012) and the colouration of begging-related traits in particular.

The main aim of this article is to determine the type of information conveyed by the colouration of begging-related traits. Specifically, we test whether an experimental chronic increase (of eight days) of the level of hunger during development influences colouration of any of the nestling traits predicted to be involved in parent-offspring communication (i.e. gape, rictal flanges and body skin). We achieve this objective using an experimental approach, in particular the administration of an appetite stimulant, cyproheptadine hydrochloride, which acts on the hunger centre located on the hypothalamus (Chakrabarty et al. 1967). We administered cyproheptadine to half the magpie (*Pica pica*) nestlings within each nest during development and measured their colouration before and after treatment. The effect of this drug on begging behaviour and parental feeding decisions has been previously detected in magpies (Martín-Gálvez et al. 2011, 2012). We also found that nestlings fed with cyproheptadine fledged in better physical condition than their control nest mates, probably due to experimental nestlings receiving more food during development (Martín-Gálvez et al. 2011, 2012). Because of the important differences between bird and human vision (see e.g. Kelber et al. 2003, Tanaka 2015), we quantified colouration of begging-related traits as perceived by parents, i.e. by using visual modelling reflectance of nestling traits and nest background, and estimating visual contrasts following the opponency model of Vorobyev and Osorio (1998) developed for the tetrachromatic visual system of birds.

Since colouration of nestling traits involved in parent-offspring communication (i.e. gapes, rictal flanges and body skin) may convey information about hunger and/or condition of nestlings (**H1**, Figure 1), we predict an effect of the experimental treatment with cyproheptadine on colouration of such traits (**P1**, Figure 1). Additionally, as we expect that the experimental treatment would alter the association between hunger sensation and condition of experimental nestlings, we try to discern the type of information (hunger level *vs*. physical condition) conveyed by the colouration of each begging-related trait. This is achieved by including nestling body mass (as a proxy for physical condition) in our analyses and exploring the association between body mass and colouration of each nestling trait for experimental and control nestlings (see Figure 1). If hunger level is the responsible for the commonly detected association between condition and trait colouration (**H2**, Figure 1), we would expect that the relationship between body mass and visual contrasts should differ between experimental and control nestlings (**P2**, Figure 1). On the other hand, if colouration of a nestling trait is a direct consequence of body condition irrespective of hunger level (**H3**, Figure 1), the relationship between body mass and visual contrasts for that trait should be similar between experimental and control nestlings (**P3**¸ Figure 1).

FIGURE 1 ABOUT HERE OR NEARBY

METHODS

Nests used in this study were a subset of that used in Martín-Gállvez et al. (2011), and thus a more detailed explanation of the study area, species and general field procedures can be found there. Fieldwork was performed during the spring of 2008 in Iznalloz (37º25' N 3º33' W), southern Spain. Magpie nestlings were weighed after 2–4 days of hatching (Pesola spring balance, accuracy 0.1g). They were ranked according to their weight and, starting with the heaviest nestling, we alternated treatments of nestlings following the brood body mass hierarchy. Experimental treatment consisted of the oral administration (with a plastic 1ml syringe) every two days of 0.1mg of cyproheptadine hydrochloride (Acofarma, Inc., Barcelona, Spain) diluted with 0.25ml of mineral water (i.e. 0.05mg/day). Control nestlings were administered with 0.25ml of mineral water. After the first dose (at first weighing, 2–4 days old), we revisited the nests every two days to recolour the tarsi, weigh the nestlings and dose them with cyproheptadine or water. Surviving nestlings (see below) received the treatment with cyproheptadine or water on five alternate days i.e. until they were 10–12 days old.

Colour measurements

Nestling colour patterns were characterized by measuring the spectral reflectance (300nm to 700nm) of three different traits: mouth (gape or palate), rictal flange and body skin. This was done using the procedures and equipment described in Avilés et al. (2008).

Reflectance of nestling traits was measured twice during their growth, at the beginning and end of the treatment with cyproheptadine, i.e. at 2–4 and 10–12 days old. All measurements were repeated three times and, since high repeatability has been previously demonstrated for this kind of measurements (e.g. Avilés and Soler 2009), we used mean values in our analyses. Afterwards, we corrected the individual average spectra by a triangular smoothing (i.e. a floating mean with weights) with a triangular distance of 10nm as performed by AVICOL v5 software (Gomez 2006) (Figure 2).

The black-billed magpie builds characteristic large domed nests from twigs lined with mud and vegetation (Birkhead 1991). The nest dome drastically reduces the light inside the nest and thus affects the perceptual processes involved in detection of visual cues (Avilés et al. 2015). In order to take into account the characteristic light environment inside the magpie nests, we measured the irradiance in 10 active magpie nests that were not used in this study. Measurements were performed between March and April of 2009, and between 09:00am and 11:00am in a close magpie population, following Avilés et al. (2008). Briefly, we took three readings per nest and placing the probe above the nest cup pointing vertically toward the dome. Since there is a remarkable consistency in the level of irradiance among magpie nests (Avilés et al. 2015), and since different light environments yield similar visual modelling results (Dugas and Rosenthal 2010), mean values were calculated across nests to obtain the average irradiance spectrum. Average spectral reflectance of magpie nest backgrounds (consisting of mainly fibrous roots and dry grass, Birkhead 1991) was obtained from Avilés et al. (2008) as these measurements were taken from a neighbouring magpie population with nest backgrounds of similar characteristics (pers. obs.).

FIGURE 2 ABOUT HERE OR NEARBY

Avian colour space modelling

Discriminability of each begging-related trait was calculated relative to the nest background, taking into account magpie vision and the ambient light in the nests. It was calculated by using the colour opponency model of Vorobyev and Osorio (1998) developed for the tetrachromatic visual system of birds in its log form (Vorobyev et al. 1998) as implemented in AVICOL v5 software (Gomez 2006). This model calculates both chromatic and achromatic (luminance) contrasts expressed in *jnd* (just noticeable differences) between two coloured patches within the visual space of a receiver.

Evidence suggests that the magpie has short-wavelength sensitivity biased toward violet (violet sensitivity, VS, Odeen and Hastad 2003). Consequently, and because no such data are available for magpies, we used the spectral sensitivity data from the peafowl (*Pavo cristatus*) as representative of the violet-sensitive system (e.g. Hastad et al. 2005, Avilés and Soler 2009) and the proportions for cone photoreceptors of 1:1.9:2.2:2.1 (VS : Short-Wavelength-Sensitive (SWS) : Medium-Wavelenght-Sensitive (MWS) : Long-Wavelength-Sensitive (LWS), Hart 2002). We assumed that the signalling noise by each cone was independent of light intensity.

Furthermore, we explored the colour components of the begging-related traits). It was because high visual contrasts between nestling traits and nest background may be achieved by either increasing or decreasing reflectance of nestling traits at different wavelengths depending on nest background colouration. In short, we focussed on the wavelength regions previously associated with variation in colouration of these nestling traits, i.e. 550–625nm (yellow) for carotenoid-based colourations, and 300–400nm (UV) for carotenoid-based and structural colourations (e.g. Hunt et al. 2003, Jourdie et al. 2004, Thorogood et al. 2008, Dugas and Rosenthal 2010). For these two regions, we calculated the mean brightness (Yellow–brightness, $R_{550–625,}$ UV–brightness, $R_{300–400}$) and the chroma (Yellow–chroma, $R_{550–625}/R_{300–700}$, UV–chroma, $R_{300–400}/R_{300–700}$) following the procedures of Thorogood et al. (2008). These calculations were done using AVICOL v5 software (Gomez 2006).

Statistical analyses

We performed Linear Models in the Statistica software v8 (StatSoft 2008) to analyse the effect of cyproheptadine (**P1**, Figure 1) on the discriminability for each nestling trait. Random variation due to differences between nests was removed from body mass and visual contrasts by equalizing within-nests mean values to zero, while maintaining original within-nest variance. Afterwards, we included the residuals of chromatic or achromatic contrasts for each nestling trait as the response variable in the Linear Model; the experimental treatment (experimental vs. control) and the residuals of nestling body mass were included in the statistical model as a fixed factor and as a covariate, respectively. We also included the interaction term between experimental treatment and residuals of body mass in order to test if the relationships between body mass and colouration (i.e. residual of the chromatic or achromatic contrasts differed between control and experimental nestlings (**P2** and **P3**, Figure 1). Although this analysis could have been done using a mixed model, we decided to do it manually (i.e. correct for within-nest variation) because the calculations of these residuals were needed for the analyses and graphical representations of the relationships between body mass and colouration separately for experimental and control nestlings. Later, we did the same analyses for Yellow–brightness, Yellow–chroma, UV-brightness and UV–chroma for each nestling trait.

For analysing the effect of cyproheptadine on body mass of nestlings during growth, we used an LMM performed using R software v2.13.2 (R Development Core R Development Core Team 2012) with *lme4* (R package v. 0.999375-42, Bates and Maechler 2011) and fitted by the restricted maximum likelihood (REML) approximation. The body mass of nestlings from four different visits was included in the model as the response variable, and treatment with cyproheptadine and age as two fixed factors. Nest identity and nestling identity (nested within nest identity) were introduced as two random factors in the LMM to control the non-independence of body mass measurements from the same nestling and from nestlings from the same nests (i.e. repeated measures design). The effect of experimental treatment on body mass during growth was tested by the interaction between age and treatment on body mass measurements. Afterwards, we used Markov-chain Monte Carlo (MCMC) simulations performed using the *pvals.fnc* command of *languageR* (R package v.1.2. Baayen 2011) to compute the highest-posterior-density (HPD) 95% confidence intervals of the model estimates and p-values. The chain length for MCMC sampling was fixed at 10,000.

Sample sizes and ethical considerations

We started our study with a sample size of 142 nestlings from 29 different magpie nests, but the final sample size was 96 nestlings from 25 nests with data for both colouration and weight. This decrease in sample size was because one brood (of 4 nestling) was depredated during the experiment, and we did not use data from three nests where at least one experimental and one control nestling did not survive until the final dose (16 nestlings). Moreover, from 122 hatched nestlings in the 25 magpie nests included in the analyses, 24 starved during the study, one was not weighed after treatment, and the colour measurements of another nestling were not saved.

Magpie territories are located in a rural area routinely used by farmers and shepherds so our nest visiting did not cause additional disturbance to the magpie pairs. Cyproheptadine is a drug widely used as an appetite enhancer for both humans (e.g. Periatin©) and animals, including birds (e.g. Vita-Vrot-c©). Although minimal side effects consisting primarily of transient drowsiness have been described (e.g. Homnick et al. 2004), results from our previous study (Martín-Gálvez et al. 2011) confirmed a generally positive effect of the treatment on phenotypic condition of magpie nestlings. As far as we could determine, nestling mortality observed during this study was not influenced by our activities as none of the magpie pairs deserted and only one magpie brood was depredated, nor by our experimental treatment as there was not significant difference in the starvation rate between experimental and control nestlings (29 %, N = 69 and 21 %, N= 69, respectively; Chi-square = 0.96, d.f. = 1, P = 0.33).

Fieldwork was carried out under licence (ref.: SCFFS-AFR-CMM) from the "Consejería de Medio Ambiente de la Junta de Andalucía" (the Environmental Agency of the local Government of Andalusia).

RESULTS

Effect of experimental treatment on colouration of begging-related traits

Before experimental treatment with cyproheptadine (i.e. when nestlings were 2–4 days old), nestlings assigned to the control and experimental groups did not differ in chromatic or achromatic visual contrasts of all measured traits (treatment effect: $0.19 < P < 0.82$, data from surviving nestlings that eventually died during development were included in these analyses (see Methods)). Nonetheless, at the end of the experiment (10–12 days after hatching), the nestlings with an experimentally increased hunger level during growing showed flanges chromatically more conspicuous (relative to nest background) than those of their control nestmates (treatment effect in Table 1). We did not detect a significant effect of experimental treatment for achromatic contrasts for rictal flanges, and for chromatic or achromatic contrasts of further measured nestling traits (treatment effects in Table 1).

Regarding colour components, we found that flanges of experimental nestlings had greater values of UV–chroma (LM, treatment effect: $F_{1,92}= 4.98$, $P = 0.028$) and smaller values of Yellow–chroma (LM, treatment effect: $F_{1,92}= 4.02$, $P = 0.048$) than their control nestmates. Instead, gapes of experimental nestlings had less UV–chroma (LM, treatment effect: $F_{1,92}= 4.25$, $P = 0.042$) and more Yellow–chroma (LM, treatment effect: $F_{1,92}= 5.39$, $P = 0.02$) than controls. We did not find differences for any colour components of body skin, and for UV–Brightness and Yellow–Brightness of rictal flanges and gapes (results not shown).

Effect of experimental treatment on the gain of body mass

Contrary to the expected (i.e. a greater food ingest for experimental nestlings (see introduction)), our experimental treatment with cyproheptadine had no effect on the increase of body mass in nestlings from 2-4 days until 10-12 days after hatching (LMM, interaction between nestling age and treatment: HPD 95% confidence interval: -1.87 to 1.81 mg/hour, $P = 0.93$).

Experimental effects on the relationships between colouration of begging-related traits and body mass

We found statistically significant relationships between body mass and the chromatic and achromatic contrasts for both rictal flanges and body skin (Table 1). Furthermore, in the case of body skin, these relationships differed between control and experimental nestlings (interaction terms between treatment and body mass, Table 1).

When exploring the relationships between body mass and colouration of body skin and rictal flanges separately for control and experimental nestlings, we found that the positive relationship between chromatic contrasts of body skin and body mass appeared for the control group only (Figure 3, and interaction term in Table 2). The rest of contrasts (chromatic and achromatic contrasts of rictal flanges, and achromatic contrasts of body skin) were negatively related with body mass only in experimental nestlings (see Figure 3).

TABLE 1 AND FIGURE 3 ABOUT HERE OR NEARBY

The results with the physical colour measurements were similar for rictal flanges and body skin. Experimental treatment with cyproheptadine affected the relationship between UV–chroma of rictal flanges and nestling body mass (interaction term: $F_{1,92}$ = 5.59, P = 0.020). We found a negative relationship for the experimental (r = -0.33, P = 0.021), but not for the control nestlings (r = 0.11, P = 0.44). Regarding Yellow–chroma of rictal flanges, we found a positive relationship with the body mass in experimental nestlings (r = 0.34, P = 0.016) but not in controls (r = 0.06, P = 0.71), but interaction term did not reach statistical significance (interaction term: $F_{1,92}$ = 2.87, P = 0.09).

Concerning the body skin colouration, experimental treatment also affected the relationship between UV–chroma and nestling body mass (interaction term: $F_{1,92}$ = 6.06, P = 0.016). In this case, we found UV–chroma of body skin were positively associated with body mass for control nestlings (r = 0.55, P < 0.001) but this was not the case for experimental nestlings (r = 0.12, P = 0.41). In addition, the relationship between Yellow–chroma and body mass was also affected by our experimental treatment (interaction term: $F_{1,92}$ = 6.03, P = 0.016, experimental nestlings: r = 0.27, P = 0.06; control nestlings: r = -0.21, P = 0.13). On the other hand, we found also some significant relationships between the gape coloration and body mass not reported when using visual contrast. Namely, we found positive relationships for UV–chroma (F =

39.63, P < 0.001) and UV–brightness (F = 16.67, P < 0.001); and negative relationship for Yellow–chroma (F = 20.21, P < 0.001)), however, none of them differed between experimental and control nestlings significantly (interaction terms: $F_{1,92} < 0.52$, P > 0.47).

DISCUSSION

We found that our experimental treatment with the appetitive stimulant during nestling development had an effect on conspicuousness of one of the measured begging-related traits. Specifically, experimental nestlings had more conspicuous rictal flanges relative to nest background than their control nest mates. These differences might be referable to the flanges of experimental nestlings tending to be more UV coloured and less yellow coloured than those of control nestlings. We also found that our treatment with cyproheptadine also affected colour components of gapes, experimental nestlings possessed a gape more yellow coloured and less UV coloured than their control nestmates. Moreover, the relationship between body mass and some colour measurements of both rictal flanges and body skin was also modified by our experimental treatment.

At least two different explanations may account for these results in a scenario of parent-offspring communication. Magpie nestlings might indicate to parents their levels of short-term food requirements by means of displaying colourations of rictal flanges and/or gapes, as nestlings of canaries and other seed-regurgitating finches do by the redness of their gapes (Kilner 1997, Kilner and Davies 1998). In this case, the experimental increase of hunger levels directly would affect colouration of these traits.

The second possibility explaining the effect of cyproheptadine on colourations of rictal flanges and gapes would be associated with the previously detected positive effect of the drug on the physical condition of experimental magpie nestlings (Martín-Gálvez et al. 2011). This is because physical condition and colouration of begging traits of nestlings are usually related (see the Introduction section). This is probably the cause of the effect of our treatment on colouration of nestling gapes (probably by inducing changes in concentration of carotenoids, see below), as suggested by the detected significant relationships between body mass and gape colourations (positive for UV–chroma and UV–brightness, and negative for Yellow–chroma), which were similar for experimental and control nestlings (P3, Figure 1). However, when considering colouration of rictal flanges, there are several arguments, discussed below, which suggest that there was an effect of our experiment that was independent of the relationship between body mass and colouration, probably by affecting the structural colouration of this trait.

We did not detect an effect of the treatment with cyproheptadine on body-mass gain experienced by nestlings during the experiment (from 2-4 days until 10-12 days after hatching). This result can be conciliated with the positive effect of cyproheptadine on body mass obtained in our previous study (Martín-Gálvez et al. 2011) because the effects were only statistically appreciable after nestlings were 12 days old (Martín-Gálvez 2006). Nonetheless the detected negative relationship between body mass and the chromatic contrasts of rictal flanges allow us to discard the possibility that differences in nestling condition could be responsible of the increased conspicuousness of this trait in experimental nestlings.

Additional pieces of evidence suggesting a role of hunger sensation experienced by nestling during development in the colouration of begging-related traits come from results exploring the effect of the experiment on the relationship between begging-related trait colouration and body mass. For rictal flanges and body skin, we found that the relationships between some colour components of these traits and body mass differed between control and experimental nestlings (P2, Figure 1). Experimental lighter nestlings showed flanges and body skin more conspicuous and more UV-coloured than lighter control nestlings (Figure 3). In other words, experimental nestlings, primarily those with relatively poor body condition, showed an exaggerated level of hunger via the colouration of flanges and body skin, which varied according to their condition.

We also observed a positive statistical association between body mass and yellow–chroma in experimental nestlings for body skin and rictal flanges. These results together with those for the UV-chroma (see above) suggest that the effect of our experiment on the relationships might be mediated by change in concentration of carotenoids. This is because carotenoid concentration in a given tissue is positively related to yellow-chroma and negatively related to UV-chroma, such as has been previously reported for rictal flanges of house sparrow (*Passer domesticus*) nestlings (Dugas and McGraw 2011).

It has been suggested that colouration of flanges (Ewen et al. 2008, Dugas and McGraw 2011, Dugas 2012, Romano et al. 2011) and body skin (Jourdie et al. 2004, Bize et al. 2006, Soler et al. 2007, Avilés et al. 2011) plays a role in parent-offspring communication as signalling nestling quality of several avian species. Our results therefore suggest that for these traits the experimental increase of chronic hunger level de-coupled the association between colour and condition of nestlings commonly detected in nature. Consequently, these results are in agreement with the possibility that level of hunger directly affects colouration of developing begging-related-traits of nestlings. That would be the case independently of the relationships between level of hunger and condition of nestlings on the one hand, and between condition of nestlings and colouration of the other hand. It should be noted here that by concluding in favour of a direct effect of our experiment on flange colouration we are not asserting that cyproheptadine directly acts as the proximal physiological mechanism determining the colour of flanges. Although we cannot completely discount that possibility, as far as we could determine, we did not find any association in the literature between the molecular targets of cyproheptadine (see http://www.drugbank.ca/drugs/DB00434) and possible metabolic pathways involved in the colouration of these nestling traits. Consequently, and considering evidence previously reported (see Introduction section), the most plausible cause of the detected colour differences between experimental and control nestlings would seem to be the differences in hunger levels provoked by cyproheptadine.

Previous studies suggest that flange colouration plays an important role in parent-offspring communication and that magpie parents could allocate food according to conspicuousness of nestling flanges (Gil et al. 2008, Soler and Avilés 2010). In our previous work (Martín-Gálvez et al. 2011), we observed that nestlings administered with cyproheptadine begged for food more frequently than control magpie nestlings and were preferentially fed by parents. The experiment did not affect any other begging variables studied (e.g. relative height, location and order of nestlings whilst begging, Martín-Gálvez et al. 2011) and thus variation in rictal flanges of magpie nestlings might be one of the traits determining the preferential feeding of experimental nestlings by adult magpies. Nonetheless, a direct modification of colouration of this trait would be needed to confirm the casual link between the parental preferential feeding and the colouration of rictal flanges in magpies.

Summarizing, our experimental results suggest that the colour of rictal flanges and body skin of magpie nestlings indicates sensation of chronic hunger during development, and that this effect is at least partially independent of the relationship between physiological conditions and colouration of begging related traits of nestlings. Furthermore, we found evidence suggesting that colouration of nestling gapes would indicate the physical condition of nestlings.

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Legend to figures

Figure 1.- Hypotheses and predictions of the function of colouration of begging-related traits of magpie nestlings that were tested experimentally by an appetite stimulant, the cyproheptadine hydrochloride (see more details in text).

Figure 2.- Average (± SD) spectral reflectance (300 nm to 700 nm) of measured nestling traits.

Figure 3.- Scatterplots and Pearson's correlation coefficients of the relationships between the residuals (nestling values minus the average value per nest) obtained for body mass, and chromatic and achromatic contrasts against nest background for rictal flanges and body skin. Open circles and dotted lines refer to control nestlings, filled circles and continued lines refer to experimental nestlings. The sample sizes were 48 experimental and 48 control nestlings from 25 different nests.

Figure 1

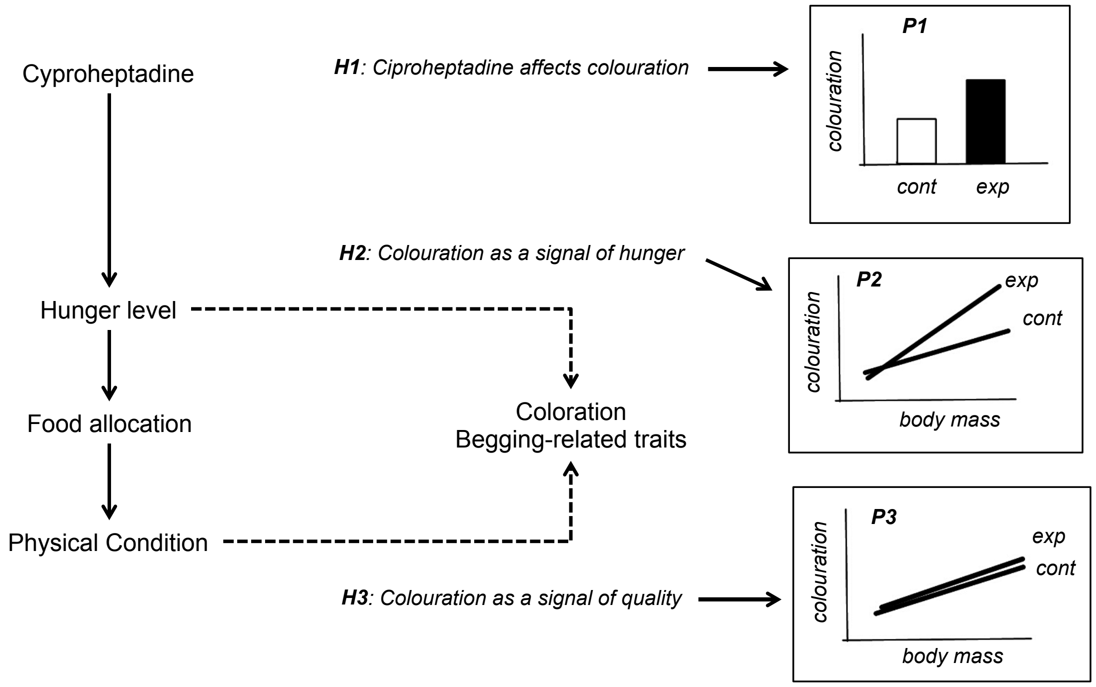
Cyproheptadine
Hunger level
Food allocation
Physical Condition
Coloration
Begging-related traits
H1: Ciproheptadine affects colouration
H2: Colouration as a signal of hunger
H3: Colouration as a signal of quality
P1
colouration
cont
exp
P2
colouration
exp
cont
body mass
P3
colouration
exp
cont
body mass

Figure 2

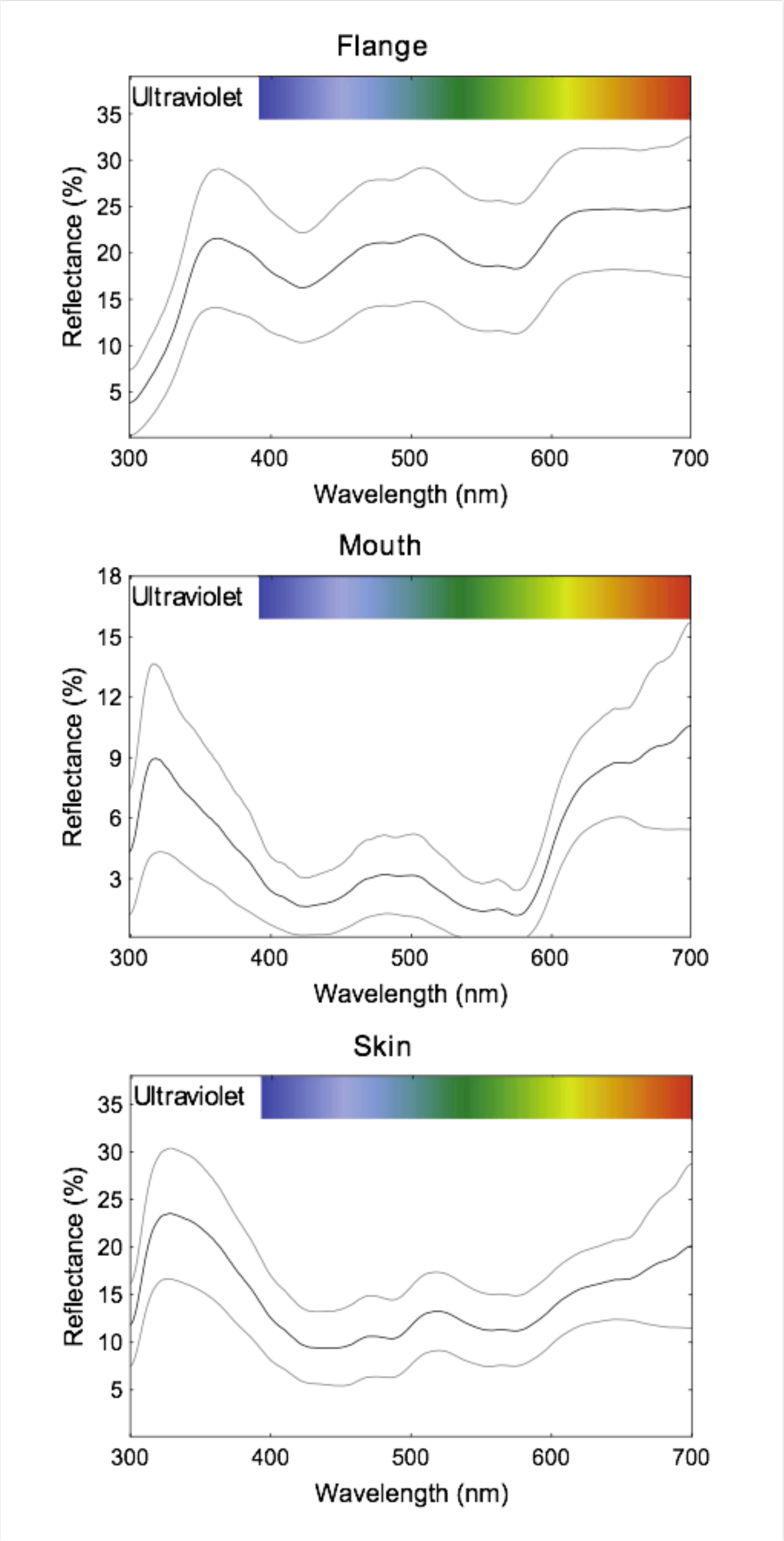
Flange
Ultraviolet
Reflectance (%)
5
10
15
20
25
30
35
300
400
500
600
700
Wavelength (nm)
Mouth
Ultraviolet
Reflectance (%)
3
6
9
12
15
18
300
400
500
600
700
Wavelength (nm)
Skin
Ultraviolet
Reflectance (%)
5
10
15
20
25
30
35
300
400
500
600
700
Wavelength (nm)

Figure 3

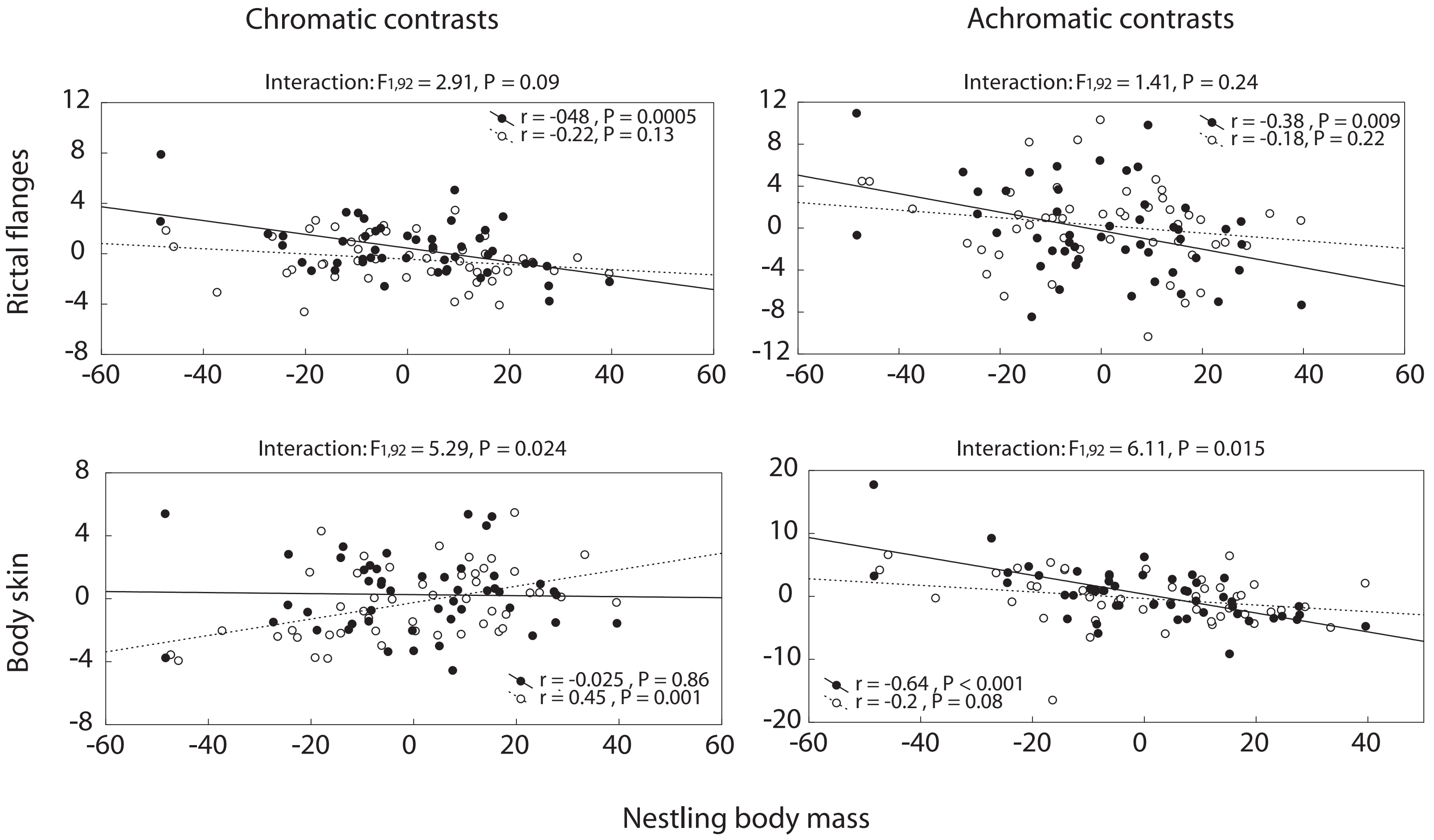
Chromatic contrasts
Achromatic contrasts
Interaction: F1,92 = 2.91, P = 0.09
r = -048 , P = 0.0005
r = -0.22, P = 0.13
Interaction: F1,92 = 1.41, P = 0.24
r = -0.38 , P = 0.009
r = -0.18, P = 0.22
Interaction: F1,92 = 5.29, P = 0.024
r = -0.025 , P = 0.86
r = 0.45 , P = 0.001
Interaction: F1,92 = 6.11, P = 0.015
r = -0.64 , P < 0.001
r = -0.2 , P = 0.08
Rictal flanges
Body skin
Nestling body mass
12
8
4
0
-4
-8
-12
20
10
-10
-20
-60
-40
-20
0
20
40
60

Table 1.- Results from the analyses to test the effect of the experimental treatment with cyproheptadine and body mass on chromatic and achromatic conspicuousness (relative to nest background) of nestling traits.

	Chromatic contrasts				Achromatic contrasts			
Factors	**df**	**MS**	**F**	**P**	**df**	**MS**	**F**	**P**
Flanges								
(Intercept)	1	<0.01	<0.01	0.98	1	0.01	<0.01	0.98
Treatment	1	18.43	5.46	0.022	1	6.01	0.37	0.54
Mass	1	48.40	14.34	<0.001	1	131.76	8.17	0.005
Treatment × Mass	1	9.84	2.91	0.09	1	22.74	1.41	0.238
Error	92	3.38			92	16.12		
Mouth								
(Intercept)	1	0.04	<0.01	0.98	1	<0.01	<0.01	1.00
Treatment	1	27.32	0.44	0.51	1	68.00	1.87	0.17
Mass	1	0.43	0.01	0.93	1	14.38	0.40	0.53
Treatment × Mass	1	138.79	2.26	0.14	1	1.88	0.05	0.82
Error	92	61.40			92	36.38		
Body Skin								
(Intercept)	1	0.01	<0.01	0.97	1	0.02	<0.01	0.97

Treatment	1	6.14	1.24	0.27	1	11.12	0.84	0.36
Mass	1	20.37	4.13	0.045	1	346.48	26.09	<0.001
Treatment × Mass	1	26.12	5.29	0.024	1	81.16	6.11	0.015
Error	92	4.94			92	13.28		

Analyses are Linear Models for data from 96 magpie nestlings.

* Chromatic and achromatic contrasts and body mass were corrected by differences among nests (25 different nests) by equalling the within-nests mean values to be zero while maintaining original within-nest variance.