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Experimental pyrethroid treatment underestimates the effects of ectoparasites in cavity-nesting birds due to toxicity

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Nest-dwelling ectoparasites may result in costs for nestlings of cavity nesters in terms of compromised growth and condition before fledging. The reduction or elimination of nest ectoparasites to study their effects on avian hosts can be conducted through physical methods such as heat-treatment or through chemical methods using insecticides. Pyrethroids are the most frequently used of the latter, although some studies have shown that they may compromise the development and future survival of birds. In this study conducted in central Spain we analysed the differences between a group of fumigated Pied Flycatcher *Ficedula hypoleuca* nests and a heat-treated group, both rendered ectoparasite-free by these treatments. We also compared these ectoparasite-free nests with a control group with natural ectoparasite loads. Our aim was to test the possible effects of a pyrethroid-based insecticide on reproductive success, parental care behaviours and body condition of adult females and nestlings. We also determined the effects of treatment on a biochemical biomarker, the total glutathione (tGSH) level, involved in detoxification of xenobiotics and considered the most important intracellular antioxidant. Although behavioural variables were not affected by treatment, results showed lighter 3-day-old chicks and shorter tarsi and wings in nestlings shortly before fledging in fumigated nests, together with depletion of tGSH levels in both females and nestlings. Fumigation with pyrethroids in ectoparasite load reduction experiments may introduce undesired systematic variability associated with toxicity, leading to underestimation of the effects of ectoparasites on avian hosts.

Keywords: body condition, glutathione, heat treatment, insecticide, nest-dwelling parasites, Pied Flycatcher.

Avian cavity-nesting has been traditionally associated with selective pressures derived from benefits in terms of the thermal environment and the impact of nest predation (Hansell 2000). Although protected from weather and relatively safe from predators, nesting cavities constitute microclimatically stable environments that may offer excellent breeding and growth conditions for bacteria, decomposers and detritivores due to the presence of faeces and food remains of breeding birds, and for ectoparasites that feed on blood, skin and feathers of avian hosts (Collias & Collias 1984).

Parasites may be an important ecological and evolutionary factor affecting avian life histories and behaviour (Atkinson & Van Riper 1991, Møller 1997). Nest-dwelling ectoparasites may result in costs for nestlings of cavity nesters in terms of compromised growth and condition before fledging (Heeb *et al.* 2000, Tomás *et al.* 2008, Brommer *et al.* 2011, Cantarero *et al.* 2013a). They may also affect adult behavioural responses directed towards

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minimizing their negative effects (Christe *et al.* 1996, Heeb *et al.* 1998, Tripet *et al.* 2002, Cantarero *et al.* 2013a).

Effects of nest-dwelling ectoparasites on hosts are frequently studied through correlational approaches by quantifying directly the number of parasites in nests, or through experimental manipulations of ectoparasite loads (Moss & Camin 1970). These manipulations can be accomplished by increasing ectoparasite loads, specifically by the addition of certain parasites to nests, or by reducing them. The reduction or elimination of nest ectoparasites can be conducted through physical methods (direct extraction of parasites, freezing or heat-treating nests) or through chemical methods using insecticides. In these experimental manipulations, it is common to compare a sample of treated nests and another of unmanipulated nests as a control group. The assumption behind these treatments is that only ectoparasites are affected by the manipulation and that other environmental variables remain unaffected, thereby allowing the effect of the parasites to be deduced from the results. Therefore, it is important to control undesirable effects arising from handling that would alter our results.

The most frequently used insecticides in such experiments are pyrethroids (Szép & Møller 2000, Heylen *et al.* 2009), highly active synthetic insecticides derived from natural pyrethrins (Vijverberg & Van den Bercken 1990) produced by the flowers of pyrethrums (*Chrysanthemum cinerariaefolium* and *Chrysanthemum coccineum*) that constitute the majority of commercial household insecticides. Most of them are composed of permethrin, sometimes accompanied by tetramethrin, at concentrations below 0.5%. The International Programme on Chemical Safety of the World Health Organization included some pyrethroids in 1990 in its Environmental Health Criteria documents and described their effects as highly toxic to fish and other aquatic organisms but of low or very low toxicity to birds and mammals. However, some studies have demonstrated their negative effects on poultry by disrupting cellular function through adverse effects on the activities of enzymes that contribute to the detoxifying activity of glutathione (GSH) (Ezeji *et al.* 2012), one of the most efficient cellular tools for detoxification of drugs and xenobiotics in general (Pompella *et al.* 2003). Reported effects of pyrethroids include haematological and biochemical alterations and damage to tissues such as kidney and liver in avian, fish and mammalian species (Khan *et al.* 2012 and references therein) and negative effects on GSH-related metabolism in rats (Otitoju & Onwurah 2005).

These effects may compromise the development and future survival of some organisms. Altricial nestlings of cavity-nesting birds could be especially vulnerable to these risks given the closed nesting environment in which pyrethroids are released and their still underdeveloped detoxification mechanisms. In a previous study with the same set of nests as we heat-treated here, we demonstrated and discussed the negative effects that ectoparasites have on nestling body condition when compared with control nests (Cantarero *et al.* 2013a). Here we assess the effects of insecticide treatment on variables commonly reported in ectoparasite manipulations, such as reproductive success, parental care behaviour, body condition of adult females, and development and condition of nestlings. We also assess the effects of pesticide exposure on a biochemical biomarker related to detoxification, the intracellular total glutathione level (tGSH). tGSH level is expected to decrease in the pyrethroid-exposed treatment due to the disruptive effect of the drug on GSH metabolism (Ezeji *et al.* 2012). In accordance with the assumption that the use of insecticides, due to their toxicity, neutralizes the positive effects of reducing nest ectoparasite loads, our predictions were that the effects on nestling condition and growth of the two most widely used treatments to reduce ectoparasite loads on nests (heat and insecticides) differ from each other, and that the fumigation-treated nests do not differ from controls with natural ectoparasite loads. To that end, we studied a breeding population of the Pied Flycatcher *Ficedula hypoleuca*, a typical cavity-nester with high prevalence of ectoparasite infestations.

METHODS

General field methods

The study was conducted during the spring of 2012 in a montane deciduous forest of Pyrenean Oak *Quercus pyrenaica* in Valsaín, central Spain (40°54′N, 4°01′W; 1200 m elevation), where long-term studies on cavity-nesting birds have been ongoing since 1991 (see Sanz *et al.* 2003 for general description). In the study area there are 552 nestboxes (see Lambrechts *et al.* 2010 for dimensions, structure and placement of nestboxes) occupied by Pied Flycatchers, Great Tits *Parus major*, Eurasian

Nuthatches *Sitta europaea* and Blue Tits *Cyanistes caeruleus*. We followed breeding activities from the early stages of nest construction to fledging in nestboxes occupied by Pied Flycatchers. Egg-laying in this Pied Flycatcher population typically begins in late May, and the modal clutch size is six. The female incubates and broods alone (Moreno *et al.* 2011). No brooding is observed after nestlings attain 7 days of age (Sanz & Moreno 1995) and chicks usually fledge at the age of 17 days.

At the age of 3 days we weighed all nestlings in each brood together on a digital balance to the nearest 0.1 g to determine mean chick mass. On day 13 (hatching day = day 1), nestlings were ringed, weighed and measured. Body mass was obtained with a Pesola® spring balance (Pesola AG, Baar, Switzerland) to the nearest 0.25 g, tarsus length was measured with a digital calliper (precision 0.01 mm) and wing length with a stopped ruler. We took a blood sample of about 120 μL from the brachial vein that was collected in heparinized microcapillaries. Blood samples (n = 333) were stored in Eppendorf tubes in an ice-box until returning to the laboratory on the same day. Plasma was separated from blood cells by centrifugation (10 min at 14 000***g***) and then both fractions were stored at –80 °C until analysed for assaying tGSH levels (see below).

Parents were captured in their nestboxes with traps while feeding nestlings of 7–8 days, ringed if necessary or identified by their ring. Females (n = 66) were also measured and blood-sampled in the same way as nestlings.

During blood collection, some samples may suffer rupture of erythrocytes, possibly due to changes in pressure during extraction. Haemolysis could cause a possible efflux of intracellular molecules into the extracellular environment that could affect the results of analyses. Thus, we controlled for haemolysis levels in plasma samples by detecting visually the red colour of plasma that occurs as a consequence of release of haemoglobin from red blood cells, and marking samples on a gradient from 0 (no haemolysis) to 2 (high degree of haemolysis). To minimize inter-observer variability only one person noted haemolysis degree.

Protocol of experimental reduction of ectoparasites

Of the 91 nestboxes occupied by Pied Flycatchers, we selected birds whose laying date was between dates 45 and 50 (April 1 = day 1, mean ± 1 se laying date = 47.94 ± 0.18). We applied different methods to reduce ectoparasite loads in nests during the egg-laying period by randomly allocating nests to the following three treatments: (1) a heat-treated group (n = 19) using a microwave oven to reduce ectoparasite loads, (2) a fumigated group (n = 14) sprayed with an insecticide, and (3) an unmanipulated control group with natural ectoparasite loads (n = 33).

In the first treatment, nests were heat-treated for about 30 s at 750 W. This treatment ensured that experimental nests did not contain live arthropods when placed in the nestbox (Rendell & Verbeek 1996), although some arthropods may colonize the nest material after the treatment. To avoid the loss of water during the heat treatment, the nests were placed into a hermetic plastic container. Furthermore, before returning the original nest, the flame from a butane jet torch lighter (Microtorch GT-3000, Blazer Products, New York, USA) was passed across the walls of the nestbox to kill ectoparasites that might remain there. While the original nests were removed from the nestboxes for treatment (around 30 min), a fresh substitute nest was introduced into the nestbox (these nests had been collected in previous seasons after being abandoned prior to laying and kept frozen at −20 °C until use).

In the fumigated group, nests and nestboxes were sprayed with a commercial pyrethroid-based insecticide (Itec Spray, Natural Granen SA, Belgium; 0.3% permethrin, 0.2% tetramethrin and 1% piperonyl butoxide) for about 5 s and then aerated for 30 s. Chicks were previously removed from the nests and kept in a container with a cotton base during treatment.

To prevent recurrence of ectoparasite colonization, a total of three repetitions of the treatments were made: (1) 7 days after clutch completion, (2) when nestlings were 2 days old and (3) when nestlings were 8 days old. Nests in the control group were visited on the same days and handled in a similar way to experimental ones but without applying any treatment.

Ectoparasite abundance estimation

The most common ectoparasites in nests of Iberian Pied Flycatcher populations are mites *Dermanyssus gallinoides*, fleas *Ceratophyllus gallinae* and the larvae of blowflies *Protocalliphora azurea*. Mites have the strongest effect on nestling growth and

mortality in some populations (Merino & Potti 1996, 1998, Moreno *et al.* 1999, 2008, 2009, Potti *et al.* 1999, 2002, Lobato *et al.* 2005, 2008, Potti 2007, Martínez-de la Puente *et al.* 2009, 2010) and their prevalence, often exceeding 60%, is usually higher than for blowflies and fleas (40–50%) (Merino & Potti 1995, Tomás *et al.* 2007, 2012, Moreno *et al.* 2009, Cantarero *et al.* 2013b). Blowfly larvae are parasites that feed intermittently on the blood of nestlings and otherwise dwell in the nest material (Bennett & Whitworth 1991, Remeš & Krist 2005). Flea larvae are not haematophagous, but the adults need blood to produce eggs (Tripet & Richner 1997). Therefore, the number of larvae in nests indicates the fecundity of adult fleas (Eeva *et al.* 1994). In mites, both adult and some nymphal stages are haematophagous (Merino & Potti 1995).

At 1 or 2 days after nestlings fledged (17 days after hatching), all nests were removed in sealed plastic bags and taken to the laboratory, where they were subjected to arthropod removal in Berlese funnels for 48 h until nests were thoroughly dried and no arthropods were moving in the nest material. Ectoparasite identification was made with the aid of a stereoscopic microscope (Olympus SZX7, Olympus Iberia, Barcelona, Spain); for arthropod collection and abundance estimations see Moreno *et al.* (2009).

Determination of tGSH levels

tGSH levels in red blood cells were determined according to Galván and Alonso-Álvarez (2008) with a few modifications. Red blood cell samples were diluted (1 : 20 w/v) and homogenized in a stock buffer (0.01 M phosphate-buffered saline and 0.02 M EDTA) using a Mini-BeadBeater (BioSpec Products, Bartlesville, OK, USA) and mixed with an equal volume of 10% trichloroacetic acid. The mixture was vortexed three times for 5 s each bout within a 10-min period. The mixture was then centrifuged (2000***g***, 15 min, 6 °C), and the supernatant was separated. Three working solutions were made up in a reaction buffer (125 mM Na-phosphate and 6.3 mM EDTA) as follows: (1) 0.3 mM NADPH, (2) 6 mM DTNB and (3) 50 U GSH reductase/mL. Solutions 1 and 2 were mixed at 7 : 1 volume. The next steps were performed on a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). To 75 μL of sample (supernatant) we added 240 μL of the mixture of solutions 1 and 2. Then, 20 mL of solution 3 was added after 15 s and the absorbance at 405 nm was monitored after 15 and 45 s. The change in absorbance was used to determine the intracellular tGSH concentration by comparing the output with the results from a standard curve generated by serial dilution of GSH from 0.5 to 0.031 mM. Only one 12-well row was used from the plate at a time. A subset of samples assayed in duplicate showed high repeatability ($R = 0.929$, $n = 44$, $P < 0.001$).

Behavioural data

At 2 and 8 days after the day of hatching of the young, we recorded nest activity inside nestboxes for about 90 min with a cold white light (5-mm LED) powered by a 3-V battery and a camera (GoPro HD Hero1, Woodman Labs, San Mateo, CA, USA) mounted on the roof inside the nestbox. All films were recorded between 08:00 and 17:00 h (for more details see Cantarero *et al.* 2013a,b) and no differences between groups with respect to time of filming were found (general linear model (GLM) analyses: early nestling phase: $F_{2,63} = 2.056$, $P = 0.136$; late nestling phase: $F_{2,63} = 1.517$, $P = 0.227$).

From recordings during the early nestling phase we obtained hourly provisioning rates by males and females and brooding attendance, estimated as the proportion of time spent by the female inside the nestbox.

From recordings during the late nestling phase we obtained hourly provisioning rates by males and females.

Statistical analysis

Statistical analyses were conducted using STATISTICA (version 8.0; StatSoft, Inc., Tulsa, OK, USA). We first tested for success of treatments to reduce or eliminate ectoparasite loads on nests using Kruskal–Wallis tests and then for the relationship between treatment and breeding parameters in three groups with ANOVA (laying and hatching dates) or Kruskal–Wallis tests (clutch size) depending on the distribution of the dependent variable.

Brood sizes at days 3 and 13 and hatching (proportion of eggs that hatched) and fledging successes (proportion of hatched chicks that fledged) were analysed with Kruskal–Wallis tests. Mean

body mass per chick when nestlings were 3 days old was analysed with GLM with treatment as fixed factor. All behavioural data and adult female body mass and tGSH levels were analysed in the same way. Nestling morphological and biochemical variables measured before fledging (day 13) were analysed with GLM including nest identity nested within treatment as random factor and treatment as fixed factor.

Although degree of haemolysis in blood samples of nestlings and females was not affected by treatment (Kruskal–Wallis test: females: $H_2 = 0.562$, $n = 63$, $P = 0.755$; nestlings: $H_2 = 0.246$, $n = 329$, $P = 0.884$), haemolysis may affect tGSH levels, thereby confounding interpretation of results. Thus, we controlled for this factor in all tGSH analyses.

These analyses were performed to test differences between three groups. *Post-hoc* analyses were performed when models were significant in order to explore pair-wise comparisons of means by Fisher's least significant difference (LSD) tests.

RESULTS

Ectoparasite loads and breeding parameters

The three types of ectoparasites were greatly reduced or eliminated in heat-treated and fumigated groups in comparison with control nests (Table 1). The three treatments did not differ with respect to laying date, hatching date and clutch size (Table 1).

Reproductive success

The three treatments did not differ with respect to brood size (Kruskal–Wallis test: brood size at day 3: $H_2 = 0.657$, $n = 66$, $P = 0.720$; brood size at day 13: $H_2 = 1.902$, $n = 66$, $P = 0.386$), hatching success (Kruskal–Wallis test: $H_2 = 0.686$, $n = 66$, $P = 0.709$) or fledging success (Kruskal–Wallis test: $H_2 = 2.523$, $n = 66$, $P = 0.283$).

Parental care behaviours

Neither brooding attendance nor hourly provisioning rates by females and males at early nestling phase were affected by treatment, as were provisioning rates before fledging (all $P > 0.20$).

Body condition

Mean body mass per chick on day 3 was significantly higher in the heat-treated group than in the fumigated nests (Table 2). At fledging, chicks in heat-treated nests had longer tarsi and wings than chicks in fumigated nests (Table 2). Nestling body mass at fledging was not different between ectoparasite reduction treatments (Table 2). No morphological parameter of females and nestlings differed between control and fumigated nests (Table 2). Female body mass was affected by treatment, being significantly higher in the fumigated than in the heat-treated group (Table 2).

Total GSH levels

Nestlings showed differences in tGSH between treatments, with lower levels in fumigated nests (Fig. 1a; $F = 11.272$, $n = 329$, $P < 0.001$; pair-wise comparisons: all $P < 0.001$) after controlling by haemolysis score ($F = 14.174$, $P < 0.001$). Females showed significantly higher levels of tGSH in heat-treated nests than in the fumigated group (Fig. 1b; $F = 3.334$, $n = 60$, $P = 0.043$; pair-wise comparisons: control–fumigated: $P = 0.722$; fumigated–

Table 1. Differences in ectoparasite abundance and breeding variables (mean ± 1 se) between three treatments and results of Kruskal–Wallis and GLM tests.

	Control	Heat	Insecticide	Statistic	P
Ectoparasites					
Blowflies	5.54 ± 1.17	0.68 ± 0.43	0.00 ± 0.00	$H = 22.221$	< 0.001
Mites	3713.58 ± 815.35	274.05 ± 208.06	83.86 ± 35.49	$H = 25.006$	< 0.001
Fleas	27.82 ± 16.19	0.00 ± 0.00	0.00 ± 0.00	$H = 11.504$	0.003
Breeding variables					
Laying date (1 = 1 April)	48.15 ± 0.25	47.37 ± 0.34	48.21 ± 0.39	$F = 2.040$	0.139
Hatching date	66.45 ± 0.24	65.95 ± 0.32	66.50 ± 0.37	$F = 1.000$	0.387
Clutch size	5.67 ± 0.11	5.84 ± 0.15	5.57 ± 0.18	$H = 1.116$	0.572

Table 2. Results of GLM analyses for effects of treatment in nestling morphological variables at early and late phases (treatment as fixed factor at day 3; nest nested within treatment as random factor and treatment as fixed factor at day 13) and in adult female body mass (treatment as fixed factor) after controlling by brood size and laying date.

	Control	Heat-treated	Fumigated	Statistic (F)	df residual	Partial η^2	Model P-value	*Post-hoc P*-values F-C	F-H	C-H
Nestlings day 3										
Mean body mass (g)	3.36 ± 0.10	3.76 ± 0.13	3.23 ± 0.15	4.453	63	0.124	0.015	0.461	0.009	0.016
Nestlings day 13										
Body mass (g)	13.97 ± 0.05	14.16 ± 0.06	14.12 ± 0.08	0.342	258	0.011	0.712	–	–	–
Tarsus length (mm)	17.48 ± 0.03	17.77 ± 0.04	17.61 ± 0.05	4.128	255	0.113	0.020	0.093	0.022	< 0.001
Wing length (mm)	47.07 ± 0.13	48.67 ± 0.16	47.59 ± 0.20	4.739	245	0.129	0.012	0.398	< 0.001	< 0.001
Female										
Body mass (g)	12.79 ± 0.14	12.32 ± 0.19	13.29 ± 0.23	5.224	61	0.146	0.008	0.085	0.009	0.168

C, control group; H, heat-treated group; F, fumigated group.

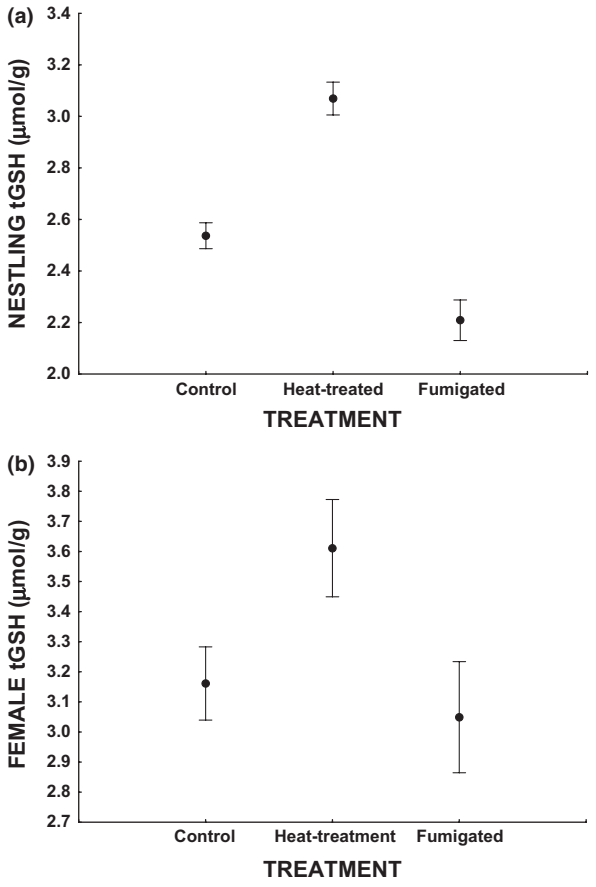

Figure 1. Mean ± 1 se tGSH levels in relation to treatment in (a) nestlings and (b) adult females.

heat-treated: $P = 0.022$; control–heat-treated: $P = 0.017$) after controlling by haemolysis score ($F = 6.309$, $P = 0.015$).

DISCUSSION

We conducted an experimental study to test the effects of two different treatments to reduce nest ectoparasite loads on parental care and body condition of females and nestlings in an Iberian Pied Flycatcher population. Our results showed that both types of randomly applied treatments were effective in greatly reducing or eliminating ectoparasite loads, with both treatments being similarly efficient. Although parental care behaviours were not affected by treatments, results confirmed our initial hypothesis about the negative effects of pyrethroid-based insecticides, as expressed by lighter 3-day-old chicks, reduced skeletal and integumentary development shortly before fledging, and a reduction of tGSH, an important biomarker of redox status with an essential role in cellular detoxification.

Shortly before fledging, nestlings showed reduced tarsus and wing lengths in fumigated nests compared with heat-treated ones, without differences between fumigated and control nests. Thus, nestling growth was negatively affected by ectoparasites in the control group (Cantarero *et al.* 2013a) and by the insecticide in fumigated nests. Tarsus length of Pied Flycatcher nestlings has been related

to their recruitment probability (Alatalo & Lundberg 1986), so these negative effects may affect the future fitness of nestlings. Wing development at fledging may also be crucial during the first post-fledging days (Nilsson & Gårdmark 2001). The lack of an effect of treatment on nestling body mass may be due to favourable conditions for breeding during the year of study (largest body masses since 1991, Cantarero *et al.* 2013a) affecting nestling mass more strongly than structural and integumentary growth.

Female body mass differed between treatments in the opposite direction to that predicted if toxicity impaired maternal condition. We have no convincing explanation for this striking result, although it may suggest a reallocation of maternal resources to survival instead of current reproduction where offspring have been affected by toxicity.

At the physiological level, GSH is a very important detoxifying agent, enabling the body to get rid of undesirable toxins and pollutants and playing an important role as an antioxidant and in detoxification and elimination of xenobiotics (pesticides) (Meister & Anderson 1983, Otitoju & Onwurah 2007). The depletion of tGSH in individuals exposed to pyrethroid-based insecticides could be explained in two non-mutually exclusive ways. On the one hand, GSH reacts with a large number and variety of foreign compounds with an electrophilic centre to form GSH conjugates. The interaction of foreign compounds with GSH may be spontaneous or catalysed by GSH *S*-transferases (Meister & Anderson 1983). GSH-adducts formed after conjugation of electrophiles are then actively secreted from the cell, eventually resulting in the depletion of cellular GSH (Pompella *et al.* 2003). Thus, reduction in GSH level is an indication that detoxification is taking place (Ezeji *et al.* 2012). Alternatively, GSH operates in the reduction of the disulphide linkages of proteins and in the protection of cells against oxidative damage, effectively scavenging free radicals and other reactive oxygen species (Meister 1991, Wu *et al.* 2004). At normal levels of oxidative stress there is essentially no net loss of GSH through oxidation (Griffith 1999). However, if pro-oxidant levels increase sufficiently, GSH protects cells by reacting rapidly with peroxides and producing GSSG (glutathione disulphide, an oxidized form of GSH). Because GSSG is not taken up intact by cells but rather is degraded extracellularly, GSSG efflux from cells contributes to a net loss of intracellular GSH (Griffith 1999, Wu *et al.* 2004). Thus, the depletion of cellular tGSH levels in individuals from fumigated nests may indicate an increase in reactive oxygen and nitrogen species (RONS, and, as a consequence, an increase in oxidative status of individuals related to treatment (Kale *et al.* 1999). In nestlings, the decrease in tGSH levels was even higher in fumigated nests than in controls, which may indicate that the insecticide has stronger adverse effects than ectoparasites during development. In the case of adult females, the depletion of intracellular tGSH suggests a direct negative effect on female physiology, probably suffered through direct exposure during the incubation and brooding phases, and not only due to the work overload associated with increased nestlings needs.

The depletion of tGSH shown in our results is in accordance with several studies based on pesticide effects (e.g. Della Morte *et al.* 1994, Kale *et al.* 1999, Ezeji *et al.* 2012, Fetoui & Gdoura 2012). Oxidative stress is a key cost that limits rates of growth in the wild (Hall *et al.* 2010). An adequate availability of antioxidants has been shown to enhance both pre- and post-hatch growth, reduce susceptibility to pathogens, and increase the ability of chicks to withstand oxidative damage (Surai 2002). Thus, a depletion in GSH levels, considered the most important intracellular antioxidant (Meister 1991), might be involved in the trade-off between self-maintenance and growth rate of nestlings and between self-maintenance and breeding effort in adult individuals during nestling development phase.

We also demonstrated that the negative effects on nestling condition exposed to a pyrethroid-based insecticide were explained by its toxicity and not by changes in behaviours related to parental care, as these were not related to treatment. Our results suggest that the choice by researchers of method to reduce ectoparasite loads to test their effects on organisms could affect the conclusions derived from the experiments. This is the first study to our knowledge that shows the different effects of two experimental methods used to reduce ectoparasite loads in avian nests. The use of pyrethroid insecticides can introduce in this type of experiment undesired systematic variability associated with toxicity that leads to underestimations of the effects of ectoparasites on avian hosts.

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