Running head: *Pyrethroids underestimate effects of ectoparasites.*

Experimental pyrethroid treatment underestimates effects of ectoparasites in cavity-nesting birds due to potential 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 like heat-treatment or through chemical methods using insecticides. Pyrethroids are the most frequently used, although some studies have shown that they may compromise the development and future survival of birds. In this study conducted in central Spain we analyzed the differences between a group of fumigated Pied Flycatcher *Ficedula hypoleuca* nests and a heat-treated group, both rendered ectoparasite free by 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 as 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 in tGSH levels in both female and nestlings. Fumigation with pyrethroids may introduce in ectoparasite-load reduction

34 experiments undesired systematic variability associated with toxicity leading to
35 underestimations of the effects of ectoparasites on avian hosts.

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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 have been proposed as an important ecological and evolutionary factor affecting avian life histories and behaviour (Atkinson & Van Riper 1991, Møller 1997). Thus, 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 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 since Moss and 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 usual to make comparisons between a set 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 results. Therefore, it is important to control undesirable effects arising from handling that would alter our results.

In the case of insecticides, the most frequently used 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 *C. 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 in 1990 in its Environmental Health Criteria documents some pyrethroids and described their effects as highly toxic for fish and other aquatic organisms and of low or very low toxicity for birds and mammals. However, some studies have shown 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), GSH being 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 like 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 under-developed detoxification mechanisms. In a previous study with the same set of nests as 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), so in

the present study we test the negative effects of insecticide treatment on variables commonly reported in ectoparasite manipulations such as (1) reproductive success, (2) parental care behaviour, (3) body condition of adult females, and (4) development and condition of nestlings. We also test the effects of pesticide exposure on (5) 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 disrupting 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, neutralize the positive effects of reducing nest ectoparasite loads, our predictions were (1) 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 (2) that the fumigation treated nests do not differ from controls with natural ectoparasite loads. To that end, we have 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 Valsain, central Spain (40° 54' N, 4° 01' W, at 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 nest-boxes (see Lambrechts *et al.* 2010 for dimensions, structure and placement of nest-boxes) occupied by Pied Flycatchers, Great Tits (*Parus major*), Nuthatches (*Sitta europaea*) and Blue Tits (*Cyanistes caeruleus*). We followed breeding activities from the early stages of nest

construction to fledging in nest-boxes occupied by Pied Flycatchers. Egg laying in the Pied Flycatcher population under study 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 an average mass per chick. On day 13 (hatching day = day 1), nestlings were ringed, weighed and measured. Body mass was obtained with a Pesola® spring balance to the nearest 0.25 g, tarsal 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 lab in the same day. Plasma was separated from blood cells by centrifugation (10 min at 12000 rpm) and then both fractions were stored at –80 °C until analysed for assaying tGSH levels (see below).

Parental individuals were captured in their nest-boxes 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 extracellular environment that could affect the results of analyses. Thus, here we controlled haemolysis levels in plasma samples, that were noted by a visual detection of red colour of plasma, as a consequence of release of haemoglobin from red blood cells, in a gradient from 0 (no haemolysis) to 2 (high degree of haemolysis). Only one person noted haemolysis degree in order to minimize inter-observer variability.

Protocol of experimental reduction of ectoparasites

Of the 91 nest boxes occupied by Pied Flycatchers we selected those whose laying date was between dates 45 and 50 (April 1 = day 1, mean $\pm$ SE laying date = 47.94 $\pm$ 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 during about 30 s at 750 W. This treatment ensured that experimental nests did not contain live arthropods when placed in the nest- box (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) was passed across the walls of the nest-box to kill ectoparasites that might remain there. During the time that the original nests were removed from the nest-boxes for treatment (around 30 min), a fresh substitute nest was introduced into the nest-box (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 nest-boxes were sprayed with a commercial pyrethroid-based insecticide (Itec Spray, Natural Granen SA, Belgium; 0.3 % permethrin, 0.2 % tetramethrin and 1 % piperonyl butoxide) during 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 (hatching day = day 1) 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 of different virulence 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, Potti *et al.* 1999, 2002, Potti 2007, Lobato *et al.* 2005, 2008, Moreno *et al.* 1999, 2008, 2009, 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, Moreno *et al.* 2009, Tomás *et al.* 2007, 2012, Cantarero *et al.* 2013b). Blowfly larvae are parasites that feed intermittently on the blood of nestlings and in the meantime 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).

One or two 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), (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 some particular 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, Oklahoma, USA) and mixed with an equal volume of 10 % trichloroacetic acid. The mixture was vortexed three times during 5 s each bout within a 10 min period. Afterward, the mixture was centrifuged (3000 ref, 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.). To 75 µl of sample (supernatant) we added 240 µl of the mixture of solutions 1 and 2. Afterward, 20 µL 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 a high repeatability ($R = 0.929$, $N = 44$, $p < 0.001$).

Behavioural data

Two and eight days after the day of hatching of the young, we recorded nest activity inside nest-boxes for about 90 min with a cold white light (LED 5 mm) powered by a 3 V battery

and a camera (GoPro HD Hero1) mounted on the roof inside the nest-box. All films were recorded between 08:00-17:00 h (for more details see Cantarero *et al.*, 2013) and no differences between groups with respect to time of filming were found (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”, meaning the proportion of time spent by the female inside the nest-box.

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

Statistical analyses

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

Brood sizes at days 3 and 13 and hatching (proportion eggs that hatched) and fledging successes (proportion hatched chicks that fledged) were analyzed with Kruskal-Wallis tests. Mean body mass per chick measured 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 analyzed in the same way. Nestling morphological and biochemical variables measured before fledging (day 13) were analyzed 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 LSD tests.

RESULTS

Ectoparasite loads and breeding parameters

Three types of ectoparasites were severely 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 well as provisioning rates near 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 (Figure 1.a; $F = 11.272$, $N = 329$, $p < 0.001$; 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 (Figure 1.b; $F = 3.334$, $N = 60$, $p = 0.043$; pair-wise comparisons: Control-Fumigated: $p = 0.722$; Fumigated-Heat-treated: $p = 0.022$; Control-Heat-treated: $p = 0.017$) after controlling by haemolysis score ($F = 6.309$, $p = 0.015$).

DISCUSSION

We have 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 show that both types of randomly applied treatments were effective in drastically reducing or eliminating ectoparasite loads, with both treatments being similarly efficient. Although, parental care behaviours were not affected by

treatments, results confirm 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 effects 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 more strongly nestling mass than structural and integumentary growth.

We found that 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 in detriment of reproduction effected 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 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 center to form GSH conjugates. The interaction of foreign compounds with GSH may be spontaneous or catalyzed 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 going on (Ezeji *et al.* 2012). On the other hand, GSH operates in the reduction of the disulfide 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 reacting rapidly with peroxides and producing GSSG (glutathione disulfide, an oxidized form of GSH). Because GSSG is not taken up intact by cells, but is rather 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 to increased nestlings needs.

The depletion of tGSH shown in our results is in accordance with several studies based on pesticide effects (e.g. Kale *et al.* 1999, Della Morte *et al.* 1994, Ezeji *et al.* 2012, Fetoui & Gdoura 2012). Oxidative stress is a key cost which 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 as 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 have 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 behaviors 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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Table 1. Differences in ectoparasite abundances and breeding variables (mean $\pm$ SE) between three treatments and results of Kruskal-Wallis and GLM tests.

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

* Significant difference, $p < 0.05$

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

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	<i>Control</i>	<i>Heat-treated</i>	<i>Fumigated</i>	<i>Statistic</i> (<i>F</i>)	<i>Df</i> <i>residual</i>	<i>Partial</i> η^2	<i>Model</i> <i>p-value</i>	<u><i>Post-hoc p-values</i></u>		
								<i>F-C</i>	<i>F-H</i>	<i>C-H</i>
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

539 *Significant difference, $p < 0.05$; C = Control group; H = Heat-treated group; F = Fumigated group

540 | **Figure 1.** Mean $\pm$ SE [glutathione](#) tGSH levels in relation to treatment in **(a)** nestlings and **(b)** adult
541 | females.
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