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# Adverse weather conditions reduce food availability and increase glucocorticoid metabolite levels in barn swallow nestlings

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Stimuli perceived as stressful by animals increase glucocorticoid secretion over basal levels. This is an adaptive response that by altering energy metabolism and animal behavior facilitates survival during acute stress. Secretion of corticosterone increases in adult insectivorous bird species after a short period of adverse weather conditions, which may determine a decreased availability of insect prey as well as thermoregulatory stress. Hormone response to stress in altricial nestlings is not yet clear. In some species (e.g. northern mockingbird, white-crowned sparrow) early age nestlings show a reduced or null response to stress stimulation, and the hypothalamus–pituitary–adrenal (HPA) axis seems to be fully active only at an age close to independence. In other species (e.g. barn swallow, canaries) the HPA axis seems to be active even in young nestlings. However, most of the data refer to experimentally induced stress while the physiological response to natural environmental perturbations has been scarcely investigated. The main aim of our study was to evaluate corticosterone secretion response to short periods of adverse weather conditions in two different age groups of barn swallow nestlings (7–14 days old and 15–21 days old). Furthermore, stress responses were compared between medium aged nestlings, late aged nestlings and adults, to understand whether HPA axis activity changed with age. A fecal non-invasive hormone assessment method was used.

Our results showed that during adverse weather conditions, insect abundance decreased and corticosterone metabolite levels increased significantly and to a similar extent in droppings of medium and late aged barn swallow nestlings as well as in adults. Our data support previous findings, that the HPA axis is already fully functional in 7–14 days old nestlings. Further studies are required to verify the existence of a hyporesponsive period in younger barn swallow nestlings.

Keywords: development, dropping, enzyme immunoassay, HPA axis, Passeriformes

Wild animals must cope with predictable and unpredictable environmental perturbations during their lifespan (Sapolsky et al. 2000). In adult birds, sudden changes in weather conditions, food shortage or predation episodes activate the hypothalamus–pituitary–adrenal (HPA) axis that leads to the release of glucocorticoids from the adrenal gland (Romero 2000). In particular, adult aerial insect feeding birds, like barn swallows *Hirundo rustica*, may experience periods of decreased food availability due to adverse weather conditions. Ambient temperature is considered the most powerful meteorological predictor of flying insect abundance and thus of food availability for aerial insectivorous birds (Frigerio et al. 2004, Gruebler et al. 2008, Jenni-Eiermann et al. 2008). Other meteorological factors such as atmospheric pressure (Frigerio et al. 2004, Pellegrino et al.

2013), wind speed (Turner 1980, Møller 2013), precipitation (Gruebler et al. 2008) and ambient humidity (Norhisham et al. 2013) can also play an important role for insect abundance.

Adult barn swallows respond to food shortage by increased release of circulating corticosterone (Gruebler et al. 2008, Jenni-Eiermann et al. 2008), the primary glucocorticoid in birds. This increase facilitates energy mobilization, via its stimulatory effects on glycogenolysis, gluconeogenesis, lipolysis and proteolysis to facilitate survival during acute stress (Sapolsky et al. 2000). Food shortage may also induce behavioral responses, such as inhibition of sexual behavior, stimulation of escape behavior and abandonment of a breeding attempt (Wingfield et al. 1995).

The nestling stage is a critical phase of life, especially for altricial birds. In these species, nestlings depend entirely on energy provided as food by their parents during their first weeks of life (Schifferli et al. 2014), and the energy and nutrients supplied to the brood determine nestling growth rates, body condition at fledging and survival (Saino et al. 1997, Almasi et al. 2009, Fairhurst et al. 2012).

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Hormonal responses of nestlings to challenging events have only been investigated in the last two decades (Schwabl 1999, Saino et al. 2003, Suorsa et al. 2003, Wada et al. 2007, Lobato et al. 2008, Crino and Breuner 2015) and further studies are required to understand how altricial nestlings perceive and respond to external stressors (Tilgar et al. 2009). Scarce comparative data are available on species-specific characteristics concerning the onset and the developmental pattern of stress response (Pravosudov and Kitaysky 2006, Tilgar et al. 2009). Sims and Holberton (2000) hypothesized the existence of an HPA axis hyporesponsive period in northern mockingbird *Mimus polyglottos* nestlings as observed in mammals (reviewed by Sapolsky and Meaney 1986). Young nestlings of northern mockingbird and white-crowned sparrow show a reduced HPA axis response to capture and handling (Sims and Holberton 2000) or to restraint (Wada et al. 2007) compared to adults. Only when nestlings approach independence (i.e. close to fledging age) they show a robustly increased secretion of corticosterone similar to that of adults, following a stress condition. Conversely, other studies show how in canaries (Schwabl 1999) and barn swallows (Saino et al. 2003) the HPA axis is fully functional in very young nestlings in basal and induced stress conditions, albeit with considerable variation among individuals.

In most studies, stress response of nestlings was artificially induced – i.e. by restraint, handling, food deprivation, nest size manipulation – (Saino et al. 2003, Wada et al. 2007, Lobato et al. 2008, Crino and Breuner 2015). In the wild, few studies deal with the stress response to environmental events, such as inclement weather or predation (Suorsa et al. 2003, Lobato et al. 2008). Such studies would be useful, considering that the hormone response of animals to environmental stress can differ both in magnitude and duration compared to artificially induced stress (Wingfield and Romero 2011, Crino and Breuner 2015).

The assessment of steroid hormone levels through serum or plasma is a standard method used in research (Sheriff et al. 2010, Romero and Fairhurst 2016). This method provides snapshot hormone assessments (i.e. the point in time of sampling) and requires expert manipulation to collect blood samples from captured animals, especially when applied to small wild birds (Wingfield et al. 1982). When hormone data related to a longer period of time are needed, fecal hormone metabolite assessment may be a useful approach, less likely to be affected by short episodic fluctuations or hormone pulsatile secretion (Touma and Palme 2005, Sheriff et al. 2011). Hormone metabolic pathway leads to the formation of metabolites that can differ between species (Möstl et al. 2005, Touma and Palme 2005). Thus, a pilot study was performed to assess corticosterone metabolite (CM) levels of barn swallow droppings as well as to set up and verify sampling collection and extraction procedures.

The main aim of our study was to carry out a non-invasive assessment of corticosterone secretion response of barn swallow nestlings at different development stages to short periods of adverse weather conditions, which may affect the availability of insects (Jenni-Eiermann et al. 2008). The relationship between weather conditions and aerial insect abundance was analyzed beforehand and the factors that best predicted insect abundance in our study area were identified. Furthermore, stress responses were compared between medium aged

nestlings, late aged nestlings and adults, in order to understand whether HPA axis activity changed with age.

## Material and methods

### Study species and study area

The barn swallow is an extensively studied species of conservation interest in the European farmland ecosystem that is suffering population declines in western and central Europe (Turner and Christie 2019). It is a small long-distance migratory passerine species that feeds almost exclusively on flying insects, mainly Diptera and Hemiptera (Turner 2006).

Fifty-three barn swallow clutches were monitored from brooding to fledging of nestlings during the 2016 breeding season. The study was carried out at two nesting sites (A and B) located in Tuscany, Italy, in a hilly rural landscape dominated by sheep farms, fields, pasture, hedgerows and olive groves (43°14'N, 11°3'E; Radicondoli, Siena). The distance between the two sites was 915 m. Site A and site B were similar, differing slightly in altitude (400 and 490 m, respectively). Site A held a smaller colony of barn swallows than site B.

Data collection was conducted following European guidelines for care and use of experimental animals (European Union Directive 2010/63/EU) and in accordance with Italian law (DL 26/2014).

### Weather data

Weather data (mean, minimum and maximum temperature, wind speed, humidity, precipitation, atmospheric pressure) were recorded automatically every 30 min by a weather station located on a building at 430 m a.s.l., within 4 km from the breeding sites, a distance in line with similar studies (Jenni-Eiermann et al. 2008, Schifferli et al. 2014). The study area is characterized by a seasonal progressive increase of daily temperature and a decrease in precipitation, from mid-April to mid-July. For this reason, the sampling period was divided into shorter periods of 15-days and the mean values  $\pm$  1SD of the weather factors most related to insect abundance were calculated for each period. Sampling days within a given period, with average values within 1SD from the mean values of that period, were considered 'normal weather' days. Sampling days with values below the mean  $-$  1SD of the period, were defined as 'adverse weather' days. Finally, sample days with values above the mean  $+$  1SD of its period were defined as 'warm weather' days. Delta maximum temperatures were also included in the analyses of the relationship between weather conditions and aerial insect abundance, and were calculated as the difference between the maximum diurnal temperature of the insect counting day and the maximum diurnal temperature of the preceding day.

### Insect counts

Point counts of flying insects were carried out two times per week during the study period (mid-April–mid-July 2016). At the two breeding localities, five sampling stations within 100 m of the nesting site were chosen randomly using a map.

The minimum distance between sampling stations was 40 m. We used the point count method applied by Gruebler et al. (2008) and Jenni-Eiermann et al. (2008), adapted from Flaspohler (1998). At each sampling station, a whiteboard (0.7 × 0.5 m) was mounted on a tripod about 0.1 m above the vegetation. All insects passing the board were counted for a period of 3 min using binoculars (10 × 42) from a 10 m distance. Insect counts were performed in the afternoon, between 14:00 and 18:00 h, the day before dropping samples were collected. The sequence of visits at the insect counting stations was always the same and the same observer performed the counts to ensure that comparable data were obtained among different days at the same station.

## Pilot study

Barn swallows usually build nests protruding from a wall or a beam inside buildings. Roosting or brooding adults and nestlings older than six days mainly defecate out of the nest (Turner 2006). This allows the collection of droppings below the nests. A pilot study was conducted to set up the sampling protocol and the hormone extraction method.

To optimize the collection of droppings, we first recorded the timing throughout the day of dropping excretion by adults and nestlings older than six days using camera traps. The analysis of the photos showed that most droppings excreted by adults were produced at night, whereas nestlings excreted most droppings during afternoon and night (Supplementary material Appendix 1 Fig. A1). A laminated paper sheet (57 × 46 cm) was placed under active nests between 17:00 and 20:00 h. The following day, between 9:00 and 12:00 h, droppings that had fallen on the sheet under each nest were counted and collected in tubes. Considerable variation was observed in the liquid content of droppings excreted. Droppings collected from 10 nests were visually categorized as wet or dry, stored in two different tubes for each nest (Supplementary material Appendix 1 Fig. A2). Samples were immediately placed in a portable icebox and stored at  $-20^{\circ}\text{C}$  within 2 h, and analyzed separately.

The extraction procedure used and validated in mammals and avian species (Wasser et al. 1994, 2000, Sheriff et al. 2011) was slightly modified in our laboratory (Cinque et al. 2017, 2018). For hormone extraction of barn swallow droppings, water content was increased in the solvent solution to facilitate the extraction of the high portion of polar metabolites found in bird droppings (Palme 2005). Droppings were mixed manually. An aliquot of 100 mg of non-dehydrated wet and dry droppings was dissolved in ethanol 80% (v/v), homogenized, shaken for 40 min at  $85^{\circ}\text{C}$  in a mixer evaporator and centrifuged at 500 g for 10 min at  $10^{\circ}\text{C}$ . The supernatant was collected. The residual pellet was suspended again in ethanol 80% to extract hormone metabolites eventually still present in the droppings, shaken for 10 min at  $45^{\circ}\text{C}$  and centrifuged at 500 g for 10 min at  $10^{\circ}\text{C}$ ; the supernatants were pooled and evaporated under warm airflow ( $80^{\circ}\text{C}$ ). Steroids were suspended with 200  $\mu\text{l}$  of ethanol 50% (v/v) and shaken again at  $45^{\circ}\text{C}$  for 40 min. The extracts were kept at  $-20^{\circ}\text{C}$  until assessment. A commercially available corticosterone EIA kit was used to assess CM levels in droppings. CM levels of non-dehydrated wet and dry droppings were positively correlated ( $r^2=0.49$ ,  $p < 0.05$ ,

$n=10$ ), but CM levels were significantly higher in dry compared to wet droppings ( $t=-8.01$ ,  $p < 0.001$ ,  $n=10$ , paired t-test), (Supplementary material Appendix 1 Fig. A3). Since it has been demonstrated that drying fecal samples results in more consistent CM measurements (Wasser et al. 1993), aliquots of the wet and dry samples were dehydrated and compared with the non-dehydrated CM values. As a result, while CM levels in non-dehydrated and dehydrated wet and dry droppings were still positively correlated ( $r^2=0.35$ ,  $p < 0.01$ ,  $n=20$ ), CM concentrations were similar in dehydrated wet and dry droppings ( $t=-1.21$ ,  $p=0.26$ ,  $n=10$ , paired t-test) (Supplementary material Appendix 1 Fig. A4). These findings demonstrated the importance of dehydration of droppings to obtain reliable and comparable hormone assessments.

A hormone stability experiment was performed to verify if CM levels were affected by the time-lag between excretion and collection. This consisted in the collection of fresh droppings (excreted maximum 2 h earlier) under a few nests, obtaining eight pooled dropping samples. These were aliquoted and left at ambient temperature at the sampling sites for different time periods (0, 2, 4, 8, 12, 16, 24 h) before being stored at  $-20^{\circ}\text{C}$  until hormone extraction and assessment. Statistical analysis revealed similar CM levels among the aliquots ( $F_{6,42}=2.00=0.09$ ,  $n=8$ , ANOVA for repeated measures) (Fig. 1), indicating substantial stability of samples. These data showed the reliability of our dropping sampling method for hormone metabolite measurements in barn swallows.

## Dropping collection

Barn swallow droppings were collected on the same two weekdays throughout the study with the same protocol applied in the pilot study, but dry and wet droppings were pooled in the same tube and stored at  $-20^{\circ}\text{C}$  within 2 h, until analysis.

Barn swallow hatchlings and very young nestlings (up to 5–6 days old) defecate inside fecal sacs that the adults remove from the nest to avoid soiling and attracting predators or pests (Turner 2006). Therefore, droppings collected during brooding and until the nestlings had reached 5–6 days of age, were assigned to adults. When nestlings were more than six days old, most of the droppings collected were assigned to nestlings. The few droppings excreted by adults while roosting or provisioning were identified thanks to the

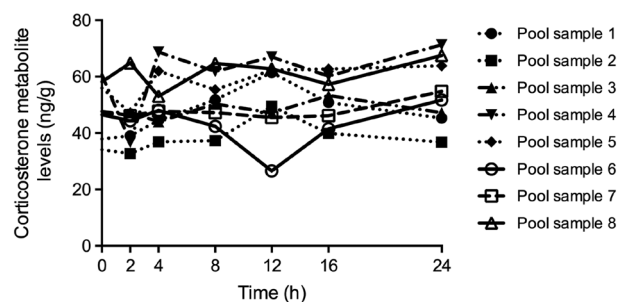


Figure 1. CM level stability of droppings left at ambient temperature from 0 to 24 h after their excretion. ANOVA repeated measures.

noticeable difference in consistency, form and dimension between these droppings and those of nestlings as well as for their position on the collection sheet (Supplementary material Appendix 1 Fig. A5). These droppings were collected separately.

In order to evaluate if hormonal response of nestlings to different weather conditions was influenced by age, nestlings were classified as 'medium aged' when 7–14 days old and 'late aged' when 15–21 days old, i.e. the usual age of fledging. Samples from nestlings were pooled on each sampling day, and the same was done with droppings from adults.

At site A, droppings excreted by adults were collected from 13 nests. Those excreted by medium aged and late aged nestlings were collected from 14 and 12 nests, respectively. At site B, droppings excreted by adults were collected from 24 nests. Those excreted by medium aged and late aged nestlings were collected from 14 nests.

### Hormone extraction

The extraction protocol was the same applied in the pilot study but the 100 mg aliquot of well mixed droppings was completely dried in a mixer evaporator, weighed again to determine its dried weight and only after the aliquot was dissolved in ethanol 80% (v/v). This step allowed the comparison between samples.

### Hormone assessment

A corticosterone EIA kit (KO14, Arbor Assays, USA) was used to access CM levels in droppings. Cross-reactivity of corticosterone antibody in this kit was as follows: 100% corticosterone, 12.30% desoxycorticosterone, 0.76% tetrahydrocorticosterone, 0.62% aldosterone, 0.38% cortisol, 0.24% progesterone, 0.12% dexamethasone, < 0.08% cortisone and estradiol. The sensitivity was 18.6 pg ml<sup>-1</sup>. This kit has already been validated for CM level assessments in droppings from other avian species (Alm et al. 2014, Costa et al. 2016, Sharma et al. 2017). To achieve a reliable hormone assessment for our species, the EIA kit utilized was analytically validated. ANCOVA and analysis of linear regression were used to test whether a pool of dropping extracts of adults and nestlings diluted from 1:10 to 1:640 was parallel to a dilution series of corticosterone standards. The lack of a significant effect in ANCOVA ( $F_{1,11}=0.16$ ,  $p=0.69$ ) is indirect evidence of the parallelism of the two curves. Analysis of linear regression gave further indication of parallelism between the curves (standard curve  $y=-36.83x+149$ ,  $r^2=0.98$ ; sample curve  $y=-38.12x+152.9$ ,  $r^2=0.98$ ). The slope of the standard curve had a standard error of 2.26, while the sample's slope standard error was equal to 2.22. Thus, the observed difference between the coefficients was well inside the confidence interval.

The intra-assay coefficient of variation of high and low concentrated quality controls ranged between 2.8% and 4.4% for adults while it ranged between 1.5% and 4.9% for nestlings (five adult and five nestling samples, each assessed in 16 wells). The inter-assay coefficient of variation for breeding adults ranged between 3.1% and 7.5% and between 4.9% and 9.9% for nestlings (five adult and five nestling samples in duplicate in eight plates).

Hormone extraction efficiency was measured in adult and nestling droppings by assessing two aliquots for each sample ( $n=5$ ). A known amount of corticosterone (5 ng) was spiked in one aliquot to calculate the percentage of hormone recovery. A high level of hormone recovery was found in samples for both adults and nestlings (mean  $\pm$  SD, adults:  $101.4 \pm 4.7\%$ ; nestlings:  $104.2 \pm 7.0\%$ ). The findings of analytic validation showed the suitability of the chosen corticosterone EIA kit for the assessment of CM levels in barn swallow droppings.

Samples were assayed in duplicate and reanalyzed if the coefficient of variation exceeded 10%. Assay data were analyzed employing a four-parameter logistic fit using Gen5 Data Analysis Software; CM concentrations were expressed as ng g<sup>-1</sup> of dropping mass.

### Weight of droppings

Adverse weather conditions might affect the amount of dropping excretion due to reduced food intake or to its metabolization and lead to an incorrect estimate of hormone metabolite concentrations (Goymann et al. 2006, Goymann 2012). To obtain reliable CM level assessments all droppings collected during normal and adverse weather conditions were dehydrated and their weights were compared within each age group.

### Statistical analysis

The influence of weather predictor variables on insect abundance in our study area was analyzed with a generalized linear mixed model (GLMM) using R ver. 3.5.2 (<[www.r-project.org](http://www.r-project.org)>), lme4 package (Bates et al. 2015). Mean diurnal temperature, maximum diurnal temperature and minimum diurnal temperature, as expected, were highly correlated. Only maximum diurnal temperature was included in the GLMM, given that this is considered a more representative measure of diurnal temperatures than the minimum or mean temperature (Lobato et al. 2008). The insect counts were used as response variable. The distribution was assumed to follow a Poisson distribution. Delta maximum temperature, maximum diurnal temperature, humidity, wind speed, precipitation, atmospheric pressure and day length were the independent variables. Days and sites of sampling were the random factors.

Insect abundance in different weather conditions at the two sampling sites was analyzed using two-way ANOVA (weather condition  $\times$  site).

The effects of different weather conditions on CM levels and weight of droppings of adults, medium aged and late aged nestlings were analyzed by means of GLMM utilizing R ver. 3.5.2, lme4 package and lsmeans package (Lenth et al. 2015). Samples from each nest were included in the analysis only if at least one hormone assessment for each of the two weather conditions was available for the same 15-day sampling period. Coefficients were estimated using the maximum likelihood method (Laplace approximation). Nest and sampling site were included in the model as random factors; weather conditions, age of the animals and their interaction as fixed factors. A gamma distribution was assumed for CM levels while dropping weights values were log-transformed to

fit a gaussian distribution. The weights of nestling droppings were normalized, based on the number of nestlings found in the nest at the time of collection.

The intra-nest mean variance of CM levels was found applying a descriptive statistical approach using StatView ver. 5.01 only for nests with two or more samples for each weather condition, separately for adults and nestlings.

The influence of weather conditions, insect abundance and log transformed weight of droppings on CM levels was analyzed with a GLMM utilizing R ver. 3.5.2, lme4 package (Bates et al. 2015). The distribution was assumed to follow a gamma distribution. Day, sampling site and age were the random factors.

## Results

### Relationship between weather factors and insect abundance

Delta maximum temperature and atmospheric pressure were positively correlated with insect abundance (Table 1). No significant correlation was found between the other variables and insect abundance, suggesting that these variables did not induce noticeable effects (Table 1) (Supplementary material Appendix 1 Table A1). The variance of the two random factors was low, showing no significant effect (mean  $\pm$  SD, days:  $0.05 \pm 0.23$ , sites of sampling:  $0.01 \pm 0.12$ ). Considering these results, the mean values  $\pm$  1SD of the delta maximum temperature and atmospheric pressure for each 15-days period were calculated. It was found that all sampling days had values that placed them in either the 'normal' or 'adverse' weather groups.

### Effects of weather conditions on insect abundance

The number of insects recorded was significantly lower in adverse weather days ( $n=10$ ) compared to normal weather days ( $n=11$ ), ( $F_{1,41}=49.21$ ,  $p < 0.001$ , two-way ANOVA) regardless of sampling sites ( $F_{1,41}=3.57$ ,  $p=0.07$ , two-way ANOVA) (Fig. 2) (Supplementary material Appendix 1 Fig. A6).

### Corticosterone metabolite levels in droppings of adult and nestling barn swallows during normal and adverse weather conditions

A significant increase in CM levels was found in droppings collected after adverse weather days compared to lev-

Table 1. Effects of the predictor weather variables on insect abundance in the GLMM analysis ( $n=210$ ).

Variable	Estimate	SE	z-value	p-value
Delta maximum temperature	0.235	0.066	3.547	< 0.001
Atmospheric pressure	0.226	0.078	2.902	0.004
Day length	0.207	0.115	1.795	0.073
Wind speed	-0.079	0.078	-1.010	0.313
Rain	0.065	0.072	0.898	0.369
Humidity	-0.141	0.174	-0.810	0.418
Maximum temperature	-0.010	0.190	-0.529	0.597

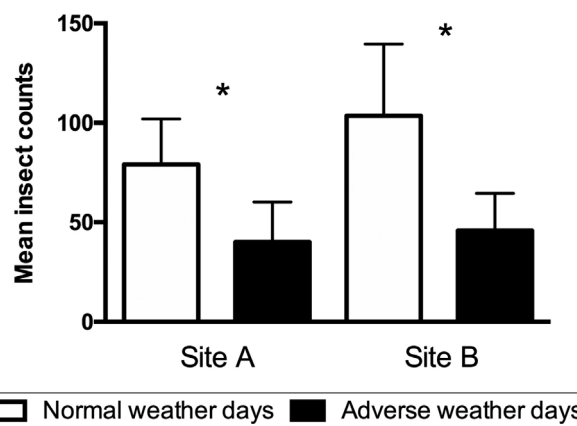


Figure 2. Insect counts in normal and adverse weather conditions in the two sampling sites. Mean insect counts ( $\pm$  SD) in normal and adverse weather conditions, \* $p < 0.05$  versus normal weather days, two-way ANOVA.

els found in droppings collected under the same nests after normal weather days ( $t=-3.25$ ,  $p=0.001$ , GLMM) (Fig. 3). No difference in CM levels was found between adults, medium aged and late aged nestlings either in adverse or normal weather conditions (Table 2).

### Intra-nest mean variance of CM levels in droppings of adult and nestling barn swallows during normal and adverse weather conditions

The intra-nest mean variance of CM levels was higher in droppings collected during adverse weather days compared to the mean variance of CM levels observed in samples collected during normal weather days, both in adult and nestling barn swallows (Table 3).

### Weight of adult and nestling droppings

The mean weight of dehydrated droppings excreted by adults, medium aged and late aged nestlings did not vary significantly between different weather conditions ( $t=0.80$ ,

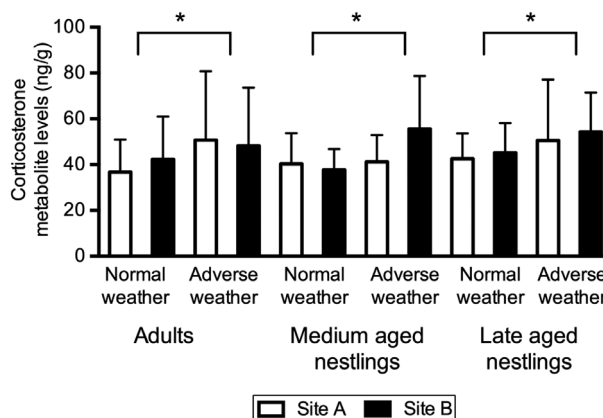


Figure 3. CM levels of dehydrated droppings in normal and adverse weather conditions in the two sampling sites. Mean CM concentrations ( $\pm$  SD) in adult, medium aged nestlings (7–14 days old) and late aged nestlings (15–21 days old), \* $p < 0.05$  versus normal weather days, Tukey's pair-wise multiple comparison test.

Table 2. Multi comparison tests of CM levels in droppings of adult and nestling barn swallows in normal and adverse weather conditions.

Tukey's pair-wise multiple comparison test			
Age	Normal weather		
	Adults	Medium aged nestlings	Late aged nestlings
Adults (n=66)	–	–	–
Medium aged nestlings (n=31)	z = 0.56, p = 0.58		–
Late aged nestlings (n=26)	z = -0.97, p = 0.33		z = 1.35, p = 0.18
Adverse weather			
Adults (n=64)	–	–	–
Medium aged nestlings (n=33)	z = 0.82, p = 0.41		–
Late aged nestlings (n=33)	z = -0.82, p = 0.41		z = 1.47, p = 0.14

p = 0.42, GLMM; adults, medium aged and late aged nestlings: z ratio = -0.80, p = 0.97 normal versus adverse weather, Tukey's pair-wise multiple comparison test) (Table 4).

### Relationship between weather conditions, insect abundance, weight of droppings and corticosterone metabolite levels

Weather conditions were negatively correlated with CM levels, i.e. CM levels were lower during normal weather days ( $t = -3.98$ ,  $p < 0.001$ , GLMM). No significant correlation was found between CM levels and insect abundance or weight of droppings ( $t = -0.07$ ,  $p = 0.94$ ;  $t = 1.68$ ,  $p = 0.09$ , respectively, GLMM).

## Discussion

Results showed that during adverse weather conditions insect abundance significantly decreased, whereas CM levels significantly increased, in medium and late aged nestlings as well as in adult barn swallows. We did not find a significant relationship between insect abundance and CM levels. This might be explained by the typically less pronounced and shorter-lasting weather fluctuations during spring–summer seasons in central Italy, in comparison to more northerly regions across Europe, where stronger declines in ambient temperature lead to a significant correlation between these three factors (Jenni-Eiermann et al. 2008). In our study area, the short periods of adverse weather could determine a less pronounced decrease in insect abundance. It is possible to hypothesize that part of flying insects had moved near leeward areas close to trees and bushes. The reduced availability and the patchy distribution of insects required

Table 3. Intra-nest variance of CM levels (mean  $\pm$  SD) in droppings of adult and nestling barn swallows during adverse and normal weather conditions.

Age	Intra-nest mean variance CM levels $\pm$ SD	
	Normal weather	Adverse weather
Adults (n=18)	284.86 $\pm$ 471.97	677.18 $\pm$ 1255.55
Nestlings (n=15)	108.85 $\pm$ 168.81	266.85 $\pm$ 427.28

Table 4. Weight (mean  $\pm$  SD) of the dehydrated adult, medium and late aged nestling barn swallow droppings.

Age	Mean weight $\pm$ SD (mg)	
	Normal weather	Adverse weather
Adults	135.58 $\pm$ 101.89 (n=66)	134.22 $\pm$ 148.59 (n=64)
Medium aged nestlings	262.84 $\pm$ 228.78 (n=31)	394.04 $\pm$ 416.62 (n=33)
Late aged nestlings	436.50 $\pm$ 372.19 (n=26)	315.52 $\pm$ 236.11 (n=33)

more energy and time (and perhaps, experience) for barn swallows to catch them (Turner 1980, Møller 2013). This condition induced a stress response through the activation of the HPA axis in adult barn swallows that experienced difficulty to collect enough food for themselves and their nestlings (Jenni-Eiermann et al. 2008). Thus, in our study area even if nestlings were still fed by parents during adverse weather conditions, most probably they received food with some delay, and this, together with possible solicitations for thermoregulation, might have led to the observed stress response in nestlings.

Further studies in geographical regions with more severe weather fluctuations during barn swallow breeding season, could be useful to verify our hypothesis and to increase knowledge of the physiology of barn swallow nestlings. The similarity found for all age groups between the weight of droppings excreted in both weather conditions, can partially corroborate the hypothesis that, in less extreme weather conditions, adult barn swallows may compensate short-term reduced food availability by drawing energy from body stores, through lipid catabolism, losing body mass (Jones 1987, Jenni-Eiermann et al. 2008). In this way parents may divert most of the collected insects to their offspring ensuring them nourishment to a certain extent (Jones 1987). In our data, the absence of correlation between CM levels and weight of droppings following adverse weather conditions rule out the possibility of an incorrect estimate of hormone metabolite concentrations, due to a different metabolization time of the hormones in the intestinal tract during periods of food shortage (Goymann et al. 2006, Goymann 2012).

In our study, the observed similar significant increase in CM levels in medium aged, late aged nestlings and adults, following adverse weather, indicates that the HPA axis is developed and fully functional already in the second week of the barn swallow's life. The existence of a hyporesponsive period in the first week of barn swallow nestlings' life could not be verified in the present study, as the protocol we used did not allow us to evaluate HPA axis activity during that period. This information could be relevant, considering that some bird species do not exhibit an HPA axis hyporesponsive period, as observed by Adams et al. (2008) in grey-faced petrel chicks.

The increased secretion of corticosterone following adverse weather conditions is an adaptive mechanism that may promote survival until conditions improve (Holmes and Philips 1976, Kitaysky et al. 1999). The higher CM levels observed in adults and nestlings during adverse weather conditions could be due, at least in part, to the increased metabolic demands for thermoregulation (Paladino 1989, Winkler et al. 2013). Moreover, the increased corticoste-

rone secretion following adverse weather is likely to have a functional meaning in barn swallow nestlings, which have the lowest fat reserves among Hirundine nestlings (Turner and Bryant 1979). The increased corticosterone levels might induce an increased begging activity, probably the only form of foraging behavior for nest-bound chicks to solicit parental food provisioning (Kitaysky et al. 2001). This suggests that the stress response in nest-bound nestling might be highly adaptive.

Within a clutch, nestlings might be in different health conditions and show different stress responses to similar stimuli even though they are kin. Besides, during food shortage, nestlings may compete for parental resources (Kitaysky et al. 2001), although competition can be reduced in barn swallow nestlings, thanks to observed cooperation between fed and unfed individuals (Romano et al. 2012). In our study, droppings from nestlings of the same nest were pooled on each sampling day. Consequently, each nest provides information on the mean response of nestlings to environmental modifications that occurred during the sampling period and it was not possible to investigate the individual stress response. However, the intra-nest mean variance within CM levels of droppings collected during adverse weather days was higher in comparison to the mean variance of CM levels of samples collected during normal weather days. This observation may suggest differences in how clutch and individual nestlings respond to adverse weather conditions.

In conclusion, our results showed that during short-term adverse weather conditions, insect abundance decreased and corticosterone metabolites levels increased significantly and to a similar extent in droppings of medium and late aged barn swallow nestlings as well as in adults. This secretion may help adults to mobilize energy, increasing the chance of nestling survival. The increased secretion of CM levels in nestlings may solicit parental food provisioning and also be important for the increased thermoregulatory demands. Our data also showed that nestlings' HPA axis was fully functional in the second week of life, strengthening the findings of Saino et al. (2003), through procedures that did not involve nestling manipulations. Further studies are required to verify the existence of a hyporesponsive period in younger barn swallow nestlings.

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Supplementary material (available online as Appendix wlb-00747 at <[www.wildlifebiology.org/appendix/wlb-00747](http://www.wildlifebiology.org/appendix/wlb-00747)>). Appendix 1.