

## Article

### To change or not to change experimenters: caveats for repeated behavioural and physiological measures in Montagu's harrier

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Animals facing anthropogenic disturbances may exhibit different anti-predator responses depending on their perception of the risk. Experimental measures of behavioural and physiological traits may be impacted by the disturbance due to experimenter handlings and activities. In this study, we assessed the behavioural and physiological responses of Montagu's harrier chicks *Circus pygargus* from 14 nests, visited four times from hatching to fledgling by either the same (group I) or different (group NI) experimenters. Escape, aggressiveness and stress-induced corticosterone concentration increased for both groups over time but chicks from group I showed a higher increase in their behavioural and physiological responses than chicks from group NI. This increase occurred after the second visit when experimenters took the first blood sampling on nestlings. Handling by the same experimenters throughout the study did not result in habituation but in sensitization. Our results suggest that Montagu's harrier nestlings may discriminate between the people who visit their nest and respond to experimenters' identity. Therefore, we urge researchers working on behaviour and/or the physiology of stress including repeated measurements (such as animal personality studies for instance) to consider whether the experiments should or should not be conducted by the same experimenters (as usually done to minimize bias) as it can influence the outcomes of the experiments.

Keywords: aggressiveness, corticosterone, habituation, heterospecific recognition, sensitization

## Introduction

Animals often consider human a potential predator (Frid and Dill 2002, Blumstein 2006, Samia et al. 2015). Human presence in animal environment and disturbances caused by anthropogenic activities may result in behavioural changes in animals such as increased escape behaviour and/or physiological stress levels through the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Romero and Romero 2002, Gill 2007). To date, interactions between wild animals and humans were mostly studied in urban environments (Fernández-Juricic et al. 2001, Atwell et al. 2012, Bókony et al.



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2012, Sol et al. 2013, but see Ditmer et al. 2018) or in the context of recreational activities (Rodgers and Smith 1995, Steidl and Anthony 1996, Müllner et al. 2004, Steven et al. 2011). For example, during hunting days, little bustards *Tetrax tetrax* increase their vigilance and flight behaviours and also exhibit higher concentrations of corticosterone (stress hormone, Casas et al. 2009, Tarjuelo et al. 2015). However, if animals are repeatedly exposed to the same human-stimulus then the intensity of their behavioural/physiological response may either increase or decrease (Chace and Walsh 2006, Baudains and Lloyd 2007, Viblanc et al. 2012, Shutt et al. 2014, Lee et al. 2017).

Habituation consists in a decrease in the intensity of the behavioural and physiological response of animals repeatedly exposed to the same stimulus, presumably as they consider such stimuli harmless to them (Nisbet 2000, Cyr and Romero 2009, Blumstein 2016). Conversely, sensitization is characterized by increasing behavioural and physiological responses to a repeated stimulus (Blumstein 2016). Both processes were studied to understand the disturbance effects of tourists on wildlife in highly frequented areas. For example, Galápagos marine iguanas *Amblyrhynchus cristatus* show lower stress-induced corticosterone response in tourist areas than individuals in undisturbed areas, suggesting a habituation to human presence (Romero and Wikelski 2002). Animals repeatedly exposed to humans, may thus either habituate or sensitize to them depending on the context resulting in different behavioural and physiological responses (Rousing et al. 2005, Levey et al. 2009).

Research and conservation activities often imply repeated interactions (i.e. handling and/or exposition) of the same animal. Some effects of investigator disturbances over repeated interactions have been documented on breeding success and chick growth rate (Sandvik and Barrett 2001, Bolduc and Guillemette 2003, Blackmer et al. 2004, O'Dwyer et al. 2006, Carey 2011) and on behavioural and physiological responses (van Oers and Carere 2007, Shutt et al. 2014). In a long-term study, great tits *Parus major* that experienced more repeated handling and bleeding were more docile and easier to catch thirty days after the experiment, but had a higher breath rate than individuals that were less disturbed (van Oers and Carere 2007). However, the potential habituation/sensitization phenomena to experimenters throughout repeated measures have rarely been explored on wildlife (but see Levey et al. 2009, Marzluff et al. 2010, Lee et al. 2011, 2016, Davidson et al. 2015). In research and conservation activities, the commonest procedure is to rely on the same experimenter to measure all individuals from the study population and to take repeated measures on a same individual. This recommendation is supposed to limit potential bias of measures and handling between different experimenters. However, in this case, habituation or sensitization phenomena could occur over repeated handlings and thus impact the behavioural and physiological responses of individuals. This is especially worrying for certain kind of research topics that imply repeated measures, e.g. studies of ageing

(Love et al. 2003, McCleery et al. 2008), animal personality (which rely on several behavioural measurements on the same individuals, Carere and Maestripieri 2013 for a review) or capture–mark–recapture (Miller et al. 2005, Petit and Valiere 2006, Ryder et al. 2011).

In the present study, we investigated the impact of repeated interactions with the same versus different experimenters on the behaviour and physiology of naïve individuals in field conditions. We took advantage of our Montagu's harrier nest monitoring research program to assess the effect of repeated visits and handling on the behaviour, stress of the chicks during the nestling phase and body condition at fledgling which is an important parameter that determines the post-fledging survival in birds (Wiens et al. 2006, Schwagmeyer and Mock 2008, Naef-Daenzer and Gruebler 2016). Two different modalities of nest visits were done: nests were visited by either the very same experimenters during the whole survey or by different experimenters from one visit to the next. We hypothesized that although the physiological and behavioural responses of the nestlings may globally increase during the monitoring due to ontogenetic changes, these responses should increase less for those repeatedly manipulated by the same experimenters than for those visited by different experimenters under a habituation process. We expected that chicks visited by different experimenters throughout the study should have higher behavioural and physiological stress responses than those visited by same experimenters. As corticosterone secretion has an impact on fledgling condition (Spencer and Verhulst 2007, Tilgar et al. 2017), chicks visited by different experimenters should exhibit a lower body condition.

## Material and methods

### Study site and model species

This study took place within the LTSER Zone Atelier Plaine & Val de Sèvre (western France, 46°110N, 0°280W), covering ca 435 km<sup>2</sup> of farmland where the population of Montagu's harrier *Circus pygargus* has been monitored since 1994 (Bretagnolle et al. 2018). The Montagu's harrier is a farmland flagship and protected species that nests on the ground mainly in cereal crops (Arroyo et al. 2002), making chicks and adults vulnerable to agricultural work (harvests) but isolated from human presence (Arroyo et al. 2017). The Montagu's harrier population is decreasing in France probably due to the intensification of agricultural practices in the last few decades, thus making them of high conservational priority but also limiting our sample size (Butet and Leroux 2001, Comolet-Tirman et al. 2015, Le Rest et al. 2015). Adult females lay up to six eggs according to the year and available resources which consist mainly of common voles *Microtus arvalis* (Millon et al. 2008). The most common pattern in this species is an average of two-days interval between laying consecutive eggs and one-day interval on average at

hatching (Arroyo et al. 2004). The incubation period lasts 29 d and 30–35 d for the rearing period (García and Arroyo 2001, Arroyo et al. 2007).

## Experimental design

### Overall design

Montagu's harrier nests found in 2017 were visited twice during incubation and four times during the chick rearing period (from hatching to fledgling) every week (lag between visits:  $7 \pm 2$  d,  $n = 96$  visits) (Fig. 1a). To plan nest visits during the rearing period, the hatching date was estimated based on egg biometrics taken during the incubation period visits following Arroyo et al. (2017). Before the first nest visit, nestlings had never been manipulated by humans. As nests were discovered progressively during the breeding season, they were alternately assigned to each experimental group (or randomly assigned to each group if discovered on the same day), so that time of breeding did not differ between the two experimental groups. In the first group called thereafter group I (for identical), the experimenters were always the same three people with the very same role at each visit and for each nest (Supplementary material Appendix 1 Table A1): experimenter A captured the chicks at the nest and took notes during the chick handling by experimenters B and C who performed the measures simultaneously on two chicks from the same nest (i.e. one chick per experimenter). Chicks were randomly handled (i.e. manipulated and measured) by the experimenter B or C from one visit to the next to avoid handling and measurement bias. In the second experimental group, called thereafter group NI (for non-identical), a combination of three experimenters was selected for each visit among nine qualified manipulators including the three of the experimental group I (A, B and C) to prevent potential measurement bias for behavioural scores (Supplementary material Appendix 1 Table A1). Each experimenter combination

for the group NI depended on people availability and was changed from a visit to the next. The roles of experimenters in the group NI were randomly assigned at each visit (i.e. one experimenter captured the chicks at the nest and took notes and two experimenters performed the measures simultaneously on two chicks, Supplementary material Appendix 1 Table A1). In both experimental groups, the experimenters were qualified and trained to manipulate this species and to take blood sampling. They were all wearing grey fishing pocket vests over sober-colours t-shirt thus making grey visible part of their body to chicks homogeneous. Thus, the identity effect that we tested relies on visual/olfactory/auditory cues due to the people doing the experiments as well as the way they manipulated the birds.

During the monitoring program, all nests found in the study area were monitored, which represents 19 Montagu's harrier nests. We excluded five of them which failed to breed due to chick predation before fledging. In the 14 remaining nests, twelve chicks were excluded: seven chicks died before fledging (because of starvation and/or predation, group I = 5 dead chicks and group NI = 2 dead chicks; Fisher test:  $p = 0.40$ ); five chicks (group I = 2 chicks and group NI = 3 chicks) hatched with a delay of at least four days thus getting one more exposure to human (they were too young at the nest visit 2 for blood sampling, thus the experimental design of visit was delayed for them). Consequently, in nests with more than two chicks, only the two first chicks were considered in this study. Group I thus consisted of seven nests and 11 chicks (mean  $\pm$  standard deviation, SD:  $1.57 \pm 0.53$  chicks per nest) and group NI consisted of seven nests and 13 chicks ( $1.86 \pm 0.38$  chicks per nest). Nestlings were individually identified at the first visit with water paint colours on the back of their head. They were banded at 15 d-old during visit 2 with a numbered aluminium ring from the Muséum National d'Histoire Naturelle (Fig. 1a). Chicks were sexed by iris

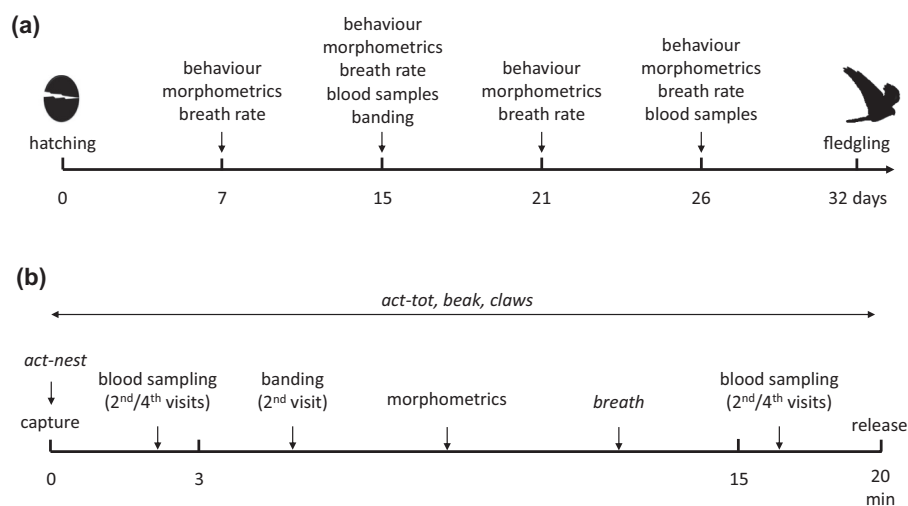


Figure 1. Summary of experimental design with: (a) the schedule of the four nest visits during the rearing period depending on the estimation of chick age ( $\pm 2$  d); (b) the schedule of behavioural, physiological and morphometric measures on chicks at each nest visit.

colour at 15 d-old (Arroyo 2002). The sex ratio within each experimental group was balanced (group I: seven females and four males, binomial test:  $p=0.55$  and group NI: four females and nine males,  $p=0.27$ ) with no difference between groups (Pearson's Chi-squared test:  $\chi^2=1.44$ , 1 df,  $p=0.23$ ). During the rearing period, four nest visits of ca 20 min (mean  $\pm$  SD, group I:  $20.0 \pm 10.56$  min; group NI:  $20.53 \pm 11.13$  min) were conducted with the same sequence of measures on chicks at each visit: behavioural scores, morphological measurements (wing, tarsus, tail length and body mass) that we used to characterize chick body condition, breath rate and blood sampling for corticosterone titration (Fig. 1b). Visits with blood sampling were longer (visits 2 and 4:  $27.27 \pm 9.84$  min) than visits without blood sampling (visits 1 and 3:  $13.31 \pm 6.34$  min).

All nest visits were made during sunny days (no rain) mainly either early in the morning (before 11:00 am) or after 04:00 pm to avoid thermal stress for the chicks, which could impair their development and their corticosterone concentrations (Lobato et al. 2008, Pérez et al. 2008, Rodríguez and Barba 2016).

### Behavioural measures

At each visit, one behaviour was measured when the experimenter approached to the nest, captured the chicks and placed them in cotton bags (Fig. 1b). 1) Their activity at the nest approach (thereafter 'act-nest') was measured: chicks stayed immobile = 0, or displayed a retreat movement = 1, or ran away = 2. Three behaviours were also scored during handling (Fig. 1b): 1) total activity score (thereafter 'act-tot'): chicks either stayed immobile = 0, or displayed rare movements = 1, or regular movements = 2, or continuous movements = 3; 2) rate of beak attacks per minute (thereafter 'beak'); 3) rate of attacks with claws per minute (thereafter 'claws'). To summarize, each nestling was scored for four behavioural traits: act-nest, act-tot, beak and claws.

### Morphometrics

At each visit, nestlings were measured twice for right tarsus length with a digital calliper (accuracy:  $\pm 0.1$  mm), right wing length (maximum chord) and tail length using a ruler (accuracy:  $\pm 1$  mm). At visit 4, left tarsus length was also measured. Before their release at the nest, chicks were weighed with spring scale (Pesola 500 g, accuracy:  $\pm 5$  g). Body condition was estimated by the scale mass index developed by Peig and Green (2009) as follows:

$$SMI_i = Mass_i \times \left( \frac{\text{Mean tarsus length of the group}}{\text{Tarsus length}_i} \right)^{b_{sma}}$$

where  $b_{sma}$  is the slope of the major axis regression of log (body mass) on log (mean of the two measures of the left and right tarsus length) following the standard major axis method for each chick  $i$  (using 'smatr' package, Lenth 2016).

### Physiological measures

At each nest visit, breath rate of chicks (thereafter 'breath') was assessed after morphological measurements as an indicator of a physiological stress response (Carere and van Oers 2004, van Oers and Carere 2007, Torné-Noguera et al. 2014; Fig. 1). Chicks were handled in back position and the number of thoracic movements were counted during 15 s. Three consecutive measures were made in one continuous observation for a total duration of 45 s and averaged to give the breath score (Fig. 1b). At the second visit ( $15 \pm 2$  d old chicks) and at the fourth visit ( $26 \pm 2$  d old chicks), two blood samples (150  $\mu$ l each) were taken from the puncture of the brachial vein using heparinized capillaries at the beginning and the end of handling (Fig. 1). The first blood sample was taken within the first three minutes of the visit to measure the baseline corticosterone concentration and the second one was performed after 15 min (mean  $\pm$  SD, group I:  $16.79 \pm 2.29$  min; group NI:  $22.28 \pm 6.61$  min) of handling (Fig. 1b). We calculated for each chick the stress-induced corticosterone concentration as the difference between corticosterone concentration at 15 min and baseline corticosterone concentration (Love et al. 2003, Romero and Reed 2005). Blood samples were kept refrigerated (0–5°C) for two to three hours and then centrifuged (10 min at 9000 rpm, Bio Lion XC-LED12K) to obtain the plasma, and then were stored at  $-20^\circ\text{C}$  until titration by radioimmunoassay following Lormée et al. (2003). Among the 24 chicks, some individuals were not included in the analyses as we failed to collect enough blood for titration. Thus, two chicks (one from each group) were excluded for baseline corticosterone analysis and eight individuals (two from group I and six from group NI) for stress-induced corticosterone analysis.

### Statistical analyses

Chick behavioural and physiological responses were analysed according to the group of experimenters and across the four nest visits.

First, two principal component analyses (PCA) were carried out to get two synthetic variables reflecting chick global activity and chick aggressiveness toward experimenters respectively. The PCAs were performed using a singular value decomposition of the centred and scaled (standardized) data matrix (Crawley 2012). The first PCA included act-nest and act-tot to obtain a synthetic score of global activity. The first axis of the PCA, PC1-activity was positively correlated with act-nest ( $r=0.76$ ) and act-tot ( $r=0.76$ ). We retained it as its eigenvalue was above one and accounted for 57.4% of the overall variance (Supplementary material Appendix 1 Fig. A1). The second PCA included beak and claws to obtain a synthetic score of aggressiveness toward experimenters. The first axis of this PCA, PC1-attacks, was positively correlated with beak ( $r=0.83$ ) and claws ( $r=0.83$ ), and we retained it as its eigenvalue was above one and accounted for 68.4% of the overall variance (Supplementary material Appendix 1 Fig. A1).

We used PC1-activity, PC1-attacks, baseline corticosterone, stress-induced corticosterone and breath as response variables. The normality and the homoscedasticity were checked with Shapiro–Wilk and Levene tests respectively. Then, the differences between groups (I versus NI) were analysed on PC1-activity, PC1-attacks, breath, basal and stress-induced corticosterone concentrations using linear mixed-effects models (LMMs, i.e. one per response variable, using ‘lme4’ package, Hothorn et al. 2008). These LMMs included as fixed effects the experimental group, the rank of the nest visit and its interaction with the group, and the sex of chicks to account for potential effect of sexual dimorphism (Weimerskirch et al. 2002, Lormée et al. 2003, Ellenberg et al. 2009). Pre-fledging body condition was compared between groups using a LMM including sex to control for sexual dimorphism. Since chicks from the same nests were not independent, chick identity nested within nest identity were set as random effects in all models except for body condition analysis. For the body condition model, only nest identity was included as random effect (one measure per chick). For each model, the statistical significance of each parameter was assessed through model comparison with likelihood ratio-based  $\chi^2$ -statistics (Fox and Weisberg 2011). Significant effects of the rank of nest visits in interaction with the group or in single effect were then tested, using post hoc tests based on least-squares means associated with Benjamini–Hochberg’s correction for multiple comparisons, in order to control for false discovery rate (with ‘lsmeans’ and ‘multcomp’ packages, Warton et al. 2012, Bates et al. 2015 respectively, Benjamini and Hochberg 1995).

Statistics were performed with R software (ver. 3.5.1, R Development Core Team).

## Ethics statement

We released all birds involved in the present study at their site of capture (i.e. their nest) after each handling. Bird manipulation was allowed by a permit of the CRBPO (Centre de Recherches sur la Biologie des Populations d’Oiseaux – Museum National d’Histoire Naturelle, licence #1308). The methods used for the capture, handling, banding and blood sampling comply with French guidelines for ethical use of animals in research.

## Data deposition

Data available from the Dryad Digital Repository: <<https://doi.org/10.5061/dryad.b8r04br>> (Rabdeau et al. 2019).

## Results

### Behaviour of the chicks

During the course of the monitoring, the overall activity (PC1-activity) of the chicks increased for both sexes similarly (Table 1, Supplementary material Appendix 1 Table A3,

Table 1. Summary of LMMs for testing the effects of the experimental group (I versus NI), the rank of the nest visits and the sex of the chicks on behavioural and physiological responses of Montagu’s harrier chicks using the model comparison (including statistic values and degrees of freedom). Significant effects are in bold. If the interaction group  $\times$  visit rank was significant, simple effects (group and visit rank) cannot be tested. The estimates of each effects included in each model are presented in Supplementary material Appendix 1 Table A3.

Explanatory variables	$\chi^2$	df	p
PC1-activity			
Group	0.07	1	0.79
Visit rank	58.09	3	< 0.0001
Sex	0.03	1	0.87
Group $\times$ Visit rank	<b>28.56</b>	<b>3</b>	<b>&lt; 0.0001</b>
PC1-attacks			
Group	2.28	1	0.86
Visit rank	38.68	3	< 0.0001
Sex	0.84	1	0.36
Group $\times$ Visit rank	<b>19.93</b>	<b>3</b>	<b>0.0002</b>
Baseline corticosterone			
Group	0.26	1	0.61
Visit rank	<b>3.54</b>	<b>1</b>	<b>0.06</b>
Sex	0.0007	1	0.98
Group $\times$ Visit rank	1.51	1	0.22
Stress-induced corticosterone			
Group	3.57	1	0.06
Visit rank	26.63	1	< 0.0001
Sex	0.51	1	0.47
Group $\times$ Visit rank	<b>5.25</b>	<b>1</b>	<b>0.02</b>
Respiratory frequency – breath			
Group	1.0	1	0.32
Visit rank	0.34	3	0.95
Sex	0.007	1	0.94
Group $\times$ Visit rank	5.48	3	0.14
Body condition			
Group	1.74	1	0.19
Sex	1.02	1	0.31

Fig. 2a). However, this trend differed between the two groups of chicks (Table 1, Supplementary material Appendix 1 Table A3). Initially, at visit 1, chicks behaved similarly in the two groups (post hoc test:  $p=0.82$ ); whereas at visit 2 chicks from group NI were more active than chicks from group I ( $p=0.02$ ; Fig. 2a). At visits 3 and 4, the activity of chicks from group I increased (visit 2 versus visit 3:  $p=0.02$  and visit 3 versus visit 4:  $p<0.001$ ), but no difference was observed for chicks from group NI (visit 2 versus visit 3:  $p=0.46$  and visit 3 versus visit 4:  $p=0.48$ ). No difference in activity was detected between groups at visit 3 ( $p=0.34$ ), but at visit 4 chicks from group I were more active than chicks from group NI ( $p=0.02$ ; Fig. 2a).

The aggressiveness of the chicks toward experimenters (PC1-attacks) increased during the monitoring with no difference between sexes (Table 1, Supplementary material Appendix 1 Table A3, Fig. 2b). Similarly to chick activity, this increase differed between the two groups (Table 1, Supplementary material Appendix 1 Table A3). At visits 1 and 2, chicks behaved similarly in the two groups (post hoc test,  $p=0.89$  and  $p=0.33$  respectively; Fig. 2b). Then,

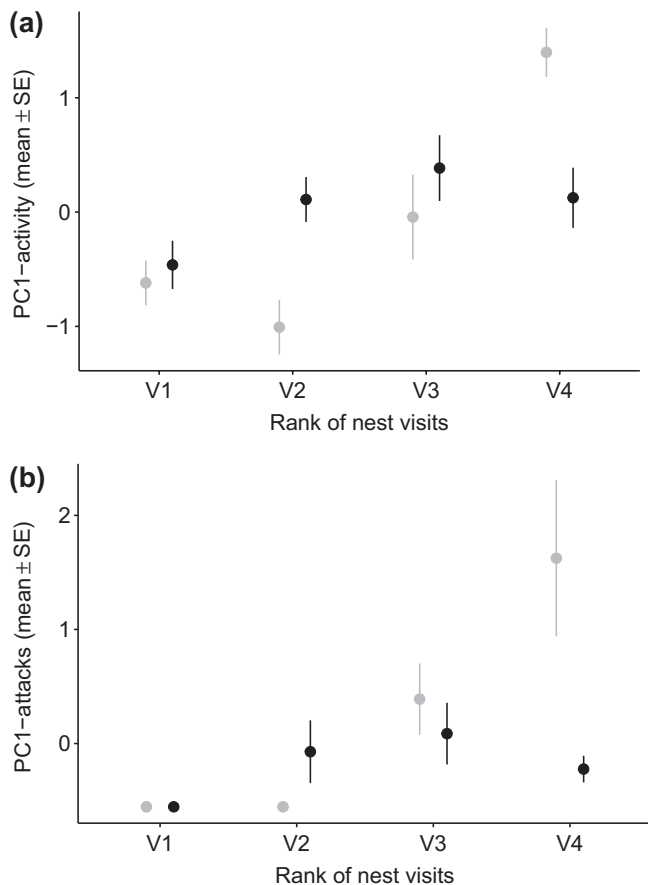


Figure 2. Effects of the group (grey=group I, seven nests and 11 chicks; black=group NI, seven nests and 13 chicks) on (a) the overall activity (PC1-activity mean  $\pm$  SE) and (b) the aggressiveness (PC1-attacks mean  $\pm$  SE) depending on the rank of nest visits.

aggressiveness of chicks from group NI did not change from visits 2 to 3 and between visits 3 and 4 (all  $p > 0.52$ ); but the number of attacks of chicks from group I tended to increase from visit 2 to 3 ( $p = 0.07$ ) and significantly increased from visit 3 to 4 ( $p = 0.01$ ; Fig. 2b). No difference in aggressiveness was detected between groups at visit 3 ( $p = 0.71$ ), but at visit 4, chicks from group I were more aggressive than chicks from group NI ( $p < 0.001$ ; Fig. 2b).

### Chick physiological stress and body condition before fledging

The respiratory frequency did not vary significantly during the monitoring neither with sex nor with experimental group (Table 1, Supplementary material Appendix 1 Table A3). The baseline corticosterone concentration tended to be higher at visit 4 than at visit 2 and was not affected by the experimental group nor the sex of chicks (Table 1, Supplementary material Appendix 1 Table A3, Fig. 3a). The stress-induced corticosterone concentration was not significantly different between the two groups at visit 2 (post hoc test,  $p = 0.38$ ) and at visit 4

( $p = 0.38$ ) (Table 1, Supplementary material Appendix 1 Table A3, Fig. 3b). However, the interaction between visit and group significantly affected the stress-induced corticosterone concentration (Table 1, Supplementary material Appendix 1 Table A3, Fig. 3b). While stress-induced concentration did not differ between the two visits for chicks in group NI (visit 2 versus visit 4:  $p = 0.23$ ), it was significantly higher at visit 4 than at visit 2 for individuals in group I (visit 2 versus visit 4:  $p < 0.001$ ). At visit 4, chicks from the two groups did not differ significantly in body condition (mean SMI  $\pm$  SD, group I:  $305.8 \pm 26.0$  g and group NI:  $300.4 \pm 22.8$  g) nor with sex (Table 1, Supplementary material Appendix 1 Table A3).

## Discussion

In this study, Montagu's harrier nests were regularly visited either by the same experimenters (group I) or by different experimenters (group NI), the only parameter that differed in our experimental design. The behaviour of the chicks was scored, and their physiological stress was estimated thus allowing to assess the difference in behavioural and physiological responses of naïve individuals between the two experimental groups. Chicks from the two experimental groups showed an increase in different behavioural and physiological responses but their dynamics were different between the two groups over repeated visits. Although the respiratory frequency is often used to measure the stress (Carere and van Oers 2004, van Oers and Carere 2007, Torné-Noguera et al. 2014), no effect was observed in the present study. Finally, the body condition of the chicks before fledging did not differ between the two groups.

### Ontogenetic changes in behaviour and physiology

Our results showed a global increase with the rank of visit in the activity and attack behaviours and stress-induced corticosterone concentrations of chicks for the two experimental groups. During the course of the monitoring, Montagu's harrier chicks, as an altricial species, developed their physical and cognitive abilities during their ontogeny (Arendt 1997, Arroyo 2002). Chicks became more active, aggressive and defensive toward the experimenters (Thomas 1977). Similarly, the development of the HPA axis led to an increase in the basal and stress-induced concentrations of corticosterone (review by Wada 2008), which is quite clear in the present case.

### Different dynamics in chick behaviour and physiology between the two groups

Chicks from the two experimental groups showed different dynamics in their behavioural responses. In the first part of the monitoring (visit 1–2), chicks manipulated by the same experimenters exhibited lower reactivity than those

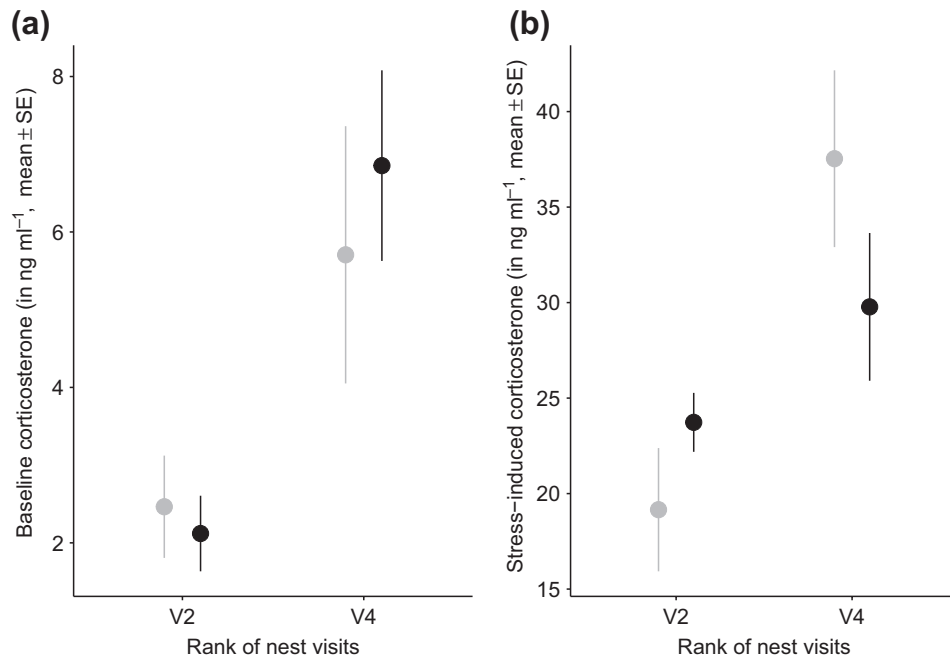


Figure 3. Effects of the group (grey = group I; black = group NI) and the rank of nest visits on (a) the baseline concentration of corticosterone (group I: seven nests and 10 chicks; group NI: seven nests and 12 chicks) and (b) the stress-induced concentration of corticosterone (group I: six nests and nine chicks; group NI: four nests and seven chicks) (mean  $\pm$  SE) in Montagu's harrier chicks.

manipulated by different people indicating lower stress. Once the second visit was done, however, the behavioural and physiological changes were more pronounced for the chicks manipulated by the same experimenters. At the visit 4, chicks from the group with the same experimenters were more active and aggressive, i.e. the reversed situation compared to second visit. This pattern is the opposite of the prediction linked with habituation. Indeed, chicks visited by identical experimenters were more stressed and aggressive at the end of the experiment suggesting a sensitization phenomenon (Blumstein 2016). Behavioural and physiological responses of chicks could be influenced by the behavioural types of adults and their calls during nest visits or environmental conditions such as anthropic disturbances (Platzen and Magrath 2004, Almasi et al. 2015, Arroyo et al. 2017). However, nests were randomly assigned to the two groups, thus these factors could not explain the differences we observed. The differences in chick behavioural and physiological responses between the two groups could result from differences in the way the experimenters handled chicks between the two groups. However, in the group with the same experimenters, chicks were randomly handled by two experimenters who were also included in the other experimental group. Although this explanation is less conceivable, this hypothesis should be properly tested to exclude such an effect. Finally, another explanation implies two major associated facts: 1) Montagu's harrier chicks identified an event during visit 2 that can be qualified as harmful for them and 2) chick could be sensitized after this visit 2.

### Blood sampling as a negative event associated with the experimenters

The differences in the behaviour and the physiology of the chicks between the two experimental groups were observed after the second visit, which differed from the first one by the realization of two blood sampling events. Blood sampling seems to be aversive for chicks (Domjan 2005), although great care was taken to minimize stress and pain during handling and sampling (only done by trained experimenters, habituated to manipulate and bleed wild birds). This phenomenon has also been evidenced in yellow-eyed penguins *Megadyptes antipodes*: individuals previously blood sampled are less likely to habituate at next interaction (Ellenberg et al. 2009). The total volume of blood collected for the first and second samples was minimized and made profitable for other further analyses (immunology, genetics, pesticide titration). Based on French guidelines, the blood samples collected at visit 2 and 4 were far below the maximal volume that is possible to collect without an impairment of the chick development (10% of the total volume of blood, which represents 6% of the body mass of nestlings of more than 150 g, i.e. 900  $\mu$ l). Moreover, recent experiments and review suggest that blood sampling (from the brachial vein like in the present case) does not alter the development of nestlings (Sheldon et al. 2008).

According to our result, blood sampling by itself is not the problem but the association with traits of the experimenters. Indeed, blood sampling alone cannot explain the pattern we observed, otherwise the large increase of the behavioural and physiological responses should be the same

in both experimental groups. Instead, nestlings would associate the blood sampling event to traits of the three experimenters through a fear conditioning mechanism and a sensitization process would occur across nest visits (see Davis 2002, Domjan 2005, Anderson et al. 2010, Marzluff et al. 2010, Blumstein 2016 for a review). This result suggests that Montagu's harrier nestlings could recognize and discriminate humans over nest visits; or at least were able to make an association between traits of our experimental group I with the manipulation that did not occur for group NI. In other bird species, individuals showed more defensive behaviours toward the humans who visited their nest or captured them, than toward neutral humans who had no previous interaction with them (Levey et al. 2009, Marzluff et al. 2010, Lee et al. 2011, 2016, Davidson et al. 2015). For example, in the American crow *Corvus brachyrhynchos*, individuals trapped by an experimenter wearing the 'dangerous mask', scold all people wearing this mask after trapping (Marzluff et al. 2010). Several species of invertebrates and vertebrates are able to discriminate among humans (Boysen 1994, Kendrick et al. 2001, Davis 2002, Davis and Heslop 2004, Adachi et al. 2007, Dittrich et al. 2010). Other studies highlighted that Montagu's harrier is able to discriminate among different species (Arroyo et al. 2001, García 2003). Heterospecific discrimination would allow adjusting the defence investment and its costs for individuals (Arroyo et al. 2001, Marzluff et al. 2010). Our results suggest that this heterospecific discrimination toward humans could be more precise; they may recognize human individual, but the exact nature of the cues they used remain unknown in the present case. Moreover, the present study was conducted on chicks, i.e. naïve individuals not exposed to humans before our nest visits compared to other field studies on adult individuals and pre-exposed to humans (Slobodchikoff et al. 1991, Levey et al. 2009, Marzluff et al. 2010, Lee et al. 2011, 2016, Davidson et al. 2015). Montagu's harriers could have cognitive and perceptual abilities to discriminate human individuals, similarly to other species known for their high cognitive ability such as pigeons, parrots and corvids. This hypothesis should, however, be properly tested and the cues (visual/olfactory/auditory/the way experimenters handled birds) used to discriminate humans should be investigated with different masks to test the use of visage cues (Lefebvre et al. 2004, Emery 2006, Iwaniuk et al. 2009, Levey et al. 2009, Marzluff et al. 2010, Belguermi et al. 2011, Lee et al. 2011, 2016, Stephan et al. 2012).

### Methodological caveats

One important outcome of the present study is to show the differential effect of the experimenters' identity. Indeed, most of the time, the impact of investigators is seen from the disturbance effect point of view either on breeding success (Bolduc and Guillemette 2003, Blackmer et al. 2004, Ibáñez-Álamo et al. 2012), growth of chicks (Sandvik and Barrett 2001, O'Dwyer et al. 2006, Carey 2009, 2011), or on behaviour and physiology (van Oers and Carere 2007)

but rarely, to our knowledge, considering the identity of the people in charge of the experiment on wildlife fauna (see, however, Slobodchikoff et al. 1991, Levey et al. 2009, Marzluff et al. 2010, Lee et al. 2011, 2016, Davidson et al. 2015). Basically, to limit bias due to experimenter effect, measures are done by the same people. Our study shows that depending on whether the model species is able to habituate or sensitize to humans, the outcomes of the behavioural and physiological measures might be either exacerbated or limited (Cibulski et al. 2014). In our study, chicks visited and handled by the same three experimenters were more aggressive and stressed than those manipulated by different people. Thankfully, the overall pattern of behavioural and physiological responses is similar in both groups suggesting that the global methodology is robust despite the limitation of the sample size. The three experimenters in the group I operated also in the group NI with six other experimenters in different combinations at each visit, which allow avoiding potential bias for behavioural scoring.

Regarding the assessment of the behavioural response to human-mediated disturbance, keeping or changing experimenters have contrasted advantages. Mostly, conserving the same experimenters limits the bias from different manipulators, but does not reflect a true exposition to humans in real life, i.e. during their life wild animals are expected to be confronted to many different humans. Thus, depending on the question (research or conservation), taking habituation/sensitization processes into account when repeated measurements are required, is unavoidable. It is particularly important to consider the sensitization process during research and conservation activities since it is linked to an increase in corticosterone due to stress during post-natal phase. Although the mechanisms are complex, an increased corticosterone secretion during nestling phase may compromise chick developmental plasticity in stopping (Spencer and Verhulst 2007) or enhancing growth rate (Tilgar et al. 2017), may modulate their future behaviour (Müller et al. 2009, Boogert et al. 2014), may promote a higher susceptibility to oxidative stress (Noguera et al. 2017) and/or may impact immunity (Chin et al. 2013, Schmidt et al. 2015, Virgin and Rosvall 2018). These consequences may alter chick survival, being critical for the conservation of endangered species. In the present case, no effect was detected on body condition before fledgling, although a link between corticosterone and body condition was highlighted in other studies (Spencer and Verhulst 2007, Tilgar et al. 2017). This is especially important in migratory species like the Montagu's harrier. However, further investigations should be undertaken since long-term effects on life history traits cannot be excluded.

### Concluding remarks

Although our sample size was limited due to the conservation status of the Montagu's harrier, our results show important differences in the response to variation in the identity of observers that are rarely considered and may bias the interpretation of the observed patterns. We



therefore urge behavioural ecologists and conservationists to 1) take into account whether changing or not experimenters in cases involving repeated interactions with the organisms in regard to the probability of habituation or sensitization, and/or to 2) consider this methodological issue when planning projects and interpreting their results. We also highlight that habituation/sensitization might also be problematic for meta-analyses and comparisons between studies.

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Supplementary material (available online as Appendix jav-02160 at <[www.avianbiology.org/appendix/jav-02160](http://www.avianbiology.org/appendix/jav-02160)>). Appendix 1.