RESEARCH ARTICLE

Repeated stimulation of the pituitary–adrenal axis alters offspring phenotype of a wild passerine

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ABSTRACT

Prolonged stress can have long-lasting effects on an individual's physiology and growth. However, the impact of chronically elevated glucocorticoids on the expression of early antipredator responses is still poorly documented. In this study, I simulated the effect of repeated acute stress on offspring phenotype in free-living pied flycatchers (*Ficedula hypoleuca*) by administering adrenocorticotropic hormone (ACTH) to nestlings for 6 days. The results showed that frequent induction of stress responses by ACTH injections, independent of parental care, adversely affected offspring final body size, wing length and baseline corticosterone levels. Nestling behavioural activity did not differ between ACTH- and saline-treated groups during exposure to control sounds, whereas behavioural activity during exposure to alarm calls was reduced in manipulated offspring only. I conclude that prolonged physiological stress may have short-term benefits to nest-bound offspring, such as more effective antipredator behaviour, but at the expense of negative effects on body size and developmental speed.

INTRODUCTION

Environmental stressors can affect the demography of free-living populations through profound changes in an individual's neurobiology, physiology and behavioural responses (Sheriff et al., 2009; Zanette et al., 2011). In vertebrates, one fundamental physiological system that links phenotypes to environmental changes is the hypothalamic–pituitary–adrenal (HPA) axis. Exposure to various stressors results in activation of the HPA axis with consequent elevation of circulating plasma glucocorticoids (GCs). The GC response, resulting from acute stress (e.g. a predator attack), is a complex mechanism that depends on several physiological components and aims at promoting immediate survival at the expense of other life-history components (e.g. reproduction) when a stressful situation occurs (Angelier and Wingfield, 2013). Short-term GC secretions are beneficial, as they assist an animal in coping with a stressor by mobilising energy reserves during the stressful situation (Sapolsky et al., 2000; Romero, 2004; Boonstra, 2013). If the stress persists over a long period, the relationship between environmental stress exposure and fitness outcomes is likely to vary with stressor severity, life-history stage and the time scale over which effects are measured (Marasco et al., 2018). Thus, moderate elevation in baseline GC levels may

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have potential beneficial effects on survival (Rivers et al., 2012), whereas the sustained high levels of GC usually have a negative impact on individual health (Siegel, 1980; Tilgar et al., 2010) and fecundity (Fuelling and Halle, 2004; Sheriff et al., 2009; Zanette et al., 2011).

The protracted effects of stress that occur during the postnatal development could be especially significant. The pathway from the perception of stressors to fitness consequences involves multifaceted phenotypic and behavioural changes (Schoech et al., 2011). Limited evidence suggests that extended secretion of corticosterone (CORT) can have harmful consequences for offspring growth in reptiles (Morici et al., 1997), birds (Spencer and Verhulst, 2007; Loiseau et al., 2008; Müller et al., 2009; Grace et al., 2017a,b; Kraft et al., 2019; but see Kitaysky et al., 2003; Schmidt et al., 2012) and mammals (Bush et al., 2003). Moreover, chronic stress or GC treatment during development influences the expression of HPA-axis regulatory genes as well as the transcriptome signature within key brain regions (Zimmer and Spencer, 2014; Marasco et al., 2016) and induces structural changes, such as the reduction of neuropil and the number or size of myelinated axons, in various brain regions (Howard and Benjamins, 1975), potentially impairing cognitive functions in birds (Kitaysky et al., 2003), rats (Gregus et al., 2005) and humans (Lupien and McEwen, 1997). Contrary to the prediction, developmental stress can have a positive effect on fitness via changes in reproductive success as shown in male zebra finches, *Taeniopygia guttata* (Crino et al., 2014). In wild animals, one fundamental question is whether stressors modify behaviours such as risk-taking or exploration, which are constantly under selection pressure. In the African cichlid *Simochromis pleurospilus*, the offspring's own experience with the offspring predator odour cues exerted lasting effects on predator avoidance behaviour (Stratmann and Taborsky, 2014). Studies on laboratory rats have shown that repeated corticosterone injections or repeated restraint stress during growth did not change activity or anxiety levels (Gregus et al., 2005), whereas 24-h-long maternal deprivation increased anxiety (Penke et al., 2001). In captive birds, experimental treatment with corticosterone reduced neophobic behaviour of nestling zebra finches (Spencer and Verhulst, 2007) and depressed avoidance behaviour, but had no effect on escape behaviour in juvenile house sparrows (*Passer domesticus*) (Grace et al., 2017a,b). To date, the effects of extended physiological stress on antipredator responses of young animals are still poorly investigated, especially in natural populations. A recent study on yellow-legged gulls (*Larus michahellis*) demonstrated that corticosterone-implanted chicks had a shorter latency to react against a potential threat than control chicks (Noguera et al., 2017).

The present study explores the effects of early-life GC exposure in a wild bird species, the pied flycatcher (*Ficedula hypoleuca*). The effect of a frequent short-term stressor was simulated by injecting ACTH into altricial nestlings over a 6-day period. I predicted that

repeated ACTH treatment would suppress body growth and alter the behavioural responses of offspring to predator cues. If high early-life stress adaptively tunes animals for stressful later life environments (Sheriff and Love, 2013), I expected that premature offspring exposed to ACTH would exhibit increased antipredator behaviour (i.e. enhanced freezing behaviour in the nest) in response to alarm calls. Alternatively, if high ACTH treatment overwhelms young nestlings, they would exhibit reduced antipredator behaviour (less effective freezing response) when compared with controls.

MATERIALS AND METHODS
Study area and animals
The study was conducted in 2013, in coniferous forests close to Kilingi-Nõmme (58°7′N, 25°5′E) in southwest Estonia. Approximately 300 nest boxes were available to pied flycatchers [*Ficedula hypoleuca* (Pallas 1764)] in forests dominated by Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*). The pied flycatcher is a small (12–13 g), short-lived and migratory cavity-nesting passerine bird that breeds in most of Europe and western Siberia (Lundberg and Alatalo, 1992). All nest boxes were checked weekly, beginning from the settlement period in early May, to obtain data on the onset of egg-laying and clutch size. From the expected hatching time, studied nests were checked daily until all chicks had hatched. Nestling body mass and tarsus length were measured on days 5 and 11 post-hatch (hatch day=day 0). Wing length was measured only on day 11 post-hatch. The body mass of chicks was measured using a Pesola spring balance with a precision of 0.1 g. Nestling tarsus length was measured to the nearest 0.1 mm using sliding callipers, and wing length was measured to the nearest 1 mm using a ruler. Nestlings were ringed on day 5 post-hatch with coloured rings to enable re-identification of individuals. During the course of the experiment, one brood died for an unknown reason, but probably owing to the depredation of the mother. Birds were ringed under the Estonian Ministry of the Environment Licence No. 11 and the study was approved by the Animal Procedures Committee (licence no. 108) of the Estonian Ministry of Agriculture.

Study design and measurements
The experimental protocol was based on a within-brood design. I randomly chose 25 nests from a larger sample (approximately 80) of available nests. Four siblings from each nest were randomly selected, of which two were assigned to the control (saline-injected) group and two to the manipulated (ACTH-injected) group (hormonal manipulation is described in detail below). Blood samples were collected on day 11 post-hatch from both groups in a random order. Samples for baseline CORT were taken within 2 min of capture to avoid any effect of handling, and blood samples for stress-induced CORT were taken within approximately 13 min (mean±s.e.m.=782.5±13.9 s) of capture. I chose this interval to be comparable with other passerine studies (e.g. Schwabl, 1999) and with my earlier studies (e.g. Tilgar et al., 2016, 2017). It has been shown in different passerine species that CORT levels reach a plateau approximately 15 min after capture (Lynn and Porter, 2008; Wada and Breuner, 2008). For ethical reasons, baseline and stress-induced levels of CORT were measured from different siblings of the same group. Breathing rates were measured for 10 s on day 11 post-hatch. Breathing rate has been proposed as an indicator of acute stress after a predatory attack, and more generally as an indicator of stress sensitivity (Carere and van Oers, 2004). All samples were collected between 08:00 and 16:00 h.

Blood samples (~70 µl), taken from the brachial veins of marked siblings, were collected in heparinized capillary tubes. Blood samples were immediately stored at +4°C and afterwards centrifuged at 2000 *g* for 10 min, in order to separate plasma from blood cells, and stored at −35°C prior to analysis. After each blood sample was centrifuged, the haematocrit was measured to the nearest 0.1 mm using sliding callipers. The haematocrit is a measure of the relative volume of red blood cells in the total blood volume. This reflects the intensity of oxygen transport, with low values potentially indicative of poor nutritional condition or infections (Potti et al., 1999; but see Dawson and Bortolotti, 1997).

Baseline and antipredator behaviour of nestlings were measured on day 12 post-hatch. Two siblings in each group were used together because complete isolation from nestmates may have an additional effect on nestling stress level. Control and experimental pairs of nestlings were immediately placed in separate nest boxes containing a flycatcher nest and transported approximately 60 m away from the original box in opposite directions to maintain an equal level of isolation from other nestmates and avoid potential parental effects on nestling stress level (Tilgar et al., 2010). Taking the chicks out of the nest and transferring them into a new nest-box can be moderately stressful, thus potentially affecting nestling baseline behaviour during the initial phase of behavioural testing. However, this effect on behaviour is supposedly marginal given that chicks were handled gently, two chicks were grouped together to avoid isolation and they had enough time (approximately 3–5 min) to recover from disturbance before the playback of control sounds was started. Visual observations also confirmed that chicks placed into a new nest calmed down quickly (1–2 min). Data from digital voice recorders (Olympus DS-50) attached to transportable nest boxes confirmed that nestlings did not attempt to communicate with their parents during the experiment. Playbacks were performed with an MP3 player using a weatherproof Yamaha speaker (PDF-B11). The speaker was placed approximately 1.5 m from the transported nest box, with the front of the speaker turned 90 deg away from the direction of the nest box. Both groups were initially exposed to a 10-min-long playback of control sounds containing a repeated series of two recordings from singing adult passerines. The control sounds were recorded in the forests of the study area. After the end of the control phase, both groups were exposed to 10-min-long playback of the pied flycatcher nestling screaming (distress) calls. These calls were recorded from 9- to 13-day-old chicks that were handled until they produced alarm calls. This type of stressor was chosen because distress calls are typically emitted by animals in extreme danger (Högstedt, 1983). It was assumed that nestlings would associate this stressor with a predation event at a nearby nest. The volume level of playbacks was standardised such that sounds could not be heard by the experimenter from a distance of 40 m. The volume level was set to sound similar to a flycatcher's distress call coming from outside the nest box. If heard by nestlings inside the nest box, these sounds are a bit muffled when compared with alarm calls given by nestlings in the nest. The rate of nestling wing flaps (the summed values of two siblings in each group) was recorded with digital voice recorders. Audio files were analysed with the software Avisoft SASLab Pro version 4.40 (Avisoft Bioacoustics). All sounds were displayed as spectrograms where wing flaps are identified by the specific pattern they produce (Figs S1 and S2). Sound spectrograms were analysed frame by frame. The length of each frame was 6 s, meaning that each 10-min-long recording contained approximately 100 frames (600 s). I counted the number of wing flaps per frame and per duration of the playback.

Hormonal manipulation
Nestlings were given intramuscular injections into the pectoral muscles near the keel of the sternum, with either an ACTH or control

solution. Two siblings from each nest received the ACTH treatment and two siblings received the saline treatment. The order of injection (ACTH first, saline second) was alternated between nests. Nestlings were injected daily with 50 µl of ACTH solution (1 IU day^{-1}) from day 5 to day 10 post-hatch (six consecutive days). It has been demonstrated that the defensive reaction in response to species-specific alarm calls first appears in pied flycatcher nestlings on day 4 post-hatch (Aleksandrov et al., 2001). Hence, I assumed that 5-day-old nestlings are physiologically able to produce GCs.

The physiological effect of the single ACTH treatment on the CORT level was tested in a separate sample of nests. Blood samples were obtained from wing veins of 11-day-old chicks 30 min after the injection. The much lower stress response of saline-injected chicks (Fig. 1) indicates that repeated short-term handling and injection procedures per se have a moderate effect on nestling stress response. Hence, the magnitude of stress response following ACTH treatment supposedly reflects the adrenal reaction of nestlings to acoustic stressors such as parental alarm calls or nestling screams (Rydén, 1980; Aleksandrov et al., 2001).

ACTH was diluted in a 10% aqueous gelatin solution. The plasma half-life of ACTH is less than 10 min in small vertebrates (López and Negro-Vilar, 1988). However, with commonly used sustained-release preparations such as gelatin, plasma levels are maintained for several hours after injection in spite of the short elimination half-life (Vigevano and Cilio, 2016). Into 50 ml of 0.9% sterile sodium chloride (NaCl), 5 mg of gelatin was dissolved. ACTH solution was injected to experimental nestlings with disposable 27 G insulin syringes, giving 1 IU (12.5 µg) of ACTH in a single dosage (50 µl). Every injection had the same concentration (1 IU ACTH per 50 µl solution), which meant that per gram of body mass, the

concentration of ACTH gradually decreased as chicks grew. The daily doses of exogenous ACTH were 1.56 µg g^{-1} (77 IU kg^{-1}) for 5-day-old chicks and 0.96 µg g^{-1} for 10-day-old chicks. These values are similar to those used in studies with other wild birds (Sims and Holberton, 2000; Wada et al., 2007) and chickens (Freeman and Manning, 1975). Control chicks were handled in the same way, except they were injected with 50 µl of a sterile solution containing 0.9% NaCl and 10% gelatin. Before injections, the skin of each nestling was disinfected with ethanol solution. Nestlings were always injected in the morning between 08:00 and 12:00 h.

Laboratory analysis

The Correlate EIA kit (catalogue no. 900-097, Enzo Life Sciences, Assay Designs) was used to measure plasma CORT levels (the assay was conducted according to the manufacturer's instructions). The optimisation involved plasma dilutions (1:20, 1:10, 1:5, 1:3) at different concentrations of steroid displacement buffer (SDB) (0, 1, 1.5 and 2.5%) per raw plasma volume. Optimising SDB concentration is critical, as higher concentrations appear to degrade antibody activity in the assay, increasing estimated corticosterone levels in wells. Endogenous plasma (50 µl of plasma+50 µl of assay buffer), spiked plasma (50 µl of plasma+50 µl standard diluent with known concentration of CORT (4000, 800 and 160 pg ml^{-1}) and standard diluent (50 µl of diluent with known concentration of CORT+50 µl of assay buffer) were used in the optimisation analysis. Spiked values were calculated by subtracting endogenous (non-spike) samples from spiked samples. Recoveries for spiked samples were calculated by comparison with a spike in the standard diluent with known concentration of CORT. The analysis showed that the spiked values with 1.5% SDB became closer to the added concentration of CORT (recovery range 91–98%) when compared with the spiked values with 2.5% SDB (90–121%). Hence, for the pied flycatcher, 1.5% SDB per raw plasma volume allowed the most reliable estimation of CORT levels from different plasma dilutions. CORT values in some baseline samples contained less than 50 pg ml^{-1} of CORT per 20 µl of raw plasma. In order to detect as small as possible concentration of CORT in baseline samples, 20 µl of plasma samples were diluted in 80 µl of the kit assay buffer (a dilution factor 5) and analysed in duplicate. Samples for baseline and stress-induced CORT measurements were distributed between different assay plates. Plasma samples from the same brood were run on the same plate. The plate effect was tested by adding plate ID as a random factor to a statistical model. However, it was removed from final models owing to insignificance. The intra- and inter-assay variations were 7.3% and 10.1%, respectively. The intra-assay coefficient of variation (CV) is a measure of variance between duplicates within an assay. Means and s.d. were calculated separately for each assay plate using five duplicates of pooled plasma per plate. The reported intra-assay CV is an average value calculated from the individual intra-assay CVs of three plates. Inter-assay CV is a measure of the variance between duplicates of internal standards on different assay plates. Means and s.d. were calculated between six duplicates on three assay plates (two duplicates per plate).

Statistical analysis

Data were analysed using R statistical software (version 3.5.3, https://www.r-project.org/). Linear mixed models (LMMs) were applied to data of body mass and tarsus length using treatment and age as fixed factors, and nestling identity and nest as random factors to account for repeated measurements and dependency between nestlings from the same brood and group (R package lme4, https://cran.r-project.org/web/packages/lme4/index.html). The wing
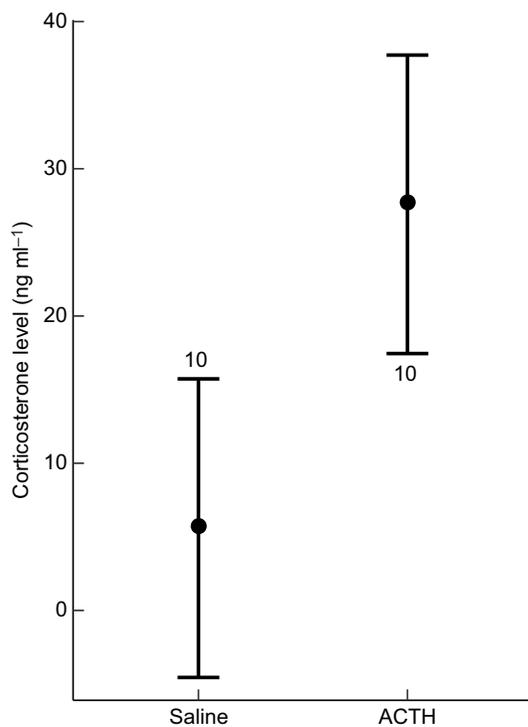
Fig. 1. Stress-induced corticosterone (CORT) levels (untransformed values shown) in adrenocorticotropic hormone (ACTH)- and saline-injected pied flycatcher nestlings on day 11 post-hatch. These nestlings were not included in the main experiment. Blood was collected from nestlings 30 min after capture. The numbers are sample sizes (nestlings); vertical bars denote means±s.e.m.

length, baseline and stress-induced CORT levels, haematocrit and breathing rate were measured only on day 11 post-hatch. Antipredator behaviour was measured on day 12 post-hatch. For these variables, LMMs were applied using treatment as a fixed factor and nest as a random factor. For ethical reasons, baseline and stress-induced CORT measurements were based on one sibling per group. For antipredator behaviour, the summed behavioural activity of two siblings from the same group (saline or ACTH-treated) was used as an independent data point. Time of day, brood size, the date of measurements and two-way interactions [e.g. age×treatment, CORT group (baseline versus acute levels)×treatment, playback type (control sounds versus alarm calls)×treatment] were also included in initial models and sequentially removed from final models if nonsignificant (P>0.05). Baseline and stress-induced CORT, breathing rate and antipredator behaviour were ln-transformed and haematocrit was squared prior to analysis to satisfy the assumptions of normality and equal variance for residuals of the model. The R package lsmeans (https://cran. r-project.org/web/packages/lsmeans/index.html) was used for *post hoc* multiple comparisons with Tukey's HSD. For LMMs, I report both marginal r^2 values (the proportion of variance explained by fixed factors alone) and conditional r^2 values (the proportion of variance explained by both random and fixed factors) obtained using the package sjstats in R (https://cran.r-project.org/web/packages/sjstats/index.html). All tests were two-tailed.

RESULTS
The effect of ACTH treatment
The effect of ACTH treatment on the level of CORT was tested in a separate sample of nestlings that were not involved in the main experiment. The administration of a single dose of ACTH (1 IU) to 11-day-old nestlings increased their CORT levels by approximately 5-fold when measured 30 min after injection, compared with siblings that received saline solution [χ^2=44.22, P<0.001, nest (N=5 nests) included as a random factor; Fig. 1].

Somatic growth
Control chicks gained more mass and grew longer tarsi during the experimental period than manipulated siblings (Table 1). No

significant differences in body mass or tarsus length were observed prior to the experiment on day 5 post-hatch (Fig. 2, Table 1). After the treatment period (on day 11 post-hatch), ACTH-treated nestlings weighed substantially less and had shorter tarsi than control siblings (Fig. 2, Table 1). Manipulated chicks also had smaller wings than controls (χ^2=21.2, P<0.001, mean±s.e.m.=41.82± 0.45 versus 43.39±0.45 mm).

Physiology
Eleven-day-old chicks were able to increase CORT levels in response to handling stress, and the interaction between treatment and CORT group (baseline versus acute CORT levels) was also significant (Table 2). Post hoc analysis showed that control chicks had lower baseline CORT levels than ACTH-treated chicks (Fig. 3, Table 2), while stress-induced levels were not affected by treatment (Fig. 3, Table 2). Control chicks had higher haematocrit levels and lower breathing rates compared with manipulated nestlings (Fig. 3, Table 2).

Behavioural activity
The rate of wing flapping was higher in the control phase when compared with that during exposure to distress calls (χ^2=4.46, P=0.035, corrected for the length of exposure, N=22 nests), and a significant interaction between treatment and playback type (baseline versus stress-induced behaviour) was also observed [χ^2=4.67, P=0.030, model r^2 (conditional)=42%]. *Post hoc* analyses showed that the rate of wing flaps in response to the playback of distress calls was significantly higher for sham-treated nestlings than for manipulated siblings (P=0.001; Fig. 4). The behavioural activity of both treatment groups was similar in the control phase (P=0.74; Fig. 4). ACTH-treated offspring displayed lower behavioural activity (wing flapping) during exposure to distress calls when compared with that in the control phase (*post hoc* test: P=0.036), whereas sham-treated siblings responded similarly to the both type of playback (*post hoc* test: P=0.35; Fig. 4).

DISCUSSION
This study demonstrates that repeated simulation of short-term stress induces complex changes in offspring phenotype. ACTH-stimulated

Table 1. The effects of treatment and age on the body parameters of control and manipulated pied flycatcher siblings

Fixed effects	Body mass (g)				Tarsus length (mm)			
	Estimate	s.e.m.	χ^2	P	Estimate	s.e.m.	χ^2	P
Intercept	12.47	0.21	88.4	<0.001	16.76	0.21	608.7	<0.001
Treatment (level 1=ACTH)	0.94	0.29	18.80	<0.001	0.45	0.18	6.06	0.014
Age (level 1=day 11)	−4.15	0.27	360.7	<0.001	−4.76	0.18	672.5	<0.001
Treatment×Age			8.10	0.004			4.41	0.036
Random effects	VC	s.d.			VC	s.d.		
Nest	0.96	0.98			0.71	0.84		
ID	<0.01	<0.01			<0.01	<0.01		
Residual	1.10	1.05			0.78	0.88		
r^2 fixed effects (marginal)	72.5%				80.6%			
r^2 random effects	12.8%				9.2%			
r^2 model (conditional)	85.3%				89.8%			
N	24 nests				24 nests			
Post hoc tests								
5-day-old				0.77				0.70
11-day-old				<0.001				0.015

N=24 nests, 47 control and 47 manipulated nestlings. *Post hoc* tests show age-specific differences between treatments. Marginal r^2 is the proportion of variance explained by fixed effects, conditional r^2 is the proportion of variance explained by both random and fixed effects, r^2 random effects is the proportion of variance explained by random effects (r^2 conditional–r^2 marginal). VC, variance component.
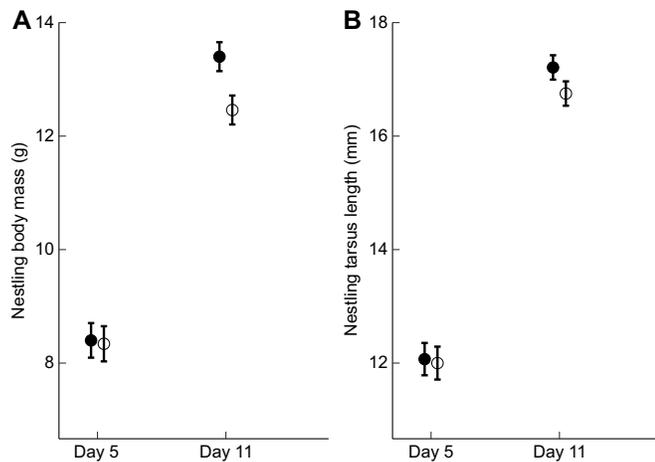
Fig. 2. Effect of treatment on somatic growth of pied flycatcher nestlings. Body mass (A) and tarsus length (B) of control and manipulated siblings on days 5 and 11 post-hatch (filled circles–saline-injected nestlings, empty circles–ACTH-injected nestlings). Sample sizes for body mass and tarsus length: N=24 nests, 47 control and 47 manipulated nestlings. Vertical bars denote means±s.e.m.

chicks attained a smaller final size, and had elevated baseline CORT levels and breathing rates. However, only manipulated siblings were able to effectively alter their behaviour in response to perceived predation risk.

There are several mechanisms mediating elevated CORT effects on the phenotype. It is well documented that during physiological stress, the need for energy increases markedly as the animal tries to cope with a stressor. Oversecretion of baseline CORT, as observed in the present study, probably enhanced antipredator behaviour but depressed growth rate by reallocatiing energy from muscles and adipose tissues to maintenance (Sapolsky et al., 2000) and inhibiting the production of growth hormone and insulin-like growth factors (Lodjak et al., 2016). Extended elevation of CORT may also increase metabolic rates as reported in lizards (DuRant et al., 2008), or reduce digestive efficiency as observed in fishes (Barton et al., 1986) and domesticated birds (Puvaldopirod and Thaxton, 2000).

Although the initial size differences between compared groups were induced by ACTH treatment, it is also possible that the parents did not feed the ACTH-injected chicks as often as the control chicks, especially if those ACTH-injected chicks were in a poor state of health. It has been shown that passerines with asynchronous hatching and a brood-reduction strategy tend to distribute food unequally among their chicks, preferentially feeding young that are in better condition (Rydén and Bengtsson, 1980; Smiseth et al., 2003). In the pied flycatcher, this is not very likely because all chicks usually hatch synchronously and typically survive to fledge (e.g. Lundberg and Alatalo, 1992). Hence, I suggest that the observed changes in nestling development are mainly due to ACTH treatment per se on nestling phenotype rather than significant changes in parental feeding behaviour.

Similar to the present study, injections of ACTH decreased body mass in young chickens (Freeman and Manning, 1975). To date, only a few studies have tested whether prolonged activation of the stress axis can affect somatic growth in free-living populations. Daily administration of CORT to nestlings of the house sparrow (*Passer domesticus*; Loiseau et al., 2008; Grace et al., 2017a,b) and the zebra finch (Kraft et al., 2019) suppressed mass gain, whereas tarsus growth was not affected (Loiseau et al., 2008). CORT implants reduced structural size as well as body mass growth in nestlings of the Eurasian kestrel (*Falco tinnunculus*) (Müller et al., 2009) and the yellow-legged gull (Noguera et al., 2017). These results show that prolonged secretion of CORT reduces growth in several bird species. In some species, these negative effects can be compensated for later in life (Grace et al., 2017a,b), whereas in others no compensatory growth has been observed (Kraft et al., 2019). Given that pied flycatchers attain their final body size and mass before fledging (Kern et al., 2001; Mänd and Tilgar, 2003), compensatory growth after leaving the nest is unlikely in this species. Previous studies in flycatchers have shown that pre-fledging body mass and structural size are good predictors of offspring recruitment rate (Lundberg and Alatalo, 1992; Potti et al., 2002), and future reproductive success (Gustafsson and Sutherland, 1988). Hence, it is likely that smaller chicks at the fledging stage, such as those injected in this study with ACTH, have reduced fitness later in life. However, the costs and benefits of phenotype alterations can be context-specific (Crino and Breuner, 2015) and there is a
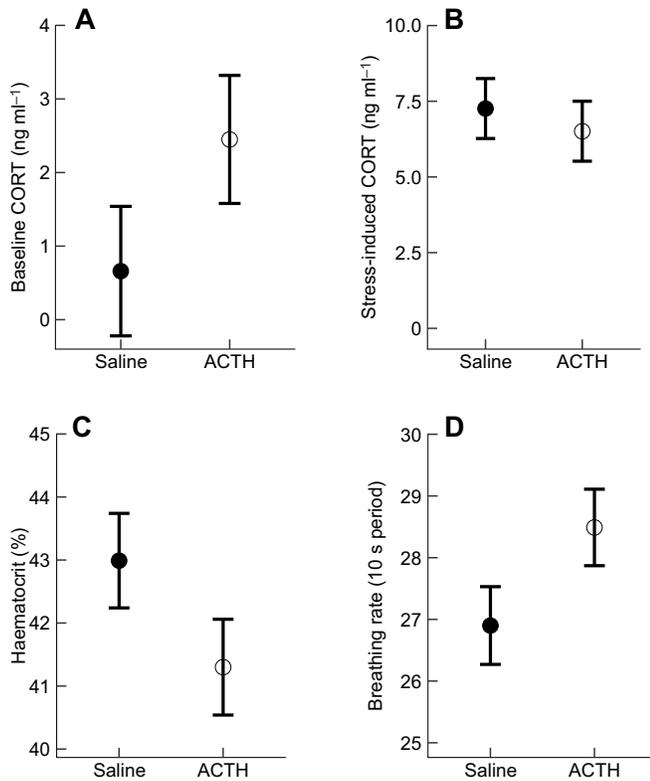
Table 2. Physiological parameters of control and manipulated pied flycatcher siblings on day 11 post-hatch

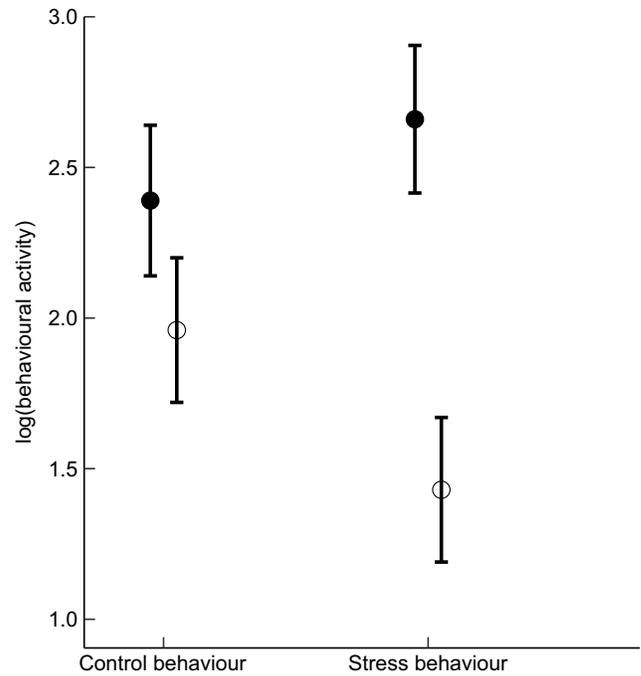| Fixed effects	CORT (ng ml⁻¹) log scale			Haematocrit (%) squared scale			Breathing rate (10 s) log scale		
	Estimate	s.e.	χ^2 (P)	Estimate	s.e.	χ^2 (P)	Estimate	s.e.	χ^2 (P)
Intercept	5.96	0.24	862 (<0.001)	1827	62.6	753 (<0.001)	3.30	0.02	490 (<0.001)
Treatment (level 1=saline)	0.42	0.22	3.44 (0.07)	−109.3	48.5	5.00 (0.025)	0.05	0.03	4.16 (0.041)
CORT group (level 1=baseline)	2.55	0.31	66.6 (<0.001)						
Treatment×CORT group			5.66 (0.017)						
Random effects	VC	SD		VC	SD		VC	SD	
Nest	0.13	0.36		66041	257		<0.01	0.05	
Residual	1.15	1.07		51648	227		0.02	0.12	
r^2 fixed effects (marginal)	47.4%			6%			8%		
r^2 random effects	5.3%			51.2%			27.3%		
r^2 model (conditional)	52.7%			57.2%			35.3%		
N	23 nests			24 nests			23 nests		
Post hoc tests									
Baseline CORT			0.006						
Acute CORT			0.74						

N=23 nests, 23 control and 23 manipulated nestlings for baseline and stress-induced corticosterone (CORT) analysis; N=24 nests, 45 control and 46 manipulated nestlings for haematocrit; and N=23 nests, 45 control and 45 manipulated nestlings for the analysis of breathing rates.

Fig. 3. The effect of treatment on physiological parameters of pied flycatcher nestlings. (A) Baseline and (B) stress-induced CORT levels, (C) haematocrit and (D) breathing rate of control and manipulated siblings on day 11 post-hatch (filled circles–saline-injected nestlings, empty circles–ACTH-injected nestlings). Untransformed values are shown; vertical bars denote means±s.e.m. Sample sizes for respective parameters: N=23 nests, 23 control and 23 manipulated nestlings for baseline and stress-induced CORT; N=24 nests, 45 control and 46 manipulated nestlings for haematocrit levels and N=23 nests, 45 control and 45 manipulated nestlings for breathing rates.

Fig. 4. Behavioural activity of control and manipulated pied flycatcher siblings (N=22 nests, in both groups two siblings per nest) on day 12 post-hatch during exposure to control sounds versus alarm calls. Filled circles, saline-injected nestlings; open circles, ACTH-injected nestlings.

need for long-term studies to evaluate the effects of developmental stress on fitness across life-history stages.

This study demonstrated that ACTH treatment suppressed nestling behavioural activity in response to alarm calls. It is important to mention that fear behaviour was measured 48 h after the completion of ACTH treatment, when behaviour was tested under normalised GC levels. Previous studies on the same species have shown that developing (11–13 days old) individuals react against a potential threat (i.e. distress calls) by exhibiting defensive (freezing) behaviour (Aleksandrov et al., 2001; Tilgar et al., 2010). Freeze behaviour supposedly reduces the predation risk of offspring because they can remain undetected by nest predators. Hence, this finding suggests an adaptive behavioural response of manipulated chicks towards predatory cues. Consistently, CORT-implanted chicks of the yellow-legged gull were faster in crouching and hiding after listening to adult alarm calls in comparison with chicks from the control group (Noguera et al., 2017). In the present study, I did not address the mechanism by which ACTH challenge impacted nestling behaviour. It is well known that baseline (Tilgar et al., 2016) and acute (Breuner et al., 1998) elevation in GCs may increase locomotor activity. Given that ACTH-stimulated offspring had heightened baseline CORT levels but they did not produce more CORT in response to a standardized stressor, the current results suggest that passive antipredator behaviour might be related to baseline rather than stress-induced GC secretion. However, it is possible that

ACTH-treated chicks may continue to increase their CORT levels after the ~10 min sampling interval. Hence, future studies with a larger number of sampling points would be necessary to test this.

During the early postnatal days, the central nervous system is relatively immature in altricial nestlings. Hence, early-life exposure to CORT can potentially affect behavioural responses via the development of neurons and respective changes in the sensitivity of the sympathetic nervous system (Kofman, 2002). Recall in this context that ACTH-stimulated chicks had higher breathing rates (respiratory muscles are also controlled by the development of respiratory neurons and the sympathetic nervous system; Behan and Kinkead, 2011) in response to a standardised stressor than control siblings. Frequent elevation in GCs can also affect fear behaviour via bio-availability of free CORT, the density of corticoid receptors in different brain centres or decreased functionality of non-genomic membrane-bound receptors (Breuner and Orchinik, 2000; Heegde et al., 2015). It is possible that repeated activation of the neuroendocrine system during early postnatal growth can exert long-lasting (organisational) effects on offspring phenotype that are similar to maternal effects. It has been demonstrated that maternal predator exposure enhances different antipredator behaviours such as freeze responses in yellow-legged gulls (Morales et al., 2018) and tighter shoaling behaviour in sticklebacks (Giesing et al., 2011). An alternative explanation to different fear responses between groups is that control chicks switched from passive avoidance behaviour to an active escape strategy because of their more rapid maturation and a better ability to flee. However, in the present study, sham-treated offspring did not change the rate of wing flapping in response to hearing control sounds or alarm calls. Hence, it is unlikely that manipulated and control chicks adopted different behavioural tactics (passive versus active response) to escape predators. Hence, longer-term data would be very interesting to look at fitness outcome effects as a consequence of the early life hormonal manipulation on antipredator behaviour.

In conclusion, the present study demonstrates the causal link between the repeated elevation of CORT levels and the reduction in offspring structural size, body mass and altered antipredator behaviour. Although physiological stress may entail some possible short-term benefits on offspring performance by enhancing antipredator behaviour, this probably does not outweigh long-term costs related to smaller body size and elevated baseline CORT levels.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.200659.supplemental

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