

RESEARCH ARTICLE

Corticosterone implants produce stress-hyporesponsive birds

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ABSTRACT

In birds, the use of corticosterone (Cort) implants is a frequent tool aimed at simulating systemic elevations of this hormone and studying effects on biological traits (e.g. physiology, morphology, behavior). This manipulation may alter adrenocortical function, potentially changing both baseline (Cort_{BAS}) and stress-induced (Cort_{STRESS}) plasma Cort levels. However, implant effects on the latter trait are rarely measured, disregarding downstream consequences of potentially altered stress responses. Here, we analyzed the effects of Cort implants on both Cort_{BAS} and Cort_{STRESS} in nestling and adult European white storks, *Ciconia ciconia*. In addition, we performed a review of 50 studies using Cort implants in birds during the last two decades to contextualize stork results, assess researchers' patterns of use and infer current study biases. High and low doses of Cort implants resulted in a decrease of both Cort_{BAS} (31–71% below controls) and Cort_{STRESS} (63–79% below controls) in storks. Our literature review revealed that Cort_{BAS} generally increases (72% of experiments) whereas Cort_{STRESS} decreases (78% of experiments) following implant treatment in birds. Our results challenge and expand the prevailing assumption that Cort implants increase circulating Cort_{BAS} levels because: (i) Cort_{BAS} levels show a quadratic association with implant dose across bird species, and decreased levels may occur at both high and low implant doses, and (ii) Cort implants also decrease Cort_{STRESS} levels, thus producing stress-hyporesponsive phenotypes. It is time to work towards a better understanding of the effects of Cort implants on adrenocortical function, before addressing downstream links to variation in other biological traits.

KEY WORDS: Adrenocortical function, *Ciconia ciconia*, Cort, Dose–response, Phenotypic engineering, Stress response

INTRODUCTION

Glucocorticoids (the ‘stress hormones’ in vertebrates) are considered major physiological mediators of allostasis, thus allowing the maintenance of homeostasis through change (McEwen and Wingfield, 2003). Blood glucocorticoid titers can vary within a lower range of baseline levels (Cort_{BAS}) that facilitate

transitions between life stages (Wada, 2008). Superimposed on Cort_{BAS} fluctuations, plasma titers typically increase to a higher range of stress-induced levels (Cort_{STRESS}) in response to disturbances (Wingfield, 2013). Although the general function posited for corticosterone (Cort; the main avian glucocorticoid) is the regulation of energy balance, the function of elevated Cort_{BAS} and Cort_{STRESS} levels are conceptually different (Blas, 2015). Fluctuations in Cort_{BAS} allow individuals to adjust their physiology, morphology and behavior to the energy demands imposed by the predictable component of the environment (e.g. expected challenges linked to seasons, day–night cycles, high–low tides) in anticipation of change (i.e. predictive homeostasis; Romero et al., 2009). In contrast, Cort_{STRESS} levels facilitate fast stress responses to emergency situations imposed by the unpredictable component of the environment (i.e. unexpected challenges such as inclement weather, predations attempts, experimental capture and handling; Wingfield and Romero, 2001), thus allowing reactive homeostasis (Romero et al., 2009).

A large body of evidence regarding the function of Cort_{BAS} and Cort_{STRESS} in birds has been obtained through experimental studies performed over the last decades (Blas, 2015). Furthermore, the use of hormone implants has been a major tool to understand the mechanisms of control that underlie different biological traits and their potential consequences in physiological, ecological and evolutionary contexts (‘phenotypic engineering’; Almasi et al., 2012; Ketterson and Val Nolan, 1992; Williams, 1999). Cort implants are regularly used with the general purpose of simulating systemic and sustained elevations of Cort_{BAS} levels (Romero and Wingfield, 2016), and studying downstream consequences on biological traits. Although verifications of a causal link between implant treatment and Cort_{BAS} levels are typically performed (justifying the use of Cort implants; e.g. Nelson et al., 2015; Ouyang et al., 2013; Tartu et al., 2016), such validation should not preclude from questioning whether Cort_{STRESS} levels are also affected by this manipulation. In fact, several lines of evidence suggest that combined measurements of Cort_{BAS} and Cort_{STRESS} should be relevant in most Cort–trait relationship study contexts, because: (i) Cort_{BAS} and Cort_{STRESS} levels involve the same hormone, regulated by the same endocrine axis [i.e. the hypothalamic–pituitary–adrenal (HPA) axis], (ii) exogenous Cort treatment likely alters HPA axis function (e.g. through negative feedback; Dallman et al., 1992), potentially affecting both Cort_{BAS} and Cort_{STRESS} levels, and (iii) elevated Cort_{BAS} versus Cort_{STRESS} can have opposite effects on the same biological traits (Landys et al., 2006; Wingfield and Romero, 2001), potentially affecting the interpretation of results. Despite the arguments above, the quantification of Cort_{STRESS} levels following implant treatment is often neglected in both wild and laboratory conditions, and this may have introduced a bias in our current understanding of Cort–trait relationships.

Our study had three major goals. First, to understand the effects of Cort implants at two doses on Cort_{BAS} and Cort_{STRESS} levels in

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List of symbols and abbreviations

C_{Bmax}	maximum change in baseline corticosterone levels
C_{Bmean}	mean change in baseline corticosterone levels
Cort	corticosterone
$Cort_{BAS}$	baseline plasma corticosterone levels
$Cort_{STRESS}$	stress-induced plasma corticosterone levels
D_{max}	maximum possible duration of implant effects on baseline or stress-induced levels of corticosterone
D_{min}	minimum verified duration of implant effects on baseline or stress-induced levels of corticosterone
HPA	hypothalamic–pituitary–adrenal
OSM	osmotic pump implant
SIL	silastic tube implant
TRP	time-release pellet

European white storks [*Ciconia ciconia* (Linnaeus 1758)], a large, long-lived bird whose adrenocortical function has been the subject of previous studies in relation to ontogenetic, environmental and life-history factors (Blas et al., 2005, 2006, 2007). Second, to place our stork results within a general context of avian responses to Cort implants, and perform a review of the literature published on this topic during the last two decades. Such a review allowed us to evaluate dose–response relationships (i.e. effects of the amount of exogenous Cort administered on circulating Cort levels), and estimate the effective duration of the Cort treatment on plasma Cort titers. Our third and last goal was to analyze researchers' patterns of use of Cort implants (i.e. types of implants, relative frequency of use, temporal trends, model species, blood sampling protocols) and associated changes in avian plasma Cort levels, with the ultimate aim of quantifying current study biases and drawing recommendations for future research.

MATERIALS AND METHODS**Cort implant experiments in white storks**

Two sequential experiments involving subcutaneous treatment with Cort implants were performed using white storks as a study model. The Cort implants were time-release pellets (TRPs), a commercially available matrix-driven delivery system (Innovative Research of America, Sarasota, FL, USA) where exogenous Cort is fused within a cholesterol matrix and delivered through the double process of erosion of the subcutaneous pellet and diffusion of the active product. Implants were purchased in two different years (in 2010 for experiment 1, and in 2015 for experiment 2).

In experiment 1, performed in June 2010, 34 wild stork nestlings were selected from 17 nests located near Doñana National Park, Seville, Spain. Nestlings were 50.4 ± 1.3 days old (estimated through morphometric measurements following Chozas, 1983) and thus near fledging age (fledging period starts at day 60 according to Redondo et al., 1995), when storks show robust adrenocortical responses (Blas et al., 2006). One experimental and one control sibling were randomly selected from each nest. The experimental nestlings were treated with one 100 mg, 21-day release Cort pellet (theoretical dose: $1.43 \mu\text{g Cort g}^{-1}$ body mass day^{-1}). This dose was selected because previous studies in other bird species yielded systemic elevations in $Cort_{BAS}$ using both lower doses (range 0.01 – $0.53 \mu\text{g Cort g}^{-1}$ body mass day^{-1} : Bonier et al., 2007; Goerlich, 2009; Pravosudov, 2003) and higher doses (range 3.07 – $8.65 \mu\text{g Cort g}^{-1}$ body mass day^{-1} : Almasi et al., 2009; Bourgeon and Raclot, 2006; Müller et al., 2009; Stier et al., 2009). For the implantation procedure of the Cort pellet, we followed the recommendations of the manufacturer. After cleaning the

featherless, dorsal, lower-neck skin area with a solution of povidone iodine (Betadine, MEDA Pharma SAU, Madrid, Spain) and a topical anesthetic (lidocaine EMLA; AstraZeneca Pharmaceuticals, Madrid, Spain), a small (10–12 mm) skin incision was made with a scalpel. Upon inserting the Cort pellet subcutaneously, the incision was sealed using veterinary glue (cyanoacrylate 3M Vetbond, St Paul, MN, USA). Control animals underwent the identical procedure, without actual insertion of a pellet. Whether to use empty (i.e. cholesterol-filled) pellets or no pellets at all for the control group is currently debated. Some researchers argue that cholesterol is not a proper control because it provides a precursor to steroid synthesis (see pages 231–234 in Romero and Wingfield, 2016 for a discussion of this issue). Because cholesterol is not an inert substance, many researchers have avoided the use of empty pellets, thereby treating control birds as we did here (see e.g. Bourgeon and Raclot, 2006; Thierry et al., 2013). Before surgery (on day 0), and also 7 days later (on day 7), stress series (one per day) were performed on all experimental and control birds. Each stress series involved the collection of two sequential blood samples (each 0.5 ml of blood; collectively less than 0.05% body mass per stress series) from the brachial vein: a first blood sample was taken within the first 3 min post-capture (121 ± 8 s; $\text{mean} \pm \text{s.e.m.}$), and a second blood sample was taken 30 min later (1934 ± 12 s). These samples allowed the subsequent determination of plasma $Cort_{BAS}$ and $Cort_{STRESS}$ levels, as well as the changes induced by exogenous Cort treatment. Birds were held individually inside cloth bags and in the shade between sequential blood sampling (during a stress series), and were returned to their nests each day after being sampled.

A second experiment (experiment 2) was performed in March 2015 using 10 adult male white storks from a wildlife recovery center in Seville, Spain. All birds were in good health, but they were unable to fly because of permanent wing injuries (linked to accidents that occurred years prior to the experiment). The storks were housed in pairs in five visually isolated outdoor cages (dimensions $3 \times 3 \times 2$ m) exposed to natural temperature and photoperiod. Four of these cages housed one experimental and one control bird, and the fifth cage housed two control individuals. After 10 days of acclimation to this setting (with food and water provided *ad libitum* throughout the study period), each experimental bird was treated with three 200 mg (total of 600 mg), 14-day release Cort pellets (theoretical dose: $12.65 \mu\text{g Cort g}^{-1}$ body mass day^{-1}). We chose this dose based on the following information: (i) previous studies suggested a linear dose–response relationship between exogenous Cort and circulating levels (Akana et al., 1992; Pravosudov, 2003), (ii) our dose in experiment 1 was unable to cause systemic plasma elevations, and (iii) previous studies using higher doses than experiment 1 successfully produced an increase in other bird species (Fairhurst et al., 2013; Müller et al., 2009). The implant procedure was similar to that reported for experiment 1 (above), but the implantation area was located in the right ventral flank (lower portion of the right hemithorax, next to the femoral–tibia–tarsal joint) owing to the high density of feathers in the dorsal neck area of adults. Before this manipulation (on day 0) and also after implantation (3, 6, 9 and 14 days later), stress series (one per day) were performed on all experimental and control birds. Each stress series involved the collection of three sequential blood samples (0.5 ml of blood from the brachial vein, collectively less than 0.05% body mass per stress series). The first sample was taken within the first 3 min post-capture from the housing cages (118 ± 5 s; $\text{mean} \pm \text{s.e.m.}$), the second sample 30 min later (1808 ± 11 s), and the third sample at 60 min post-capture (3592 ± 9 s). We decided to

include the 60-min blood sample to test the effect of Cort implants on storks' adrenocortical responses with a finer, longer-term temporal resolution (compared with experiment 1), and because we were now sampling captive individuals and the duration of our field work could not affect the normal activities of birds in a wild colony. Birds were held individually inside cloth bags and in the shade between sequential blood sampling (during a stress series), and were returned to their cages each day after being sampled.

In experiments 1 and 2, the implantation area was palpated and visually inspected during each sampling episode, allowing us to verify the presence of Cort pellets and the absence of signs of infection. All blood samples from experiments 1 and 2 were kept cold in portable coolers until centrifuged (1252 g, 10 min) on the same day, storing the resulting plasma at -80°C until further laboratory analysis. The experimental procedures were approved by the CSIC Ethical Committee and the Andalusian Committee of Animal Experimentation (refs. CEBAEBD-11-24/12-39) to comply with Spanish and European legislation on the protection of animals used for scientific purposes.

Plasma Cort extraction was performed with diethyl ether (Fisher Chemical, Fair Lawn, NJ, USA) as described previously (Blas et al., 2005). Samples of experiments 1 and 2 were extracted separately in two batches, and the extraction efficiency was higher than 90% in each batch. Plasma Cort levels were determined by radioimmunoassay (RIA) on reconstituted extracts (300 μl of phosphate-buffered saline, 0.05 mol l $^{-1}$ and pH 7.6). Antiserum (C8784; lot 090M47520) and purified Cort (C2505, lot 22K1439) for standards were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). Samples were measured in duplicate and serial dilutions of the extracts showed displacement curves that were parallel to the standard curve. Assay variability was calculated as the coefficient of variation (CV) resulting from repeated measurement of six samples of a known amount of Cort in each assay. Samples of experiments 1 and 2 were measured in three RIAs each experiment, with intra- and inter-assay CV being 6.49% and 10.23%, respectively, in experiment 1, and 6.07% and 7.89%, respectively, in experiment 2.

The effect of Cort implants on circulating Cort levels (both Cort_{BAS} and Cort_{STRESS}) was analyzed separately for experiments 1 and 2. Linear mixed models (Zuur et al., 2009) were built using the library 'nlme' (<https://CRAN.R-project.org/package=nlme>) in R 3.2.3 (<https://www.r-project.org/>). Plasma Cort concentration was always the response variable, individual identity was treated as a random effect, and the following fixed effects were tested: experimental period (factor: before versus after implant treatment), treatment (factor: Cort-treated versus sham-treated), handling time in minutes (factor: 0 versus 30 min for experiment 1 and 0 versus 30 versus 60 min for experiment 2) as well as the interactions between fixed terms. Because our main hypothesis was that Cort implants affect plasma Cort levels, our main prediction was that the experimental groups should differ after (but not before) inserting Cort implants. We thus expected a significant treatment \times handling time \times experimental period interaction term. Secondly, experiment 2 allowed us to test the temporal dynamics of plasma Cort levels. Because the variability in sampling day was only present after implantation (i.e. we only sampled 1 day pre-implant), we built a second model to test this effect. This model included individual identity as a random term, and the following fixed effects: treatment (factor), handling time (factor: 0, 30, 60 min), days post-implant (factor: 3, 6, 9, 14 days) and the interactions between fixed terms. We checked the normality and

homoscedasticity of the residuals. Statistically significant interactions were analyzed through Tukey *post hoc* tests applying Bonferroni correction.

White stork responses in relation to previous studies using Cort TRP implants

In order to place our results from experiments 1 and 2 (above) within a general context of avian studies, we performed a quantitative analysis of previously published information using the same type of Cort implants (i.e. TRP implants). We first performed a bibliographic search of original studies in the Class Aves during the last two decades (publication period: 1996 to 2016). Using the platforms Web of Science, Scopus and USearch, the following terms were combined: corticosterone AND birds AND (time-release pellet OR pellet OR manipulation OR implant OR subcutaneous implantation OR Innovative Research of America). When two or more studies used the same experimental individuals or a subset of them, only one of the published papers was selected (the most complete or the first one), thus avoiding pseudo-replication of results. From these studies, each combination of dose \times species was identified as one 'experiment' (sample unit of analysis; see Table S1). To evaluate dose-response relationships (i.e. effects of the amount of exogenous Cort on circulating Cort levels), the dose of each experiment was standardized as μg exogenous Cort g $^{-1}$ body mass day $^{-1}$, using published data (Dunning, 2008) to extract body mass information when it was not provided in the original study or in coetaneous publications on the same model species by the same group of authors. To quantify changes in circulating Cort levels, two response variables were calculated: (i) the mean change in Cort_{BAS} (baseline mean change, C_{Bmean}) and (ii) the maximum change in Cort_{BAS} (baseline maximum change, C_{Bmax}). C_{Bmean} was the difference between post- and pre-implant Cort mean levels (positive values implying an increase). Pre-implant levels were estimated by combining control and experimental birds (or using data only from the latter, depending on the information available in each study), whereas post-implant levels considered all measures taken throughout the supposedly effective period (i.e. the theoretically expected duration of the implant according to the manufacturer's instructions) in implanted birds only. C_{Bmax} was the difference between post-implant maximum Cort_{BAS} levels (selecting the maximum values from all the samples taken throughout the theoretical period) and pre-implant mean Cort_{BAS} levels. Because we had no access to the individual data collected for each study, we resorted to the mean levels reported therein. When this information was not given in the main text or tables of the selected publications, it was obtained by approximation using the figures contained in each study. For each response (dependent variables: C_{Bmean} or C_{Bmax}) a linear mixed model was built, with species and publication as random effects (Zuur et al., 2009). These models allowed us to test the linear versus quadratic effect of the exogenous Cort dose and compare the variance explained in both cases. Linear effects were tested by including Cort dose as an independent covariate, whereas quadratic effects were tested by including also the squared term Cort dose 2 . To evaluate the fit of our results on white storks within these more general dose-response patterns, the resulting models were recalculated again, including experiments 1 and 2, and quantifying changes in marginal variance (i.e. that explained by the fixed terms; Johnson, 2014; Nakagawa and Schielzeth, 2013).

Finally, we estimated the effective duration of the treatment with TRP implants (i.e. the temporal period of significant modification of circulating Cort levels). Considering the day of implantation as 'day 0', we calculated two new variables from each experiment:

Table 1. Summary of the model explaining circulating corticosterone (Cort) levels in white storks from experiment 1, including pre- and post-implant samples (N=136)

Tested effects	Estimate (s.e.m.)	X ²	P
Intercept	11.26 (1.99)	32.14	<0.01
Treatment*	-0.75 (2.81)	0.07	0.79
Handling time [‡]	24.37 (2.51)	94.05	<0.01
Experimental period [§]	-8.09 (2.51)	10.36	<0.01
Treatment*×Handling time [‡]	0.36 (3.55)	0.01	0.92
Treatment*×Experimental period [§]	12.59 (3.55)	12.55	<0.01
Handling time [‡] ×Experimental period [§]	-20.23 (3.55)	32.41	<0.01
Treatment*×Handling time [‡] ×Experimental period [§]	16.65 (5.03)	10.97	<0.01

*Estimate for sham-treated group.

[‡]Estimate for Cort_{STRESS} levels.

[§]Estimate for post-implant experimental period.

(i) the minimum verified duration (D_{\min}) and (ii) the maximum possible duration (D_{\max}) of effects on Cort_{BAS} and Cort_{STRESS} levels. D_{\min} was defined as the latest post-implant blood sampling episode showing Cort levels to be statistically different from the control situation and was expressed as a percentage of the total theoretical duration (according to manufacturer specifications). As each experiment was unique regarding the number of blood sampling episodes and their temporal regimes, D_{\min} is a conservative estimate (i.e. the effects of the implant could have lasted even longer, but we lacked more detailed information). By definition, D_{\min} was only estimated in those experiments that verified a significant effect of the implant on plasma Cort levels (i.e. experiments showing no effect were not considered here, but see D_{\max} below). D_{\max} was defined as the first post-implant blood sampling episode that showed Cort levels to be statistically indistinguishable from the control situation and was also expressed as a percentage of the total supposedly effective duration (according to manufacturer specifications). Again, as the temporal regimes of each experiment were unique, D_{\max} is a non-conservative estimate (i.e. the effects of the implant undoubtedly lasted for shorter periods of time, but the resolution of blood sampling episodes did not allow more detailed examination). D_{\max} could only be estimated in those experiments that verified the absence or disappearance of effects during the supposedly effective period (regardless of whether the implant previously exerted any effect over this period). Both D_{\min} and D_{\max} considered both our original results (experiments 1 and 2 on storks) and the experiments selected in the bibliographic search described above.

Researchers' patterns of use of Cort implants, and associated avian responses

In order to qualitatively analyze researchers' patterns of use of Cort implants (i.e. types of implant, relative frequency of use, temporal trends, model species, blood sampling protocols) and the associated changes in avian Cort_{BAS} and Cort_{STRESS} levels, a new bibliographic search was made using the platforms described above. Because the aim was to identify studies involving Cort implants (thus excluding other types of Cort manipulations such as through food or drink), the search terms were expanded to include: corticosterone AND birds AND (time-release pellet OR pellet OR manipulation OR implant OR subcutaneous implantation OR Innovative Research of America OR silastic OR osmotic pump). The search period was restricted to the years 2005–2015 to allow us to draw conclusions that were representative for this decade. When two or more studies used the same experimental individuals or a subset of them, we only used one publication (the most complete or the first one). From these studies, each combination of dose×species was identified as one

'experiment', allowing us to extract the following information: (i) whether the protocol involved post-implant quantification of either Cort_{BAS} or Cort_{STRESS} (or both, or none of these parameters) and, when applicable, (ii) the direction of change in these plasma Cort levels (i.e. statistically significant increase, decrease or no change following treatment). Frequency comparisons were performed using Pearson's chi-squared tests.

RESULTS

Effects of Cort implants on plasma Cort levels in white storks

In experiment 1, our model revealed a significant three-way interaction involving experimental period, treatment and handling time (Table 1). To analyze this interaction, we performed multiple comparisons through Tukey *post hoc* tests (Table S2). Before implantation, Cort_{BAS} and Cort_{STRESS} levels were similar between experimental groups and there was a significant elevation in Cort levels in response to handling and restraint (Table S2). Cort_{STRESS} levels increased on average by 24.56 ng ml⁻¹ above Cort_{BAS} titers after 30 min of handling (Fig. 1; Fig. S1). After implantation, the control group displayed a significant average increase of

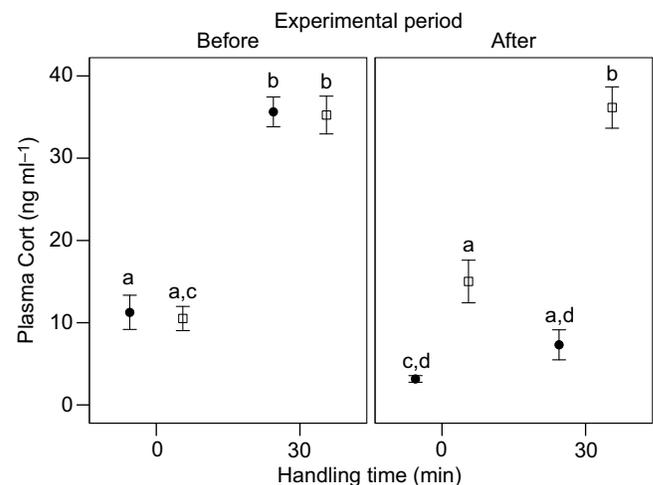


Fig. 1. Effects of corticosterone (Cort) implants on white stork plasma Cort levels (experiment 1). Baseline and stress-induced plasma Cort levels (handling time: 0 versus 30 min post-capture) in free-living white stork nestlings from experiment 1, both before (day 0) and after (day 7) the manipulation with time-release pellets (TRPs). Solid circles show Cort-implanted birds (N=17) and open squares show sham-implanted individuals (N=17). Groups sharing the same lowercase letter showed statistically indistinguishable Cort levels according to Tukey multiple comparisons tests with Bonferroni correction. Data are means±s.e.m.

21.15 ng ml⁻¹ from Cort_{BAS} to Cort_{STRESS} titers during the 30-min handling and restraint protocol (Fig. 1; Fig. S1, Table S2). However, in the Cort-treated group, the effect of handling was not statistically significant (Table S2), and the average increase was only 4.14 ng ml⁻¹ above same-group Cort_{BAS} titers (Fig. 1; Fig. S1), implying an 83% reduction in the stress response compared with controls. The average changes in Cort_{BAS} and Cort_{STRESS} levels after implantation were 8.09 and 28.33 ng ml⁻¹, respectively, lower than before implanting (implying a reduction of 72% and 79% in relation to within-group, pre-implant levels). Furthermore, Cort-treated birds showed Cort_{BAS} levels below their own pre-implant titers and below post-implant levels of control birds (Fig. 1; Table S2).

In experiment 2, our model revealed a significant three-way interaction involving experimental period, treatment and handling time (Table 2, model 1). To analyze this interaction, we performed multiple comparisons through Tukey *post hoc* tests (Table S3). Before implantation, experimental groups did not differ in either Cort_{BAS} or Cort_{STRESS} levels and all birds displayed an elevation in response to handling and restraint (Table S3). Cort_{STRESS} levels increased on average by 45.53 ng ml⁻¹ above Cort_{BAS} titers, with

similar Cort levels reached at both 30 and 60 min post-capture (Fig. 2; Table S3). After applying subcutaneous implants, control birds showed Cort_{BAS} and Cort_{STRESS} levels similar to pre-implant titers and displayed an average increase of 54.46 ng ml⁻¹ during the handling and restraint protocol. On the contrary, Cort-treated birds showed a rather slight, non-significant mean increase of 10.07 ng ml⁻¹ from Cort_{BAS} to Cort_{STRESS} levels during handling (implying an 81% reduction in the stress response compared with controls). Regardless of handling time, Cort-treated birds displayed plasma Cort levels within the baseline range of control and pre-implant birds (Fig. 2; Table S3). The average changes in Cort_{BAS} and Cort_{STRESS} levels after implantation were 8.69 and 51.00 ng ml⁻¹, respectively, lower than before implantation (implying a reduction of 31% and 63% in relation to within-group, pre-implant levels). With regards to post-implant temporal dynamics, plasma Cort levels were independent of sampling day (Table 2, model 2; Fig. S2), and the model revealed a significant interaction between treatment and handling time (Table 2, model 2). To analyze this interaction, we resorted to multiple comparisons through Tukey *post hoc* tests (Table S3), which revealed that experimental groups showed

Table 2. Summary of the models explaining circulating Cort levels in white storks from experiment 2

Model	Sample	Tested effects	Estimate (s.e.m.)	X ² (d.f.)	P
1	Pre- and post-implant (all samples; N=150)	Intercept	27.87 (9.98)	7.80 (1)	<0.01
		Treatment*	-5.84 (12.88)	0.21 (1)	0.65
		Handling time [‡]	47.89 (12.04)	25.82 (2)	<0.01
		Handling time [§]	56.90 (12.04)		
		Experimental period [¶]	-8.68 (9.52)	0.83 (1)	0.36
		Treatment*×Handling time [‡]	9.61 (15.54)	0.41 (2)	0.82
		Treatment*×Handling time [§]	2.77 (15.54)		
		Treatment*×Experimental period [¶]	20.09 (12.29)	2.67 (1)	0.10
		Handling time [‡] ×Experimental period [¶]	-39.56 (13.46)	13.35 (2)	<0.01
		Handling time [§] ×Experimental period [¶]	-45.08 (13.46)		
		Treatment*×Handling time [‡] ×Experimental period [¶]	35.90 (17.38)	6.52 (2)	0.04
		Treatment*×Handling time [§] ×Experimental period [¶]	40.51 (17.38)		
		2	Post-implant (N=120)	Intercept	15.13 (9.19)
Treatment*	9.90 (11.87)			0.70 (1)	0.40
Handling time [‡]	11.58 (10.87)			1.89 (2)	0.39
Handling time [§]	13.97 (10.87)				
Day ^{**}	15.15 (10.87)			3.14 (3)	0.37
Day ^{**}	3.80 (10.87)				
Day ^{§§}	-2.73 (10.87)				
Treatment*×Handling time [‡]	37.01 (14.03)			9.06 (2)	0.01
Treatment*×Handling time [§]	36.19 (14.03)				
Treatment*×Day ^{**}	-10.40 (14.03)			4.47 (3)	0.21
Treatment*×Day ^{**}	17.21 (14.03)				
Treatment*×Day ^{§§}	10.60 (14.03)				
Handling time [‡] ×Day ^{**}	3.96 (15.38)			2.31 (6)	0.89
Handling time [§] ×Day ^{**}	9.38 (15.38)				
Handling time [‡] ×Day ^{**}	-9.49 (15.38)				
Handling time [§] ×Day ^{**}	-12.34 (15.38)				
Handling time [‡] ×Day ^{§§}	-7.51 (15.38)				
Handling time [§] ×Day ^{§§}	-5.65 (15.38)				
Treatment*×Handling time [‡] ×Day ^{**}	12.27 (19.85)			1.27 (6)	0.97
Treatment*×Handling time [§] ×Day ^{**}	5.13 (19.85)				
Treatment*×Handling time [‡] ×Day ^{**}	12.22 (19.85)				
Treatment*×Handling time [§] ×Day ^{**}	6.51 (19.85)				
Treatment*×Handling time [‡] ×Day ^{§§}	9.52 (19.85)				
Treatment*×Handling time [§] ×Day ^{§§}	16.70 (19.85)				

*Estimate for sham-treated group.

[‡]Estimate for Cort_{STRESS} levels at 30 min.

[§]Estimate for Cort_{STRESS} levels at 60 min.

[¶]Estimate for post-implant experimental period.

**Estimate for Day 3.

**Estimate for Day 6.

§§Estimate for Day 9.

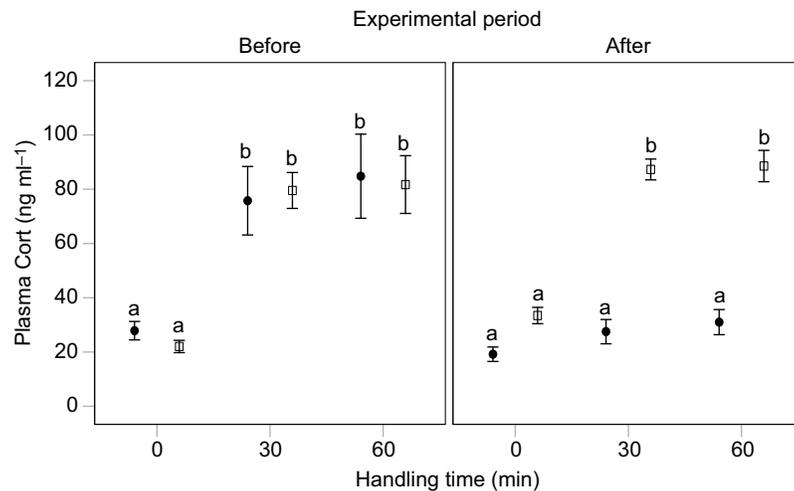


Fig. 2. Effects of Cort implants on white stork plasma Cort levels (experiment 2). Baseline and stress-induced plasma Cort levels (handling time: 0, 30, 60 min post-capture) in captive white stork adults from experiment 2, both before (day 0) and after (days 3, 6, 9 and 14 together) the manipulation with TRPs. Solid circles show Cort-implanted birds ($N=4$) and open squares show sham-implanted individuals ($N=6$). Groups sharing the same lowercase letter showed statistically indistinguishable Cort levels according to Tukey multiple comparisons tests with Bonferroni correction. Data are means \pm s.e.m.

statistically similar Cort_{BAS} levels, but differed in Cort_{STRESS} levels (Fig. 2), and also that Cort-treated birds displayed plasma Cort levels within baseline range regardless of handling time (Fig. 2).

White stork responses in relation to previous studies using Cort TRP implants

Our bibliographic search yielded a total of 26 original studies published since 2003 (when the first article using TRP implants in birds was detected), and we identified 46 experiments using 12 avian species as study models (Table S1). Of these experiments, 36 provided enough information to analyze dose–response relationships. The dose of exogenous Cort administered explained the mean change in Cort_{BAS} (C_{Bmean}) through a quadratic function [model 2: marginal R^2 (R_m^2)=0.42; Table 3] better than through a linear function (model 1, R_m^2 =0.13; Table 3). The incorporation of results from experiments 1 and 2 on white storks slightly reduced the marginal variance explained by the quadratic model, but dose effects remained statistically significant (model 3, R_m^2 =0.35; Table 3, Fig. 3A). The dose of exogenous Cort also explained the maximum change in Cort_{BAS} (C_{Bmax}) through a quadratic function (model 5, R_m^2 =0.30; Table 3) but not through a linear function (Table 3, model 4). The incorporation of results from experiments 1 and 2 on white storks slightly increased the marginal variance explained by the quadratic

model, and dose effects remained statistically significant (model 6: R_m^2 =0.32; Table 3, Fig. 3B). The lack of available information regarding post-implant Cort_{STRESS} levels (i.e. $N=6$ experiments) prevented dose–response analyses on this trait.

With regards to the estimated duration of effects of TRP implants on Cort_{BAS}, D_{min} indicated a verified duration of $29\pm 4\%$ of the supposedly effective (theoretical) time (mean \pm s.e.m.; range=5–81%; $N=29$) and D_{max} indicated a maximum possible duration of $36\pm 6\%$ of the supposedly effective (theoretical) time (range=3–86%; $N=24$; Fig. 4A). The duration of effects on Cort_{STRESS} levels was slightly longer, with D_{min} and D_{max} values being $71\pm 14\%$ (range=33–114%; $N=6$) and $41\pm 20\%$, respectively, of the supposedly effective (theoretical) time (range=14–80%; $N=3$; Fig. 4B), but sample sizes were small.

Researchers' patterns of use of CORT implants, and associated avian responses

Our bibliographic search yielded 50 original studies using Cort implants on 22 bird species (belonging to 15 families and nine orders; Table S1). Three main methods of manipulation were used: commercial TRPs, hand-made silastic tube implants (SIL) and commercial osmotic pumps (OSM). In addition, one of these studies also used hand-made fat Cort implants (Goerlich, 2009), but because

Table 3. Summary of the models explaining dose–response effects of time-release Cort implants (TRPs) on circulating Cort levels in birds

Response*	Model	Effect	Estimate (s.e.m.)	X^2	P	R_m^2 †
Baseline mean change (C_{Bmean})	1 ($N=36$)	Intercept	8.64 (6.05)	2.04	0.15	0.13
		Dose	2.79 (1.42)	3.86	<0.05	
	2 ($N=36$)	Intercept	–10.79 (9.21)	1.37	0.24	0.42
		Dose	26.91 (5.70)	22.28	<0.01	
		Dose ²	–2.72 (0.76)	16.47	<0.01	
	3 ($N=38$ §)	Intercept	–3.86 (7.82)	0.24	0.62	0.35
Dose		13.82 (3.94)	12.32	<0.01		
Dose ²		–1.09 (0.33)	10.98	<0.01		
Baseline maximum change (C_{Bmax})	4 ($N=36$)	Intercept	17.33 (11.22)	2.38	0.12	0.12
		Dose	3.99 (2.55)	2.45	0.12	
	5 ($N=36$)	Intercept	–2.32 (12.89)	0.03	0.85	0.30
		Dose	30.69 (6.72)	20.89	<0.01	
		Dose ²	–3.16 (0.81)	15.30	<0.01	
	6 ($N=38$ §)	Intercept	–2.03 (12.38)	0.03	0.87	0.32
Dose		18.24 (5.50)	10.98	<0.01		
Dose ²		–1.30 (0.42)	9.45	<0.01		

*Sample unit: combination of study \times species \times dose (i.e. experiment).

†Marginal variance explained by the fixed factors.

§Including results from experiments 1 and 2 on white storks.

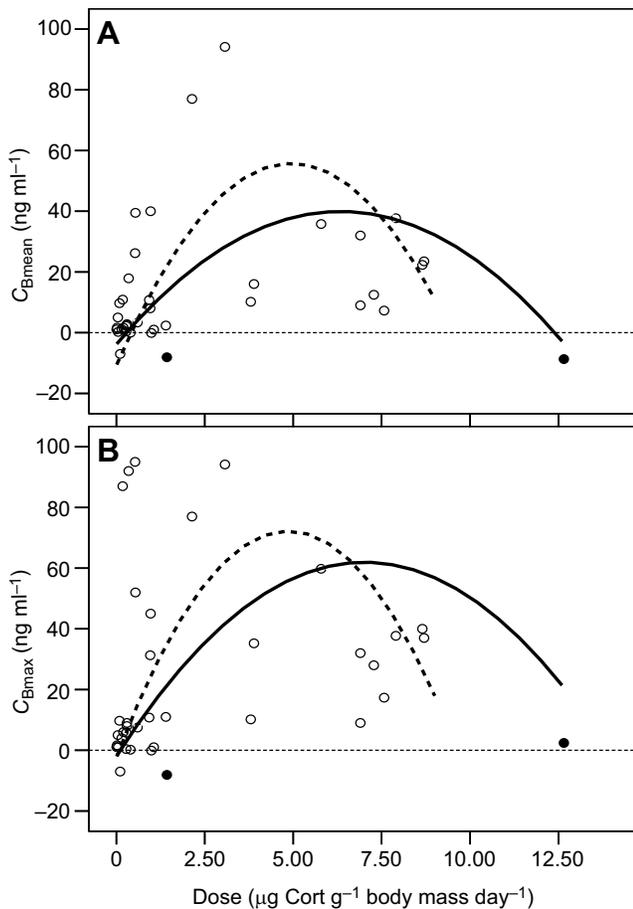


Fig. 3. Dose–response relationships of (exogenous) Cort dose on (endogenous) plasma levels. (A) Baseline mean change (C_{Bmean}) and (B) baseline maximum change (C_{Bmax}) in circulating Cort levels as a function of exogenous Cort dose in birds treated with TRPs. Open circles represent avian experiments performed during 2003–2016 ($N=36$), and solid circles indicate white stork results from experiments 1 and 2. Dashed and solid lines represent quadratic dose–response curves before and after including white stork data, respectively.

this method was anecdotal, it was not included in our analyses. The most frequent method of manipulation was SIL (50%), followed by TRPs with a statistically similar frequency (44%; $X^2_1=0.16$, $P=0.69$), and by OSM with a rather marginal use (6%; Fig. S3A). Along the study period, there was a gradual and significant increase in the relative frequency of use of TRP implants (Pearson's $r=0.64$, $P=0.04$; Fig. S3B) and an opposite but non-significant trend in the frequency of use of SIL (Pearson's $r=-0.52$, $P=0.10$).

From the original studies, we identified 74 Cort-implant experiments. A majority of these experiments only measured post-implant $Cort_{BAS}$ (68.9%), and this frequency was statistically higher than the protocols involving both $Cort_{BAS}$ and $Cort_{STRESS}$ measurements (12.2%, $X^2_1=47.12$, $P<0.01$) or those completely omitting blood sampling (18.9%, $X^2_1=35.55$, $P<0.01$; Fig. 5A). In the experiments in which post-implant $Cort_{BAS}$ was measured ($N=60$), the predominant avian response was an increase in circulating titers (71.7%; Fig. 5B), followed by the lack of effects (26.7%, $X^2_1=22.54$, $P<0.01$) and then by a decrease in plasma levels (at even lower frequencies: 1.7%, $X^2_1=13.43$, $P<0.01$). Only nine experiments measured post-implant $Cort_{STRESS}$, and the predominant avian response was a decrease in circulating levels (77.8%) followed by the lack of effects and the increase in equal

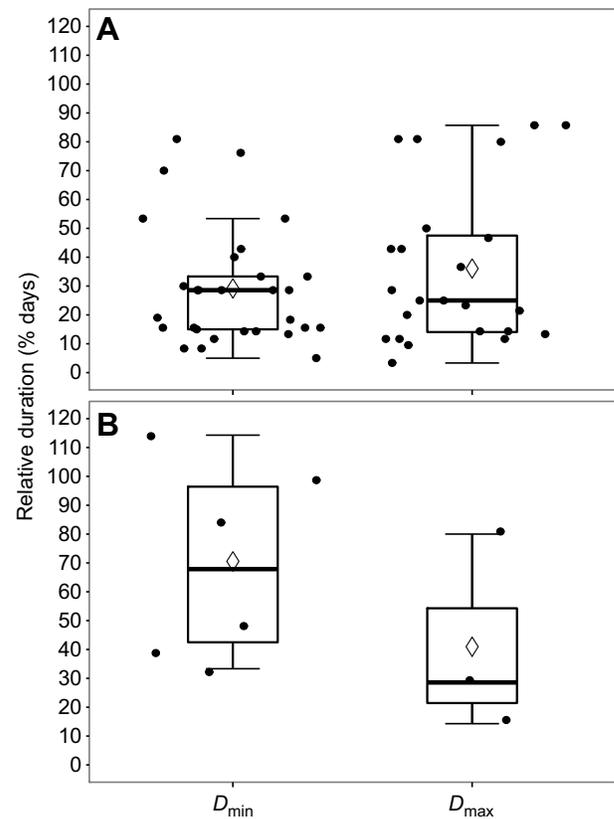


Fig. 4. Duration of implants' effects on plasma Cort levels. Minimum verified duration (D_{min}) and maximum possible duration (D_{max}) of the effects of Cort TRP implants on circulating baseline Cort (A; $N=29$ and $N=24$, respectively) and stress-induced Cort levels (B; $N=6$ and $N=3$, respectively) in birds. The duration was estimated relative to the supposedly effective (theoretical) period specified by the manufacturer and according to the information and sampling regimes available in studies published between 2003 and 2016. Black dots show original data, whiskers show non-outlier ranges, boxes show quartiles (25–75%), black lines show medians and diamonds show arithmetic means.

proportions (11.1%, $X^2_1=5.63$, $P=0.02$; Fig. 5C). The proportion of experiments that reported a decrease in plasma Cort levels was higher for $Cort_{STRESS}$ than for $Cort_{BAS}$ (i.e. 77.8% versus 1.7%; $X^2_1=37.12$, $P<0.01$; Fig. 5B,C). The relative frequency of blood sampling protocols (researchers' patterns of use) and the reported effects on plasma Cort levels (avian responses) were similar when we considered all implant methods together, or when we analyzed separately TRP or SIL implants (all $P>0.05$; Fig. 5).

DISCUSSION

The administration of exogenous Cort through TRP implants in white storks did not increase circulating levels of this hormone, at either $Cort_{BAS}$ or $Cort_{STRESS}$ concentrations. The reported decrease in $Cort_{BAS}$ was statistically significant in experiment 1, and this direction of effects was uncommon in previously published studies. In fact, our qualitative literature review only revealed one previous study reporting decreased $Cort_{BAS}$ in kittiwake gulls, *Rissa tridactyla*, treated with SIL implants (Goutte et al., 2011), which accounted for only 1.7% of all the experimental outcomes (Fig. 5B). However, when we moved beyond qualitative comparisons and performed quantitative dose–response analyses, a lack of elevation in $Cort_{BAS}$ could be expected within the overall pattern of avian responses, because (i) the effects of exogenously administered Cort doses on both C_{Bmean} and C_{Bmax} were better fitted to quadratic than

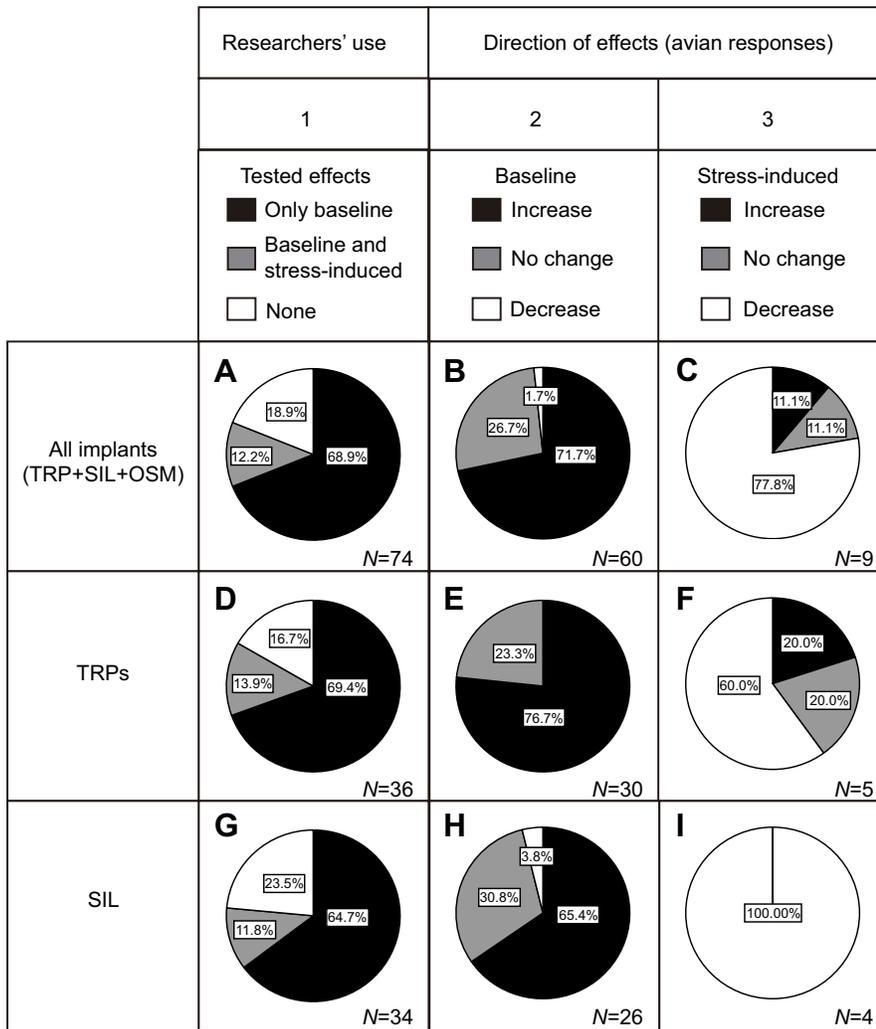


Fig. 5. Blood sampling protocols most frequently used and direction of the effects of Cort implants on circulating avian plasma Cort levels. Researchers' patterns of use in the election of blood sampling protocols following Cort treatment through implants (1), and frequency of responses regarding baseline (2) and stress-induced (3) plasma Cort levels in birds. The first row (A–C) combines all types of Cort implants (SIL: silastic tube; TRP: time-release pellet; OSM: osmotic pump), whereas the second (D–F) and third (G–I) rows show separate results for TRP and SIL implants, respectively. Sample sizes (combinations of study×species×dose) are indicated within boxes, and percentages are indicated within pie sections. Data are from experiments published during 2005–2015.

to linear functions (Table 3), and (ii) our stork experiments were performed using doses of exogenous Cort located near both ends of the dose–response curve (i.e. where the increase in plasma Cort levels is expected to be minimal; Fig. 3). In fact, the incorporation of results from our stork experiments in the dose–response models only produced a small change (positive or negative for C_{Bmax} and C_{Bmean} , respectively) in the amount of variance explained (Table 3). Despite the fact that a quadratic dose–response relationship across bird species may explain the results we obtained from white storks, our experiments lacked an intermediate dose to prove that Cort dynamics follow the same quadratic rule in our model species (i.e. Cort was never found to increase in storks treated with TRP implants in the present study). Alternative explanations such as a problem with the pellets or the way they were implanted, impregnated, etc., or the fact that stork physiology may be somewhat different from the other avian species cannot be excluded. However, we may assume that the pellets were correctly manufactured and implanted because they were purchased in two different years, implanted in two different areas, and we followed the same procedures as other studies using the exact same product and the same recommendations from the manufacturer. From a physiological perspective, decreased plasma Cort levels may suggest that Cort implants caused an over-activation of the negative feedback in the HPA axis of the treated storks, which led to a lower release of endogenous Cort from the adrenal glands (see Dallman et al., 1992). Such negative feedback has also been

suggested to occur in rodents following a constant infusion of Cort (e.g. Akana et al., 1992), and in chickens after punctual intravenous administration of this hormone (which caused upstream effects at the level of the hypothalamus through decreasing mRNA levels of corticotropin-releasing factor precursor; Vandeborne et al., 2005). Cort implants may thus be altering stork adrenocortical function (and as a consequence of this alteration, changing circulating hormone titers). We believe that it is time to expand the prevailing assumption that Cort implants only affect plasma Cort levels, and begin considering that this manipulation likely exerts more complex effects on HPA physiology. Our results highlight the need for additional experiments aimed at testing whether and how negative feedback explains the patterns we found, and we encourage future studies to combine simultaneous treatment of birds with Cort implants and dexamethasone (a synthetic glucocorticoid that competes with circulating Cort for the same receptors, thus inducing negative feedback in the HPA axis).

Regarding the effects of Cort implants on C_{STRESS} levels, our experiments on white storks reduced this parameter by 79% and 63% below those of control birds in experiments 1 and 2, respectively (Figs 1 and 2; Figs S1, S2). This result was consistent with the overall response of birds implanted with exogenous Cort: the reduction of C_{STRESS} levels was the predominant outcome in 78% of the experiments considered in our literature review (and only 11% showed an increased response to stress after implantation; Fig. 5C).

These results indicate that Cort implants generally produce stress-hyporesponsive avian phenotypes.

The combined measurement of both Cort_{BAS} and Cort_{STRESS} in the same study subjects (e.g. through a ‘stress series’) is a routine method and widespread practice in field and laboratory experiments on birds (e.g. Almasi et al., 2015; Blas, 2015; López-Jiménez et al., 2016; Rich and Romero, 2005; Walker et al., 2006). However, the number of studies that followed this protocol after applying Cort implants was small (only 12% of 74 experiments; Fig. 5A). The marked absence of this information could be explained by two alternative hypotheses. On the one hand (hypothesis 1: biased convention), post-implant measurements of plasma Cort levels may be generally pursued only to verify that implants work, and it seems that the accepted convention for an effective Cort implant is that Cort_{BAS} levels increase. Once this conventional rule is verified, there would be no further need to question whether the resulting phenotypes secrete Cort in response to stress, and further discussions about the implant consequences on biological traits are thus assumed to be only related to Cort_{BAS} levels. On the other hand (hypothesis 2: self-censorship bias), we cannot discard the possibility that Cort_{STRESS} levels are actually being measured in some implant experiments, but data are not reported because the prevailing outcome (i.e. a decrease) would be interpreted as a failed experiment.

Regardless of the reasons for this lack of information, currently, most implant studies are neglecting a major physiological mechanism (i.e. the adrenocortical response to stress) involved in the regulation of plasma Cort levels (which paradoxically constitutes the leitmotif of Cort-implant experiments). As a consequence, our current understanding of Cort–trait relationships could be biased and may need to be revisited. As a hypothetical example, we may assume that both Cort_{BAS} and Cort_{STRESS} exert a positive effect on a study trait such as locomotion (e.g. Cort_{BAS} levels naturally increase during fall migration and dispersal; Landys et al., 2006; and Cort_{STRESS} elevations promote flight responses and relocation following disturbance; Wingfield and Romero, 2001). According to our review of published results, we may predict that Cort-implanted birds will show: (i) an increase in locomotion related to higher Cort_{BAS} levels compared with control birds and also (ii) a decrease in locomotion related to lower Cort_{STRESS} levels compared with control birds. The current approach in most Cort-implant studies would only take into account the first prediction. As a consequence, the lack of change in locomotion (one possible experimental outcome) could be interpreted as a lack of Cort effects on phenotypic traits, when in fact these effects may have been cancelled by the opposed impact of implants on Cort_{BAS} and Cort_{STRESS}. Downstream consequences on biological traits could thus cancel each other out (like in this example), but also be diminished or enhanced depending on the links between each adrenocortical parameter (i.e. Cort_{BAS} and Cort_{STRESS} levels) and the specific trait under study. The potential impact of reduced Cort_{STRESS} levels on biological traits may also depend on the specific study question and experimental setup. For example, controlling exposure to stressors may be easier in captive settings compared with field conditions. However, captivity also involves exposure to stimuli (e.g. cage cleaning, food and water provisioning, interactions with conspecifics, novel environmental cues and lack of natural stimuli, capture and sampling) that may be perceived as stressors, eliciting the activation of stress responses and potentially interfering with study traits in ways that may be difficult to control. We believe that it is time to work towards a better understanding of implant effects on adrenocortical function (i.e.

integrating both Cort_{BAS} and Cort_{STRESS} measurements, and testing negative feedback) before inferring downstream links to variation in other biological traits.

Regarding the duration of TRP implant effects, experiment 2 allowed us to distribute repeated blood sampling episodes over 14 days, and thus to show that the effects on the plasma Cort_{STRESS} levels of the storks lasted, at least, for the entire supposedly effective (theoretical) period as specified by the manufacturer (Fig. S2). The intrinsic limitations of experiment 1 (using wild stork nestlings near fledging age) prevented repetition of the blood sampling episodes over a more expanded period, and we are only certain that the effects lasted for a minimum of 7 days. This methodological limitation is common in experiments on wild birds (including those considered in our literature review), and may impose a bias when estimating the effective duration of implants. Nonetheless, with the information currently available, the overall effective duration of TRP implants on avian Cort_{BAS} seems to be limited to the first third of the theoretical period (Fig. 4A). Although it is always advisable to perform pilot studies with repeated blood sampling sessions for each study model and throughout the supposedly effective (theoretical) period, the results of our analysis can be useful as a reference for designing future experiments. Regarding the duration of implant effects on Cort_{STRESS} levels and despite the small sample size, our literature review suggests that the effects last longer than on Cort_{BAS} (but still 40–70% of the supposedly effective period; Fig. 4B).

There are two complementary (but not mutually exclusive) hypotheses to explain the short duration of TRP implants in birds (based on Müller et al., 2009). On the one hand (hypothesis 1: metabolism-induced fast matrix depletion), it is possible that the effects of the implant will last only until the implant matrix is completely absorbed by the organism, with the species-specific metabolic rate determining the speed of absorption (Müller et al., 2009). As birds have a higher metabolic rate than mammals (Costantini, 2008), the absorption of the implant (which was originally designed considering metabolic rates of rodents, not birds) would end before the supposedly effective period. However, this hypothesis alone would not explain why the duration of implant effects is longer for Cort_{STRESS} than for Cort_{BAS} (nor the decrease in stress response, which is the predominant result in birds). On the other hand (hypothesis 2: short-term negative feedback adjustment period), it is possible that Cort_{BAS} elevations are only effective during a brief initial period, reflecting the re-adjustment time of the negative feedback in the HPA axis (i.e. the time required to subsequently achieve a state of lower production of endogenous Cort, aimed at compensating the exogenous input). Once this negative feedback is readjusted by the individual, Cort_{BAS} levels would remain around pre-implant titers even though the implant matrix is still active. In this case, decreased Cort_{STRESS} levels could last for as long as the negative feedback remains active (i.e. the stress response would be downregulated until the whole implant matrix is absorbed, and despite Cort_{BAS} levels having already returned to pre-implant titers). Additional experimental manipulations including dexamethasone treatment would be necessary to further understand the potential effects of implants on negative feedback function.

Note that the implications of the two hypotheses above are quite different in terms of interpreting research results. If matrix depletion (hypothesis 1) is the primary mechanism, then the result will be a large bolus of Cort at the beginning of the experiment that is then cleared prior to the completion of the experimental period. The downstream effects of exogenous Cort addition are then removed earlier than intended. In contrast, if the primary mechanism is feedback adjustment (hypothesis 2), then exogenous Cort continues

to circulate in the blood, continues to interact with receptors and thus continues to exert physiological effects. Because Cort functions as a transcription regulator, even if the titers have decreased, the effects of Cort likely last much longer – probably until the peptides whose production is stimulated by Cort also degrade. The end result will be continued downstream effects of Cort even though the measured Cort titers suggest that the hormone's physiological impacts have ended (Romero and Wingfield, 2016). It is currently unknown which hypothesis is the dominant mechanism explaining measured Cort titers (and is likely a combination because the hypotheses are not mutually exclusive), but resolution of this problem will be very important for proper interpretation of research results using implants.

Our literature review also allowed us to assess researchers' use of currently available Cort implants, and revealed a gradual temporal increase in the use of TRP implants (Fig. S3). Such an increase may be due to the following reasons: (i) TRPs are commercially available, and thus their use is a more standardized method (SIL are manually manufactured, and doses are likely subjected to higher variability within and between studies); (ii) TRPs provide a more controlled release of exogenous hormone compared with SIL (Fusani, 2008; Quispe et al., 2015), whose polymer membranes prevent Cort diffusion (Kincl et al., 1968) and force researchers to manually puncture holes in the tube or even cut the ends of it (thereby increasing variability among studies); (iii) TRPs have a pre-defined theoretical period of duration specified by the manufacturer, which may facilitate experimental design (but see our considerations above) and facilitate comparisons among studies; and (iv) TRPs are self-degrading, avoiding the need to re-capture study subjects to remove implants at the end of the experiment (which may be difficult in the wild). However, tight research budgets might limit a wider use of TRP implants, as these are substantially more expensive than SIL.

In conclusion, our results challenge and expand the prevailing assumption that Cort implants increase circulating Cort_{BAS} levels in birds because: (i) Cort_{BAS} levels show a quadratic association with implant dose, and negative values (i.e. decreased plasma levels) may occur at both high and low implant doses, and (ii) Cort implants also decrease Cort_{STRESS} levels, thus producing stress-hyporesponsive avian phenotypes. There is an urgent need to refocus research to understand Cort-implant effects on adrenocortical function, before inferring downstream links to variation in other biological traits. Furthermore, a number of gaps in our knowledge justify the following recommendations for future research: (1) incorporate stress series in the blood sampling protocols (to assess the consequences of Cort implants on both Cort_{BAS} and Cort_{STRESS}); (2) incorporate dexamethasone treatments into Cort-implant studies (with the aim of testing whether negative feedback explains implant-induced hyporesponsiveness in the HPA axis); (3) improve the resolution and duration of blood sampling protocols to obtain more accurate estimates of the effective duration of implants and associated responses (repeated sampling within and beyond the theoretically expected duration of implants is strongly recommended); (4) consider the potential effects of additional factors (e.g. life stage, experimental conditions, sex, time of year, time lag between implantation and plasma sampling, etc.) on dose–response relationships (assessing their relative contribution fell beyond the scope of our study, but we encourage further research in this area); and finally, (5) increase the number and taxonomic range of study species to ensure that the emerging patterns are truly representative of the Class Aves.

Acknowledgements

We thank S. Palacios-Ojeda, J. Bejarano, M. Ardila, J. Ayala, A. Jurado, M. Vazquez and volunteers from Estación Biológica de Doñana for help during fieldwork, and three anonymous reviewers for their comments and suggestions that allowed us to improve previous manuscripts. P. Rodríguez and M. Adrián (Cañada de los Pájaros) and M. Ardila (Ayuntamiento de Puebla del Río) allowed access to birds. The Consejería de Medio Ambiente (Junta de Andalucía) and the Agencia de Medio Ambiente y Agua de Andalucía provided housing facilities and permits to conduct the experiments.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: F.T.-M., S.C., T.A.M., M.W., L.M.R., M.H., M.C., J.L.T., J.B.; Methodology: F.T.-M., S.C., T.A.M., M.W., L.M.R., M.H., M.C., J.L.T., J.B.; Validation: F.T.-M., S.C., T.A.M., J.B.; Formal analysis: F.T.-M., J.B.; Investigation: F.T.-M., S.C., M.C., J.L.T., J.B.; Resources: T.A.M., M.W., L.M.R., M.H., J.B.; Data curation: F.T.-M., S.C., J.B.; Writing - original draft: F.T.-M., S.C., J.B.; Writing - review & editing: F.T.-M., S.C., T.A.M., M.W., L.M.R., M.H., M.C., J.L.T., J.B.; Visualization: F.T.-M., J.B.; Supervision: J.B.; Project administration: J.B.; Funding acquisition: T.A.M., J.B.

Funding

This work was supported by the University of Saskatchewan (Isabel Maria Lopez Martinez Memorial Scholarship to F.T.-M.), the Spanish Council of Science (Consejo Superior de Investigaciones Científicas; Ramon y Cajal contract 2010-07120 and i-link project 0564 to J.B.), the Spanish Ministry of Economy and Competitiveness and European funds (Ministerio de Economía y Competitividad; project CGL2012-32544 to J.B.) and the Spanish Ministry of Agriculture, Food and the Environment (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente; Organismo Autónomo de Parques Nacionales, grant 511/2012 to J.B.).

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.173864.supplemental>

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