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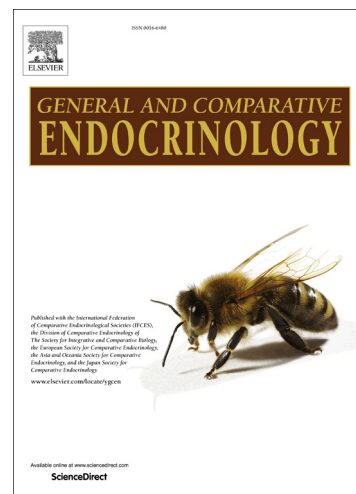
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**State of the art knowledge in adrenocortical and behavioral responses  
to environmental challenges in a threatened South American ratite:  
Implications to *in situ* and *ex-situ* conservation**

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## ABSTRACT

The Greater Rhea (*Rhea americana*) is an endemic ratite to South America, whose wild populations have undergone a remarkable decrease due to habitat degradation and fragmentation by the expansion of the agricultural frontier, poaching and predation by dogs. Anthropogenic perturbations in wild environments, as well as the management in captivity, can generate different stress responses in this species, thus, the monitoring of adrenocortical and behavioral activities are considered primary assessment tools with both conservation and welfare implications. In this review we analyze and integrate the different measurements of glucocorticoids (in plasma, feces, and yolk) carried out in different captive and wild populations, taking into account the diverse predictable and unpredictable conditions to which the Greater Rhea responds in each of those environments. In addition, the translocation of this bird is presented as an application of stress physiology in field ecology for conservation purposes, in which we evaluated how this species responds when it is released into a novel environment. Our results indicate that this ratite has a striking high sensitivity of the hypothalamic-pituitary-adrenal (HPA) axis compared to that of other bird species and shows a wide variety of adrenocortical responses depending on the environment in which it lives. This suggests that its HPA axis has a phenotypic plasticity that enables the rhea to cope with the environmental challenges. In this sense, we propose that one of the routes of this plasticity could be mediated by the maternal transfer of steroid hormones to the egg. Finally, we discuss the importance of integrating the monitoring of the adrenocortical response along with the environmental variables that define the life history of the species, in management and conservation programs *ex-situ* and *in situ*.

**Keywords:**

Conservation physiology, *Rhea americana*, stress, welfare, wildlife management

**1. Introduction****1. 1. The species**

The Greater Rhea (*Rhea americana*) is the largest South American avian species, and as a flightless bird it is included in the Ratite group or runners (Blake, 1977; Del Hoyo *et al.*, 1992). Rheas are omnivorous, although fundamentally herbivorous (Martella *et al.*, 1996; Pereira *et al.*, 2003). They are gregarious and during the autumn and winter (non-breeding season) form large flocks of 20 to 70 individuals of both sexes and different ages (Bruning, 1974). These groups disaggregate at the beginning of the reproductive season due to an intense sexual competition among males for the access to females. Their reproductive season comprises August to January, when they form reproductive groups consisting of one or two males and a variable number of females (Bruning, 1974). Their mating system is unusual among birds, as it combines simultaneous polygyny and simultaneous and serial polyandry (Handford and Mares, 1985), with a high degree of promiscuity (Martella *et al.*, 2014).

Agricultural practices have drastically fragmented and reduced the suitable habitat for the Greater Rhea (Giordano *et al.*, 2008; Martella and Navarro, 2006), leading to local extinctions (Bazzano *et al.*, 2014; Giordano *et al.*, 2010). Other factors that negatively affect their population viability have been poaching of individuals and eggs, predation by dogs and harassment by rural people (Novaro *et al.*, 2000; Rivas *et*

*al.*, 2015). As a result, the Greater Rhea has been categorized as near threatened (IUCN, 2017) and included in Appendix II of CITES (CITES, 2017).

The Greater Rhea is also reared under captive conditions, and this fact has allowed the development of *ex-situ* conservation studies and has served as a source of individuals for the reintroduction to the wild. Captive rearing has been mainly conducted under two systems (Navarro and Martella, 2011): (a) intensive, in pens < 1/4 ha or less with bare ground, where each reproductive group usually consists of several males and up to 6 females, which are daily provided with food (balanced feed ration and bale or finely fresh chopped green material), and (b) semi-extensive, on larger paddocks (1 ha or more) with natural or implanted pasture, usually atriplex (*Atriplex* sp.) or alfalfa (*Medicago sativa*), where up to 3 or 4 reproductive groups are usually kept (depending on the quality of the forage), grazing on a rotational basis and to which a ration of pelleted food can be provided as a supplement. All cases use artificial incubation and intensive breeding of the chicks (precocial) in pens with indoor areas, sometimes combined to some extent with the incubation and natural breeding in charge of a male.

## 1. 2. Ecological studies

To design appropriate management and conservation strategies, initially it was necessary to assess the situation of wild populations of the Greater Rhea, and to gather information on the evolution of population size, the use of habitat, the components of the diet throughout the year in each site, etc. Later, there also arose the need to carry out studies under captive conditions, both in experimental and in commercial farms. This is why these populations, in addition to having economic importance, are also relevant components in the global conservation scenario, as they become demographic and

genetic reservoirs and, in many cases, the only situation in which it is possible to obtain basic information about the species.

Ecological studies based on the science of conservation biology were combined and complemented, focusing on aspects pertinent to *in situ* conservation (Bellis *et al.*, 2004a, b; Bellis *et al.*, 2008) and *ex-situ* conservation (Navarro and Martella, 2008; 2011). Approaching the study of the populations from both perspectives has allowed the development of scientific studies on the characteristics of the species, the individual and population variations, and their response to different environmental conditions (Navarro and Martella, 2011). On the other hand, specific conservation actions have been addressed, reinforcing or repopulating areas where these species had suffered severe reductions or had disappeared, through the translocation of individuals produced in captivity (Bellis *et al.*, 2004a; 2004b; Vera Cortez *et al.*, 2015). In addition, data were obtained on various aspects of the ecology of wild populations, which make it possible to evaluate and forecast the outcome of the possible actions (Bazzano *et al.*, 2014; Giordano *et al.*, 2010). Since both anthropogenic disturbances in wildlife and captive management for productive or conservation purposes can induce stress responses in the rhea, it was considered crucial to gain knowledge about rheas' physiological stress responses, and the causal links between processes of environmental change induced by humans, and the influences associated with captive breeding. This information is highly relevant to improve both the conservation and welfare status of this threatened species.

### **1. 3. Hormonal studies**

Initially, our research on the adrenocortical response of Greater Rhea encompassed, as a theoretical framework, the classic concepts of eustress and distress

(Breazile, 1987). However, within the concept of eustress, it is not possible to differentiate the adrenocortical and behavioral responses to predictable environmental changes (e.g. circadian and seasonal), from those that are unpredictable (e.g. sudden onset of a predator). Therefore, our studies were framed within the "Reactive Scope Model" proposed by Romero *et al.* (2009), which combines the classical concepts of homeostasis and allostasis, and contextualizes how individuals respond to predictable and unpredictable environmental changes. In this model, different magnitude ranges are proposed for glucocorticoid (GC) levels. In the "predictive homeostasis range", baseline GC levels oscillate in response to predictable environmental changes that generate physiological demands, by which the individual can anticipate and be prepared to face as part of their normal life cycle. Above this threshold is the "reactive homeostasis range" that represents GC concentrations necessary to sustain homeostasis following an unpredictable event (disturbance or stressor) that is threatening. These facultative elevations of GC levels are also called stress responses and produce rapid physiological and behavioral changes, characteristic of the state of emergency (Wingfield *et al.*, 1998), which allow the individual to cope with the stressor. Once the perturbation disappears, GC levels return back to the predictive or baseline range, and the animal returns to its normal life cycle. However, if the stressor does not disappear and the animal does not habituate to it, the prolonged maintenance of high GC levels could lead to a state of homeostatic overload, generating deleterious effects in the individual (Romero *et al.*, 2009).

The hypothalamic-pituitary-adrenal (HPA) axis of the rhea should present a certain degree of phenotypic plasticity, in order to allow it to respond to the predictable and unpredictable conditions of the different wildlife and captivity environments in which it currently lives and reproduces. One of the mechanisms that could permit this

plasticity, is the maternal transfer of hormones to the egg. In birds, it has been proposed that through the deposition of corticosterone and other steroid hormones in the yolk, the female could influence the adrenocortical and behavioral responses of the chick, in order to allow it to deal more effectively with the conditions of the environment in which it will live (Love *et al.*, 2013). These changes would occur in an epigenetic way, that is, the genetic composition of the offspring may be the same, but the expression of different genes in response to environmental conditions may generate different phenotypes. These changes in the phenotype of the offspring due to changes in the phenotype of the female, or the environment in which she lives, are known as "maternal effect" (Mousseau and Fox, 1998).

This review presents the state of the art knowledge of the adrenocortical and behavioral response of the Greater Rhea, under different environmental conditions in wildlife and captivity, within the framework of an *ex-situ* and *in situ* management and conservation project of this species. First, we present the techniques for measuring GCs that were validated for the quantification of corticosterone in plasma and egg yolk, and glucocorticoid metabolites in feces (FGM). Then, we analyze and integrate the different measurements of GCs, carried out in different captive and wild populations, taking into account the diverse predictable and unpredictable conditions the rheas cope with, in each of those environments. Next, translocation is presented as an application of stress physiology in field ecology, evaluating the response of the rhea when facing new environmental challenges. Finally, we propose new research guidelines to further integrate the measurements of stress in conservation and animal welfare programs in general, and future perspectives in this topic that will help to understand the role of GCs in the life history of this avian species.



## 2. Techniques for quantifying glucocorticoids

### 2.1. Corticosterone in plasma and yolk

The quantification of GC levels in the blood is used as a conservation physiology tool to monitor how individuals respond to predictable and unpredictable environmental changes. However, capture and manipulation for blood sampling may be perceived as unpredictable events that trigger a rapid increase in GC levels, so the blood sample should be taken immediately after the capture and immobilization of the animal (within 2-3 min post-capture), in order to avoid an increase in GC levels due to the manipulation and restraint of the individual. In the birds studied so far, the main GC secreted by the adrenal glands is corticosterone (Romero, 2004), while in mammals it has been found that some groups secrete cortisol and other groups corticosterone, so it was essential to determine which was the main hormone released by the rhea. While corticosterone had been determined in the plasma of other ratites such as the Ostrich (*Struthio camelus*) (Mitchell *et al.*, 1996), the Emu (*Dromaius novaehollandiae*) (Blache *et al.*, 2001), and the Common Kiwi (*Apteryx australis*) (Cockrem *et al.*, 2004), GCs had not yet been quantified in rheas. In this way, the first results of our line of research allowed us to prove that the main GC released by the adrenal glands of the Greater Rhea is corticosterone. For its correct determination in plasma, the commercial radioimmunoassay (RIA) of Corticosterone-I125 (MP Biomedicals, Costa Mesa, California, USA) was validated (Lèche *et al.*, 2009). These validation studies were possible due to working with rheas in captivity, where animals were sexed and identified and were accustomed to permanent contact with humans, which facilitated

their manipulation for the capture and sampling of blood, something that is very difficult to accomplish with this species in the wild.

Since commercial RIA of Corticosterone-I125 (MP Biomedicals, Costa Mesa, California, USA) allowed us to quantify corticosterone in the plasma of the rhea, we validated it to measure yolk corticosterone in the egg of this species (Della Costa *et al.*, 2016), initiating a new line of research, which addresses the study of the maternal effects. As in the previous research, to carry out this validation we worked with captive individuals, which facilitated the daily collection of fresh eggs that were not incubated, to prevent the metabolism of the hormone by the developing embryo (Groothuis and von Engelhardt, 2005). Because the rhea is a threatened species, being able to have populations that live and reproduce in captivity allowed us to obtain eggs from them, avoiding and/or reducing collection from nests in the wild.

## **2.2. Glucocorticoid metabolites in feces**

Measurements of GCs in plasma are very useful in the field of conservation biology. However, taking blood samples is complicated in wild animals, because they are often difficult to capture and immobilize. In addition, this invasive method usually triggers an acute stress response that could even put the individual's life at risk, which can be a problem in endangered species that are very sensitive to stress, such as the rhea. In these cases, hormonal measurements in excreta are a very useful alternative, because they have the advantage that the samples can be easily obtained without disturbing the animals under study, thus avoiding an increase in the release of GCs induced by capture and handling. Another advantage of this procedure is that the collection can be extended for long periods, without affecting neither the behavior nor

the endocrine state of the animal. However, prior to its use, this non-invasive technique must be correctly validated for each species (Millspaugh and Washburn, 2004), since the excreta contains multiple GC metabolites, and little or nothing of the hormone in its native state (Palme *et al.*, 2005). The commercial RIA of Corticosterone-I125 (MP Biomedicals, Costa Mesa, California, USA) was then validated, to be able to efficiently monitor FGM levels in the rhea (Lèche *et al.*, 2011). As in the previous studies (section 2.1.), to carry out this validation, we worked with captive individuals, which facilitated the development and adjustment of the technique.

### **3. Adrenocortical and behavioral responses in captive environments**

#### **3.1. Baseline levels**

##### **3.1.1. Seasonal variation of fecal glucocorticoid metabolites**

The seasonal adrenocortical pattern of the rhea differs between sexes in captive populations raised under the intensive system (Lèche *et al.*, 2014a). In the females, FGM levels are constant throughout the seasons, while in males they increase during the breeding season (Fig. 1). These hormonal levels appear within the predictive range proposed in the Reactive Scope Model (Romero *et al.*, 2009): while in females this range is stable throughout the year, in males it oscillates over the different seasons, in response to predictable changes in the environment (e.g. breeding cycle). The higher FGM levels of the males during the breeding season could be due to the males trying to mate with as many females as possible, whereby there is strong competition among them (Bruning, 1974). During this period, plasma testosterone levels also increase

(Valdéz *et al.*, 2014), which is consistent with the observed frequent fights between males. In addition to this, the courtship, nest construction and/or incubation may explain the increase in FGM levels of males during the reproductive season, since it is necessary to mobilize energy resources. However, it is likely that these hormonal profiles are not reflected in the same way in wild environments, where larger spaces allow decompression of the social conflicts among males. The hormonal ranges reported by Lèche *et al.* (2014a) were determined in individuals from an intensive system, where environmental conditions would be more stable than in the wild. For example, in the intensive system, because the food is supplied exclusively in an artificial way, there is no seasonality in its availability. In contrast in an agroecosystem where, as we will see later, Lèche *et al.* (2014b) observed that individuals increase their FGM levels during the dry season, when food is scarce after harvest. This difference in food availability also occurs in environments with natural pastures, where its abundance and/or composition could change according to seasonal temperatures and rainfall. The confinement in pens of comparatively reduced space affects the formation and separation of more than one reproductive group, so in general in the intensive system one nest is established per pen, preventing females from copulating with males from other reproductive groups, and from laying their eggs in several nests as it occurs in the wild (Martella *et al.*, 2014). Then, females in an intensive system may not require elevating their FGM levels during the reproductive season, unlike what might happen in females that live in wild environments. Taking into account the great variability that has been reported in the adrenocortical activity of individuals from different populations of the same species (Wingfield and Romero, 2001), it could be expected that the hormonal patterns in Greater Rhea females and males will also vary between different environments.

### 3.1.2. Maternal transfer of corticosterone to the egg

Recently, it was found that environmental conditions influence the transfer of maternal corticosterone into the Greater Rhea eggs: females bred under captive conditions in an intensive system deposit higher yolk levels of this hormone than those of the semi-extensive system (Della Costa *et al.*, 2016). Moreover, it was found that the chick stress responses were associated with their embryonic exposure to maternal corticosterone (Della Costa *et al.*, 2015). When the offspring of females from the semi-extensive system were exposed to an unpredictable perturbation (isolation and restriction of movements) they increased their FGM levels, their time of ambulation, and reduced the time spent pecking in comparison with their control counterparts. In contrast, the offspring of females from the intensive system showed neither hormonal nor behavioral changes in response to the mentioned perturbation (Della Costa *et al.*, 2015). This finding suggests that maternal transfer of corticosterone to the egg could allow females to adjust the physiological and behavioral responses of their offspring according to the prevailing environmental conditions relevant for their survival. Less reactive chicks should have a better chance of survival in the intensive system rearing conditions, where they are exposed to a comparatively reduced number of stimuli (plain environment) and to forced proximity with humans and partners in a highly restricted space environment and without the possibility of getting away from them. In contrast, a more reactive chick should have a better chance of surviving in a challenging and stimulating environment such as the semi-extensive system, where the environmental conditions resemble those of wildlife, allowing the development of a greater number of behaviors (and behavioral strategies) for the species. In the semi-extensive system, the

availability of more space for ambulation and the existence of natural pastures for foraging would also increase the probability that the chick will encounter new stimuli and even possible "predators or threats". All those situations would require rapid adrenocortical and behavioral responses to allow the birds to quickly adjust and cope with stressors and challenges. In this environment, and unlike what happens in the intensive system, the availability of greater space would also allow the chicks to escape from the threat, and the presence of the adult male would allow them to seek shelter and protection.

### 3.2. Stress response levels

Lèche *et al.* (2009 and 2011) evaluated the adrenocortical response of rheas after exogenous administration of ACTH. Interestingly, both plasma corticosterone and FGM concentration after challenge were found to be around 40 times higher than the basal measurements (Fig. 1). Moreover, this exacerbated adrenocortical response was also observed in rheas that were individually transported during 30 min on daylight hours, experiencing a similar increase of about 40 times in their plasma corticosterone levels (Lèche *et al.*, 2013) and a 10 times increase in their FGM levels (Fig. 1) (Lèche, 2012). There is also evidence of a response of similar magnitude in another ratite, the Common Kiwi after a 30-min capture and immobilization protocol (Cockrem *et al.*, 2004). This is very striking when compared with the lower increases reported in other non-ratite birds studied so far (Table 1). However, in the Ostrich, it was observed that plasma corticosterone concentrations exhibited only a two-fold increase after a transportation during 4.5 h (Mitchel *et al.*, 1996), and no increase after a 18-h transportation when compared to the basal measurements (Bejaei and Cheng, 2014). Nevertheless, these

relative lower increases reported in the Ostrich can be attributed to the fact that those measurements may reflect sampling points towards the recovery of pretreatment corticosterone plasma levels, rather than the measurements of peak responses (Lèche *et al.*, 2013). The high sensitivity of the HPA axis reported in rheas and kiwis has been proposed as part of a stress response strategy of these large sized flightless birds to support an antipredator strategy that consists of a fast and prolonged race in environments where there is no place to hide or get away from predators (Lèche, 2012). In this way, the glycogenolytic action of corticosterone by which glucose is released into the bloodstream allowing its uptake by the muscles, would support an intense and frequently prolonged physical activity while escaping.

The behaviors of rheas were also altered during the first 48 h after transportation: females spent less time preening and resting and increased the time allocated to vigilance and ambulation, while males spent more time eating and drinking. At 48 h post transport, FGM levels and all behaviors, except preening in the females, returned to their baseline levels (Della Costa *et al.*, 2013). These results suggest that females and males adopt different behavioral strategies to cope with a perturbation in the environment and that females require more time to recover their behavioral dynamics after a stressful situation.

#### **4. Adrenocortical responses in two wildlife environments: agroecosystem and grassland**

Wild populations of the Greater Rhea in central Argentina have been adversely affected by the great advance of the agricultural frontier occurred in recent years, where human activities (deforestation, fragmentation, hunting, intensive agriculture) are the

main cause of the reduction of their populations (Martella and Navarro, 2006; Navarro and Martella, 2008). In this context, Lèche *et al.* (2014b) determined the impact of human activities and changes in land-use on wild populations of this species, through the analysis of the variation over two years in the levels of FGM of the individuals, in two environments with different degree of conservation and anthropic disturbance. On the one hand, the FGM levels were determined in rheas of a semi-natural grassland dedicated mainly to raising livestock, and organized in large parcels or paddocks of 300 ha each, in which there were mixed forests, natural or implanted pastures (75%), alfalfa (13%) and a lower proportion (10%) of corn, soybean and sunflower crops. On the other hand, FGM levels were quantified in rheas of an agroecosystem with extensive crops of corn and soybeans. In this study Lèche *et al.* (2014b) showed that FGM levels differed between rheas of the agroecosystem and the grassland, observing a greater oscillation over time in the first one (Fig. 2). There the greatest increases of FGM levels were registered in the dry season of the year (winter), which coincides with the post-harvest. After the harvest that usually begins at the end of March and ends in May, the fields are practically without vegetation. Consequently, the lack of food resulting from human activities (intensive agriculture) added to the low temperatures of the season (autumn-winter), where energy requirements are higher, could explain the higher FGM levels observed during those months. An alternative explanation could be that a low water feed-consumption can result in slower fecal transit time and therefore in higher FGM levels (Goyman 2012). Nevertheless, the chances of this occurring in the agroecosystem are low, taking into account that the Greater Rhea is an herbivorous species that consumes green items all year round (Martella *et al.*, 1996). Furthermore, an increase in the FGC levels has not been detected in the grassland during winter, when vegetation is dryer (see below). If the food shortage prolonged over time is the cause of a state of



homeostatic overload, where there is maintenance of high GC levels, it could, in turn, generate deleterious effects in individuals. In this way, habitat degradation generated by the expansion of the agricultural frontier would jeopardize the survival and reproduction of the rhea in the agroecosystem. However, it is also possible that in the face of prolonged exposure to anthropogenic disturbances, the rheas in the agroecosystem have become habituated to these conditions. If so, the peak FGM levels observed during the dry season could be within the predictive or baseline range for the individuals of that population, which might enable them, for example, to increase their ambulation for the search for food. In this regard, Bazzano (2010) provides evidence that would support this idea of adaptation to prevailing conditions, when she found that the same population did not decrease ( $\lambda = 1$ ) and that the number of nests, the clutch size, the number of males with chicks, and the number of chicks per male in that agroecosystem did not differ from those of a grassland population.

In the grassland, rheas had a much more constant FGM pattern than in the agroecosystem (Fig. 2) (Lèche *et al.*, 2014b). The increases in the levels of FGM occurred in the rainy season (spring-summer), coinciding with the reproductive period. Because the individuals in the grassland and agroecosystem were not identified or sexed, it was not possible to determine the adrenocortical patterns of rhea females and males separately, as was done in the captive environment of the intensive system (section 3.1.1). Although the peak observed in FGM levels of the grassland individuals during the reproductive period could be due to the increase in the hormonal levels of the males, as observed in the intensive system, we cannot rule out that in the grassland females, as well as in those of the agroecosystem, FGM levels have been increased. Because the grassland rheas would be exposed to environmental conditions that are predictable for their life history (animals live and reproduce in that environment) FGM

levels quantified by Lèche *et al.* (2014b) probably oscillate within the predictive range for that population.

## **5. Adrenocortical responses to an environmental challenge: the translocation**

Several studies (Bazzano *et al.*, 2014; Giordano *et al.*, 2008; 2010; Navarro and Martella, 2011) indicated that populations of Greater Rhea were declining and that there was an urgent need to carry out management practices to prevent local or regional extinctions. With this objective in mind, a very popular conservation strategy was used (Osborne and Seddon, 2012), as is the translocation, which involves the release of animals into a novel environment for conservation purposes. As Dickens *et al.* (2010) summarized, stress is an inevitable component of translocations, so our studies involved the assessment of adrenocortical stress responses in Greater Rheas during the different phases of translocation.

Considering that transport is always involved in translocation programs, we evaluated the effect of transport in male and female groups of rheas during a translocation study at night. The study shows that animals recovered their baseline FGM levels in a shorter period (16 h, Lèche *et al.*, 2016) than those transported individually during the day (30 h, Lèche, 2012). These results should be considered when carrying out rhea transfers.

Two translocations of adult individuals from captivity to an agroecosystem were carried out. In the first (Lèche *et al.*, 2016), rheas came from an intensive system, while in the second (Navarro *et al.*, submitted), they came from a semi-extensive system. In both cases, we observed that individuals increased their FGM levels when they were released in the new environment, and this increase was maintained during the 60 days

that lasted the hormonal monitoring (Table 2). The release into a novel environment meant that the rheas were exposed to multiple factors, such as unknown sites to be explored, different habitats, pastures, crops, contact with animals of other species (including livestock and wild and human predators) and conspecifics, the establishment of new interactions, the presence of rural workers and the existence of diverse usual anthropic activities that include the movement of agricultural machinery and other vehicles (Lèche *et al.*, 2016; Navarro *et al.*, submitted). All of them can be considered as unpredictable changes that the animals had to cope with, which could have generated an increase in their FGM levels and that these could be maintained over time (Lèche *et al.*, 2016; Navarro *et al.*, submitted). Studies such as those carried out by Franceschini *et al.* (2008) and Jachowski *et al.* (2012; 2013) show that a period of acclimatization in the area of release is necessary to allow the animals to recover their GC levels. However, how much the values of post-release GCs should descend? Should they achieve the pre-release levels, or set into a new higher value within this range, according to the new environment in which these animals live? Although the FGM levels reached by the rheas coming from the intensive system during the 60 days post-release were higher than the pre-release levels, these were lower than those reported after the exposure to different stressors (Lèche, 2012, Fig. 1), so it is probable that FGM would not be within the range of homeostatic overload. These hormonal levels would not give rise to deleterious effects on individuals but would enable them to respond adequately to the conditions of the new environment. It is likely that this bird species has some phenotypic plasticity in the HPA axis, which allows it to adapt to the conditions of its environment. Evidence in this direction was observed in captive rheas that having been subjected to different stressors (handling, transport, novelty, and isolation), triggered a physiological and behavioral stress response and when the

stressors disappeared, the animals recovered their basal levels, or if the stressor remained, they habituated to it (Della Costa *et al.*, 2013; Lèche, 2012).

When rheas were released in the novel environment, a difference was also observed in the FGM levels associated with the origin of the animals. Individuals from the semi-extensive system showed higher FGM levels than individuals from the intensive system (Lèche *et al.*, 2016; Navarro *et al.*, submitted). This variation in post-release adrenocortical response according to provenance was also observed in the Mantled Howler Monkey (*Alouatta palliata*) and in Grevy's Zebra (*Equus grevyi*) (Aguilar-Cucurachi *et al.*, 2010; Franceschini *et al.*, 2008). In the Greater Rhea, the different adrenocortical response between individuals of different origins could be associated with the levels of maternal corticosterone to which they were exposed during embryonic development. As mentioned in section 3.1.2, female rheas of the semi-extensive system transfer lower corticosterone levels to their eggs and produce more reactive chicks than females of the intensive system. Accordingly, when planning future translocations, the numerous factors that translocated individuals will face in the new environment should be considered, since those rheas that show higher GC levels would be more reactive and have a better chance of escaping from a predator or threat. Consequently, it would be advisable to translocate individuals from the semi-extensive system to the wild, instead of from the intensive system.

## 6. Conclusions

The results obtained with this new line of research in the Greater Rhea showed for the first time relevant and particular aspects about the physiology of stress in this species and have allowed the design of *in situ* and *ex-situ* management strategies that

promote the welfare of the rhea in farms and other captive environments, and conservation in wild areas.

The studies of rhea populations in the intensive system made it possible to determine the baseline adrenocortical patterns of females and males, which can be used as a diagnostic tool to establish whether a level of GCs is within the predictive range or if it is a stress response. Having these populations in captivity has also allowed experiments at different levels, with eggs, chicks, and adults. Thus, it was possible to get to use a RIA currently validated to quantify GCs in different matrices, which represented a crucial step in research on field endocrinology for rhea species. As a result, studies with the captive population allowed us to determine that there is a high sensitivity of the HPA axis of the rhea compared with that of other bird species. This constitutes a warning sign to be taken into account when designing farms and for conservation in the wild. By controlling the unpredictable stimuli to which the animals are exposed, they could be prevented from triggering a chronic release of GCs within the range of homeostatic overload.

In wildlife, rheas showed particular hormonal ranges for the agroecosystem and the grassland. This would highlight the importance of carrying out studies in different environments and demystify the idea that wildlife values can be benchmarks for assessing the welfare of this species.

The translocation showed that rheas can respond to a new environment by raising their GC levels within a range that would not be harmful. The novelty of the physiological, behavioral and management point of view is that this response could be conditioned by the embryonic exposure to maternal corticosterone. Therefore, any management strategy should consider this possibility.

In short, the various adrenocortical responses observed in the rheas from different populations would indicate that the HPA axis of this species has a certain phenotypic plasticity, which would allow it to cope with, and adapt to environmental challenges. In this sense, probably one of the routes of the maternal effect is through epigenetic modifications that result from the maternal transfer of steroid hormones to the egg. Future studies should integrate the monitoring of the adrenocortical response along with the environmental variables that define the life history of the species.

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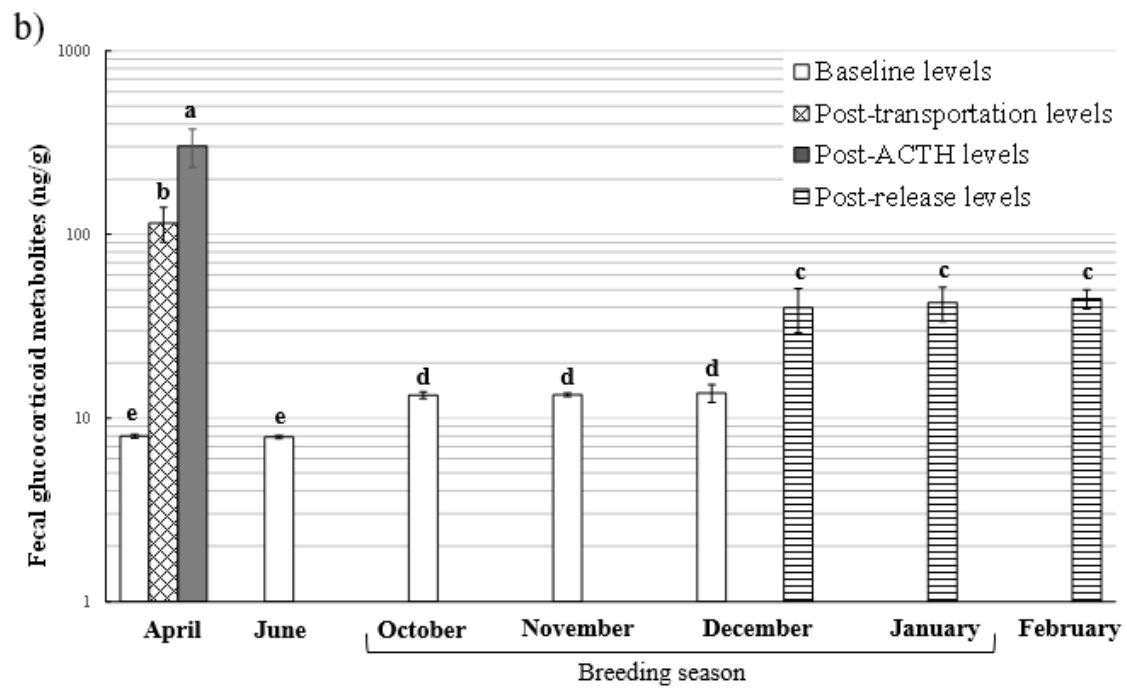
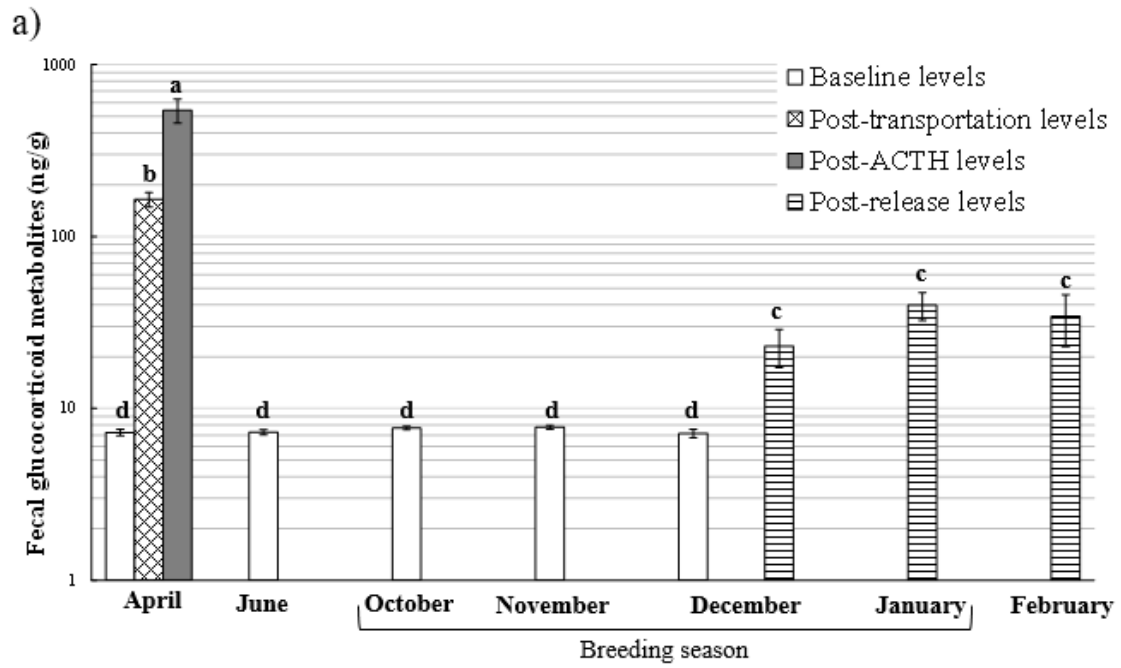
## Figures and tables

**Fig. 1:** Mean ( $\pm$ SE) fecal glucocorticoid metabolite (FGM) levels of Greater Rheas females (a) and males (b) from an intensive rearing system when they were exposed to predictable conditions (baseline levels) and unpredictable conditions (post-ACTH, post-transportation, and post-release into a novel environment). Levels not connected by the same letter are significantly different ( $P < 0.05$ ). Figure was made on a log scale with base 10.

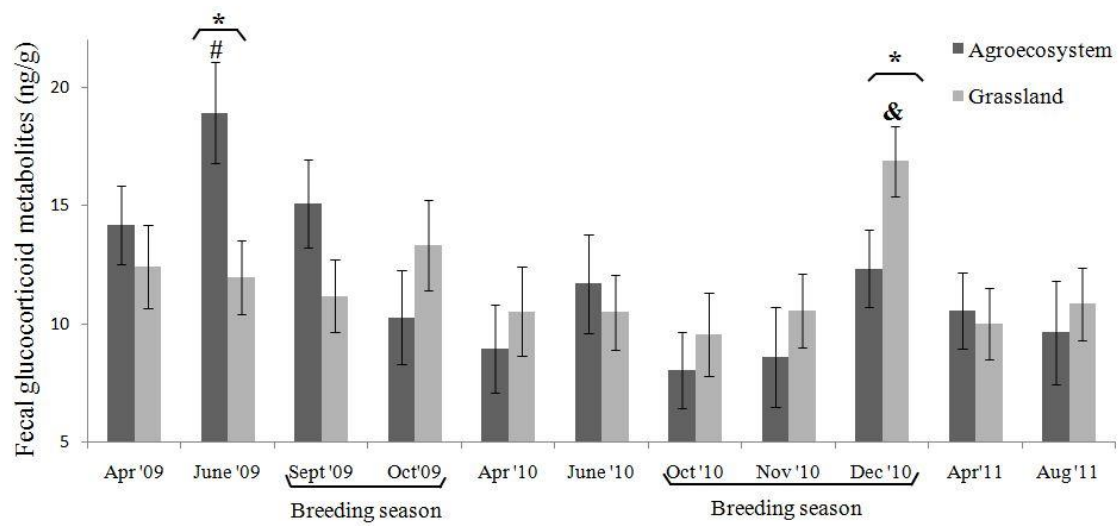
**Fig. 2:** Mean ( $\pm$ SE) fecal glucocorticoid metabolite (FGM) levels of Greater Rheas in the grassland and the agroecosystem. The following symbols indicate significant differences at  $P < 0.05$ : (#) compared with all other sampling periods in the agroecosystem; (&) compared with all other sampling periods in the grassland; (\*) between the grassland and agroecosystem. Figure modified from Lèche *et al.* (2014).

**Table 1:** Examples of the magnitude of the increase in glucocorticoid (GC) levels in plasma or feces, after exposure to a stressor (ACTH injection or capture/handling), in ratites and in flying avian species.

**Table 2:** Mean ( $\pm$  SE) fecal glucocorticoid metabolite (FGM) levels of Greater Rhea males and females from an intensive rearing system and a semi-extensive rearing system, when they were translocated to an agroecosystem. Comparisons were made between sexes, within each system. Differences were considered significant at  $P < 0.05$ .







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**Table 1:** Examples of the magnitude of the increase in glucocorticoid (GC) levels in plasma or feces, after exposure to a stressor (ACTH injection or capture / handling), in ratites and in flying avian species.

| Species   | Stressor                    | Matrix | GC fold-increase above baseline levels | References                     |
|---|-----------------------------|--------|--|--------------------------------|
| Greater Rhea ( <i>Rhea americana</i> )                            | ACTH injection              | Plasma | 40                                     | Lèche <i>et al.</i> 2009       |
|   | ACTH injection              | Feces  | 40                                     | Lèche <i>et al.</i> 2011       |
|   | Capture/restraint/transport | Plasma | 40                                     | Lèche <i>et al.</i> 2013       |
|   | Capture/restraint/transport | Feces  | 10                                     | Lèche, 2012                    |
| Common Kiwi ( <i>Apteryx australis</i> )                          | Capture/restraint           | Plasma | 50                                     | Cockrem <i>et al.</i> , 2004   |
| Japanese quail ( <i>Coturnix japonica</i> )                       | ACTH injection              | Plasma | 3                                      | Hazard <i>et al.</i> , 2007    |
| Chicken ( <i>Gallus domesticus</i> )                              | ACTH injection              | Plasma | 16                                     | Dehnhard <i>et al.</i> , 2003  |
| Great tits ( <i>Parus major</i> )                                 | ACTH injection              | Plasma | 6                                      | Baugh <i>et al.</i> , 2017     |
| House sparrows ( <i>Passer domesticus</i> )                       | ACTH injection              | Plasma | 1,5                                    | Romero, 2006                   |
| Harlequin duck ( <i>Histrionicus histrionicus</i> )               | ACTH injection              | Plasma | 6                                      | Nilson <i>et al.</i> , 2008    |
| Florida sandhill cranes ( <i>Grus canadensis pratensis</i> )      | ACTH injection              | Plasma | 21                                     | Ludders <i>et al.</i> , 1998   |
| Snow petrel ( <i>Pagodroma nivea</i> )                            | ACTH injection              | Plasma | 8                                      | Angelier <i>et al.</i> , 2009  |
| White-crowned sparrows ( <i>Zonotrichia leucophrys gambelii</i> ) | ACTH injection              | Plasma | 3                                      | Astheimer <i>et al.</i> , 1994 |
|   | Capture/restraint           |        | 6                                      | Krause <i>et al.</i> , 2014    |
| Greater sage grouse ( <i>Centrocercus urophasianus</i> )          | ACTH injection              | Feces  | 2                                      | Jankowski <i>et al.</i> , 2009 |
| Smith's longspur ( <i>Calcarius pictus</i> )                      | ACTH injection              | Plasma | 10                                     | Meddle <i>et al.</i> , 2003    |
| Lapland longspur ( <i>Calcarius lapponicus</i> )                  | ACTH injection              | Plasma | 6                                      | Romero <i>et al.</i> , 1998    |
| Chukar ( <i>Alectoris chukar</i> )                                | ACTH injection              | Plasma | 10                                     | Dickens <i>et al.</i> , 2009   |
|   | Capture/restraint           |        | 4                                      | Dickens <i>et al.</i> , 2009   |
| Eastern bluebirds ( <i>Sialia sialis</i> )                        | Capture/restraint           | Plasma | 6                                      | Lynn <i>et al.</i> , 2010      |

**Table 2:** Mean ( $\pm$ SE) fecal glucocorticoid metabolite (FGMs) levels of Greater Rheas males and females from an intensive rearing system and semi-extensive rearing system, when they were translocated to an agroecosystem. Comparisons were made between sexes, within each system. Differences were considered significant at  $P < 0.05$ .

| Translocation to agroecosystem     | FGMs levels (ng/g)  |   | References                        |
|------------------------------------|---|---|-----------------------------------|
|                                    | Pre-transportation<br>(3 days before)                     | Post-release<br>(throughout a 60-day period)              |                                   |
| From intensive rearing system      | ♂: $13.31 \pm 0.40$<br>♀: $6.99 \pm 0.27$ ( $P < 0.05$ )  | ♂: $42.34 \pm 4.81$<br>♀: $31.13 \pm 4.54$ ( $P = 0.20$ ) | Lèche <i>et al.</i> (2016)        |
| From semi-extensive rearing system | ♂: $22.26 \pm 0.66$<br>♀: $20.41 \pm 0.65$ ( $P < 0.05$ ) | ♂: $44.55 \pm 2.00$<br>♀: $53.22 \pm 6.97$ ( $P = 0.50$ ) | Navarro <i>et al.</i> (submitted) |

**Highlights:**

Adrenocortical and behavioral responses of Greater Rhea depend on the environment

Adrenocortical responses in Greater Rhea suggest phenotypic plasticity of HPA axis

Yolk corticosterone influences the stress responses in Greater Rhea

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