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Reproductive endocrinology and stress physiology in Galápagos land iguanas

Endocrinologia riproduttiva e fisiologia dello stress nelle iguane terrestri delle Galápagos

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Your reason and your passion are the rudder and the sails of your seafaring soul.

Kahlil Gibran

INDEX

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 1

General introduction

1) *Multidisciplinary is the key for conservation*

During the last century, threatening processes such as invasive species and pathogenic diseases have been working synergistically to biodiversity loss (Brook et al. 2008). Free-living animals must struggle against a variety of challenges and man-mediated alterations that can cause high stress conditions. Disruption of behaviour and reproductive physiology and the alteration of population fitness are among the most hostile consequences of prolonged stress. Thus, understanding if and how threatened free-living populations reproduce and respond to external changes is necessary to determine vulnerability and set conservation priorities. In that respect, the use of an integrative and functional approach, direct also to the comprehension of physiological and endocrine individual dynamics, can help to characterize and alleviate problems that could threaten a species, population, community.
Conservation physiology is an evolving important field of conservation science that takes advantage from this integrative approach (Wikelski and Cooke 2006). Conservation biologists are progressively using different techniques that range from endocrinology to stress physiology, to develop and choose solutions. This combination of conservation tools, with supplementary knowledge of the basic biology of organisms, is fundamental for the safety of both captive and wild populations. Multidisciplinary is the key to more efficient problem solving in conservation. For this reason, in this occasion, I quote Wildt et al. (2003) who stated: "conservation can be likened to a complex jigsaw puzzle where the puzzle pieces are issues, stakeholders or scientific disciplines themselves".

2) *Reproductive endocrinology and aims*

Reproduction is the foundation on which a species survives. Understanding the complexities of when and how individuals reproduce is basal for the perpetuation of natural populations and their future management. Therefore, knowledge about the reproductive

endocrinology of a species can fill an important gap in our understanding of timing and modalities of reproduction. Reproductive endocrinology offers the possibility to better realize factors impairing species vitality, and it may even offer early-warning signals of a risk before survivorship or reproductive rates plummet.

Much of our knowledge of vertebrate reproductive endocrinology has been collected from studies of mammals. Anyhow, there are sufficient structural and functional similarities between mammals and non-mammals to indicate that many of mechanisms regulating reproduction are probably common to all vertebrates (Crews and Silver 1985).

Reproductive activity is associated with an essential variation in circulating concentrations of the primary sex steroid hormones: progesterone (P4) and 17β-estradiol (E2) (Jones and Guillette 1982; Crews and Silver 1985; Norris and Lopez 2010). Measuring the plasma levels of these sexual steroid hormones can be useful to track the reproductive hormone profiles of free-living animals and this could be crucial for species conservation especially when direct observations on field are strongly limited by logistic constraints and *ex-situ* captive breeding programs may become necessary. This is the case of two Galápagos land iguanas species that occur in Volcán Wolf (Isabela Island): *Conolophus marthae* and *C. subcristatus*, among the most representative species of the Galápagos Islands.

Conolophus marthae, the Galápagos Pink Land Iguana (also simply known as pink iguana), was only recently described (Gentile and Snell 2009; Gentile et al. 2009) and listed as Critically Endangered in the IUCN Red List (Gentile 2012). Current data suggests that this species lives, with an extremely small population, exclusively on the top and along the northwest slopes of Wolf volcano, the highest peak (1,707m) in the archipelago. Just because only recently discovered, information about its ecology is limited to circumstantial observations and reproductive biology is completely unknown. Newly hatched individuals and juveniles of the species were never observed.

Contrary to the pink iguana, the Galápagos common iguana *Conolophus subcristatus* (for convenience here also referred to as yellow iguana) currently inhabits six islands in the archipelago, including Isabela Island and the Wolf volcano where occupies an area larger than *C. marthae*. The yellow iguana is listed as vulnerable in the IUCN Red List and experienced various disturbances by direct and

indirect human activity so that several populations became dramatically reduced in size or were extirpated (Snell et al. 1984). About this species, some studies were produced sustaining that clutch size and mating season vary across islands (Werner 1983; Snell et al. 1984). However, little is known about the reproductive biology of this species on Wolf volcano.
To pinpoint species-specific times of reproduction in the volcano and possible interspecific interactions, I examined and explained baseline steroid levels of progesterone (P4) and 17β-estradiol (E2) in both iguana species. The existence of previous studies on sex steroid hormones of the Galápagos marine iguana *Amblyrhynchus cristatus*, the sister taxon of *Conolophus* spp. (Rassmann 1997), offered a unique opportunity to use an appropriate reference model for the much less investigated land iguanas.

3) *Stress physiology and aims*

Free-living animals periodically experience a multiplicity of internal/external environmental challenges and man-mediated alterations that can produce stress condition.
The term "stress" has become popular thanks to the pioneering work of Hans Selye (1946), who described the stress condition as "a general adaptation syndrome (GAS)" in which a rapid initial reaction ("alarm") was followed by sustained glucocorticoid secretion ("phase of resistance") and eventually by a dangerous debility when corticoid output could not be sustained ("phase of exhaustion").
Stress is a term used across a broad spectrum of scientific researches; however, its definition is often ambiguous and sometimes not defined at all. Nowadays, biologists distinguish between "stressor" and "stress response". Stressor is any noxious stimulus (Romero 2004) or exceptional event that disturbs an animal's homeostasis generating the so-called emergency life-history stage (ELHS) (Wingfield et al. 1998; McEwen and Wingfield 2003). Free-living animals experience many stressors during their life including physical factors (i.e., change in temperature, oxygen, and salinity), climatic stressors (drought and storms) and biotic stressors (predation, competition and social dynamics, parasitism) that challenge their homeostasis (Romero 2004; Jessop et al. 2013). These disturbance phenomena may have effects on the ecology and evolution of organisms (Hoffmann and Hercus 2000;

Badyaev 2005; Jessop et al. 2013) and, depending on their pervasiveness, magnitude and frequency, can influence the individual fitness (Bonier et al. 2009; Busch and Haiward 2009). Thus, in response to a stressor, animals mount stress responses, which work for neutralizing the effects of the stressor to regenerate homeostasis. The stress response is constituted by all physiological, endocrinological, immunological and behavioural adaptations, which can be concurrently used to cope with the stress condition limiting the negative consequences on fitness (Wingfield et al. 1998; Wikelski and Cooke 2006).

One main feature of stress response in vertebrates is the release of glucocorticoids (i.e. cortisol and corticosterone), steroid hormones whose synthesis is regulated by hypothalamic–pituitary–adrenal axis (HPA). Fish and most mammals generally release cortisol, whereas most birds, reptiles, amphibians, and many rodents release corticosterone (Johnson et al. 1992; Sapolsky 1992; Romero 2004; Romero and Butler 2007; Crespi et al. 2013). Glucocorticoids (GCs) are the final product of the HPA axis; these stress hormones participate in the control of homeostasis activating immediate life-saving processes (Romero et al. 2009). Upon perception of stress, the hypothalamus is activated to secrete arginine vasotocin (AVT, homologous of the mammalian arginine vasopressin) and corticotropin-releasing factor (CRF), which stimulate the pituitary gland to release adrenocorticotropin (ACTH). This in turn, causes the release of glucocorticoids from the adrenal glands (Rich and Romero 2005). The cessation of the pathway leading to GCs production occurs through a negative feedback under the control of the GCs themselves. Stress-induced concentrations of GCs interact with glucocorticoid receptors in the hippocampus, hypothalamus and pituitary gland to suppress the initial steps of the HPA axis (De Kloet et al. 1998). The level at which GCs are elevated depend on the severity of the stressor; therefore, under acute stress conditions, the feedback mechanism operates efficiently and the system rapidly returns to normal; under chronic stress conditions, feedback signals are weak and the system remains activated for longer periods (Sapolsky 1992).

Generally, short-term glucocorticoid releases are helpful for organisms because stimulate emergency mechanisms such as mobilizing glucose (gluconeogenesis) and protein catabolism to immediately increase energetics availability to overcome the

perturbation (Wingfield and Ramenofsky 1999; Wingfield and Romero 2001; Wingfield 2013). However, chronic activation of the HPA axis and prolonged elevated GC concentrations may have large deleterious effects on fitness (Romero 2004; Blas et al. 2007) resulting in stress-related disease (Sapolsky 1992; Romero et al. 2009). Indeed, long-term activation of the stress response can expose the individual to a long-term overstimulation of survive mechanisms with consecutive inhibition of many fundamental functions including immunocompetence and reproduction (Sapolsky 1987; Wingfield et al. 1997; Dhabhar 2000; Sapolsky et al. 2000; Dallman and Bhatnagar 2001; Wingfield and Romero 2001).

Overall, GCs concentrations are being used increasingly in ecological and conservation studies as indices of animal well-being (Wikelski and Cooke 2006; Busch and Hayward 2009; Sheriff et al. 2011). Measuring these hormones can help to understand how specific stressors affect the survival and reproductive success of free-living animals. However, although these hormones can be measured directly from many biological matrices as blood, saliva, faeces and urine (Wasser et al. 1997; Sapolsky et al. 2000; Narayan et al. 2010; Sheriff et al. 2011), measuring them under field conditions is very difficult and may require caution.

Historically, blood is the traditional biological matrix used to assess GCs concentration. Blood collection allows the measurement of instantaneous and direct product of the adrenal cortex. Moreover, this method permits a simultaneous collection of blood components with a comprehensive assessment of the state of the animal, including indices of condition (haematocrit), immune function (leukocyte profiles), and reproductive status (reproductive hormones). The most appropriate method for blood collection varies across species. However, despite the method used, the collection of blood sample is itself invasive. Thus, when planning researches on basal-levels of stress, the effects of sample collection must be considered as they may bias the hormonal response of the examined animals. To avoid this problem blood samples are generally taken before the adrenal cortex has been activated, that is within few minutes from capture (Wingfield and Romero 2001; Romero and Reed 2005). Other, less sensitive, indicators of stress may also be used, such as leukocytes profiles or more in detail the heterophils (or neutrophils in mammals) and

lymphocytes ratio. In fact, the immune response is another part of the adaptive responses to stressful situations.

The immune system is the primary defence mechanism through which the organism protects itself from stressors represented by pathogens. Leukocytes or white blood cells (WBC) are fundamental mediators of the immune response (Lobato et al. 2005; Davis et al. 2011); they circulate continuously in the blood stream and various organs, actively destroying invading microorganisms. This circulation is essential for maintaining an effective immune defence network.

Most vertebrates have five types of WBCs: lymphocytes, neutrophils, eosinophils, basophils and monocytes, each one with specific morphology and function. The morphology of each cell type appears to be conserved across taxa, except in the case of neutrophils; indeed, in birds and reptiles neutrophils are replaced with heterophils, which perform the same immunological function (Hawkey and Dennett 1989; Jain 1993).

Neutrophils/heterophils and lymphocytes make up the highest percentage (i.e. nearly 80% combined) in WBCs of many vertebrates including reptiles (Eliman 1997; Fisse et al. 2004; Davis et al. 2008). Specifically, neutrophils/heterophils are the primary immune phagocytosing cells; they enter the tissues during the inflammatory response (Jain 1993; Campbell 1995; Davis et al. 2008) participating actively to the phagocytosis of organisms and other foreign material (Thrall et al. 2012). Lymphocytes are involved in a variety of immunological functions such as immunoglobulin production and modulation of immune defence (Campbell 1996). Generally, the numbers and proportions of leukocytes in blood provide an important representation of leukocyte distribution in the body and of the activation state of the immune system. Leukocyte profiles have a recognized predictive power for interpreting individuals' health status; indeed, the observations of variations in leukocytes numbers are particularly useful in the field of conservation biology to describe an altered health status (Wakelin 1996; Davis et al. 2004; Davis et al. 2008). Usually, during a stress condition, an increase in neutrophyls (N)/heterophils (H) and a decrease in lymphocytes (L) are observed (Maxwell and Robertson 1998; Ots et al. 1998; Davis et al. 2008). Since numbers of these types cells are affected by stress in opposite directions, the relative proportion of neutrophils/heterophils to lymphocyte (N-H/L) is commonly used as a composite measure of the

stress response (Gross and Siegel 1983; Maxwell 1993; Maxwell and Robinson 1998; Lobato et al. 2005; Davis et al. 2008; Xuereb et al. 2012; Lentfer et al. 2015). Differently from the glucocorticoids measurements, the WBC approach offers the advantage that it does not require prohibitively rapid sampling and is relatively inexpensive. Moreover, leukocyte profiles are particularly useful in the field of stress physiology because they can be directly related to stress hormone levels (Davis et al. 2008). Many studies have described the stress-induced change in leukocyte distribution mediated by hormones released by the adrenal gland (Dhabhar et al. 1996; Dhabhar and McEwen 1999). Chronically elevated glucocorticoid levels may cause long-term elevations in N-H/L ratio as they simultaneously induce a reduction in the number of circulating lymphocyte, with a redistribution from circulatory to bone marrow. At the same, they cause increase in the number of neutrophils/heterophils, by stimulating their influx into the blood and attenuating their egress from the blood to other compartments (Sapolsky et al. 2000). Therefore, trafficking and function of blood cells are altered transiently by GCs.

Because stress in animal populations is an important factor to consider when evaluating their welfare in both captive and wild condition, using different stress markers is fundamental to obtain a reliable assessment of the stress condition. For this, in this part of the PhD project, using both haematologic and hormonal profiles, I specifically explored how parasites affect iguanas' life traits as leukocyte profiles and glucocorticoids levels. In fact, the two populations of *C. marthae* and *C. subcristatus* have to overcome the strong impact of ticks, which seem to be more abundant in Volcán Wolf than elsewhere in the archipelago. The site is characterized by a massive occurrence of ticks *Amblyomma* spp., ectoparasites already described infecting marine iguanas (Wikelski 1999). Probably, ticks are the major vectors of *Hepatozoon* (Apicomplexa: Adeleorina) in Galápagos reptiles (Bataille et al. 2012). *Hepatozoon*, with over 300 species described, is the most common among intracellular blood parasites in reptiles (Telford 1984; Smith 1996). They infect host erythrocytes and their effects on host fitness are still debated.

In general, ecto and endoparasites are considered potential sources of biotic stress for all organisms (Lozano 1998). They can coexist with their hosts without causing any measurable deleterious effects, but

they can also increase in numbers and overwhelm a host already weakened by other forms of stress such as malnutrition or reproduction (Walzer and Genta 1989). Finally, they can directly provoke inflammatory responses and disease affecting individual health and fitness-related traits (Schwanz 2008). Moreover, parasites and glucocorticoid hormones interact and affect a multiplicity of processes, such as immune response and reproduction (Wingfield et al. 1997; Sapolsky et al. 2000). However, the nature of the relationship between parasitic infection and levels of glucocorticoids and their possible covariation with haematological profiles has received relatively little attention in wild animals, and with equivocal results. For this, aware of the poor knowledge about patterns of natural variation of haematological parameters and glucocorticoid levels and their relationship with parasites in reptile wild populations, in the second part of the project I analysed the relation between ecto- and endoparasites and the stress physiology of these two Galápagos land iguanas. For this purposes I used: (i) leukocyte profiles and specifically the heterophils/lymphocytes ratio (H/L), commonly used as diagnostic tool for assessing long-term stress in vertebrates (Davis et al. 2008), (ii) endocrinological markers as baseline corticosterone plasma levels, the primary adrenal glucocorticoid hormone produced in response to stressful events in reptiles (Greenberg and Wingfield, 1987).

I used a separate population of *C. subcristatus*, occurring in a coastal area where notoriously ecto-parasites and haemoparasites are marginally present (Bahia Urbina), as "blank" condition for haematologic and hormonal comparisons.

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CHAPTER 2

Plasma levels of progesterone and estradiol and their relation to reproduction in Galápagos land iguanas, *Conolophus marthae* and *Conolophus subcristatus*

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Submitted (Animal Reproduction Science)

1) Introduction

Reproductive endocrinology is a fundamental resource for scientists interested in comprehending different aspects of reproductive biology in vertebrates. Endocrinological mechanisms controlling the reproductive biology have been studied more widely in mammalian organisms than in reptiles (Bronson 1989) and birds (Tsutsui et al. 2000; Wikelski et al. 2000). Despite this, although differences that reflect the evolutionary processes among classes exist, several studies on vertebrate reproduction and endocrinological processes indicate a notable homogeneity throughout the subphylum (Nandi 1967; Bentley 1998).

Reproductive activity in reptiles is associated with an essential variation in circulating concentrations of the primary sex steroid hormones: progesterone (P4) and 17β-estradiol (E2) (Jones and Guillette 1982; Crews and Silver 1985; Wibbels et al. 1992; Edwards and Jones 2001; Taylor et al. 2004; Norris and Lopez 2010). Reproductive rhythms, sexual behaviours, physiological processes correlated to reproduction such as mating, gestation and oviposition are under a complex endocrine control which involves the activity and regulation of hypothalamic-pituitary-gonadal axis (HPG) on sex steroids hormones production (Licht 1979; Crews and Silver 1985).

In reptiles, surveys on sex steroid hormones openly declare the importance of progesterone in regulating the oocyte maturation and in maintaining gestation (Callard et al. 1992; Custodia-Lora and Callard 2002). Progesterone has a role in determining the timing of oviposition (Norris 2007), inhibits oviductal contractility (Guillette and Jones 1985; Edwards and Jones 2001), and delays parturition (Guillette et al. 1991). The pattern of progesterone production during the reproductive cycle differs between viviparous and oviparous reptiles (Callard et al. 1992). While in live-bearing reptiles the highest concentration is reached during mid-pregnancy, in those laying eggs progesterone shows a pre-ovulatory and early-pregnancy rise with a strong decrease before oviposition (Crews and Silver 1985; Taylor et al. 2004). The role of P4 in pregnancy maintenance has been well-studied especially in viviparous reptiles where its function in inhibiting follicular development and maintaining oviductal vascularity is deeply described (Guillette et al. 1981; Mead et al. 1981).

The 17β-estradiol (E2) is the primary estrogenic steroid hormone in reptiles (Norris 2007). Ovarian development and vitellogenesis process (yolk production) are usually associated with elevated plasma concentrations of estradiol in squamates (Bonnet et al. 1994), turtles (Ho et al. 1981), and alligators (Guillette et al. 1997). Vitellogenesis is clearly an estrogen-dependent process; estradiol regulates the synthesis of vitellogenin by the liver and the yolk protein accumulation in blood and oocytes (Licht 1979; Ho 1987). Moreover, estradiol plays an important role in inducing sexual behaviours during mating period (Whittier and Tokarz, 1992; Rhen and Crews 2000); indeed, exogenous administrations of estrogen are known to have a stimulatory effect on female sexual receptivity in some species of lizards (Crews 1975a; Valenstein and Crews 1977).

Thus, investigating circulatory levels of sexual hormones can be very informative of the reproductive status of wild reptiles and may prove very useful when conservation is also an issue, especially when the duration of field investigations, that allow direct observations, is strongly limited by logistic constraints. This is the case of the pink iguana from the Galápagos (*Conolophus marthae*), a species recently discovered (Gentile and Snell 2009; Gentile et al. 2009) and listed as Critically Endangered in the IUCN Red List (Gentile 2012). The species occurs only on the top and along the northwest slopes of Volcán Wolf (Isabela Island), the highest peak (1,707m) and one of the most remote and difficult field sites in the Galápagos archipelago (Fig. 1). Threatens include small population size, extremely limited distribution, possible competition with a syntopic population of *C. subcristatus*, and introduced predators (Gentile et al. 2016).

Contrary to the pink iguana, *C. subcristatus* is widely distributed across the archipelago, including Isabela Island and Wolf volcano. A third Galápagos land iguana species, *C. pallidus*, occurs only in the island Santa Fe. Little is known about the reproductive biology of these species. The available information is incomplete and regards only *C. subcristatus* and *C. pallidus* for which previous studies indicated that clutch size and mating season vary across islands (Werner 1983; Snell et al. 1984). Information on the reproductive biology and ecology of *C. marthae* is limited to circumstantial observations.

As the two syntopic species on Wolf volcano may compete for nesting sites, comprehending times and modes of reproduction is crucial to

understand whether the two populations have complete overlapping reproductive seasons. Additional needed sensible data are also the densities of reproducing females, particularly important in the light of the fact that clutch size are very different in the two species (Gentile et al. 2016). Clearly, information gained from hormonal surveys may potentially allow addressing these issues.

Unfortunately, no previous studies of sexual hormones of *Conolophus* species exist. Sexual steroids were instead investigated in the marine iguana *Amblyrhynchus cristatus* (Rubenstein and Wikelski 2005; Vitousek et al. 2010; Vitousek and Romero 2013), the sister taxon of *Conolophus* (Rassmann 1997). Such studies focused precisely on how baseline patterns of sex steroids vary during the breeding season in relation to female aggression (Rubenstein and Wikelski 2005), receptivity (Vitousek et al. 2010), and mate selection (Vitousek and Romero 2013). In *A. cristatus* physiological changes in circulating hormones affect reproductive biology. Progesterone and estradiol were reported to be associated to different reproductive processes and work independently showing distinctive patterns during mating and nesting periods. Progesterone was elevated at the beginning of mating period but decreased towards the end, increased again at the beginning of nesting period, related to pregnancy maintenance, and then incessantly decreased throughout nesting phases (Rubenstein and Wikelski 2005). Moreover, in *A. cristatus* progesterone seemed to be a potential inhibitor of vitellogenesis as its plasma levels increased during follicular atresia (Vitousek et al. 2010). On the contrary, estradiol apparently stimulated attractivity and receptivity of female marine iguanas; plasma concentration of estradiol was extremely low during all nesting phases but peaked during the mating period when it stimulated the vitellogenesis process and modulated the aggressive behaviour (Rubenstein and Wikelski 2005).

Considering the evidences obtained in marine iguanas and the sister taxon relationship between *Amblyrhynchus* and *Conolophus*, in this study we used *A. cristatus* as a reference biological system and used a combined approach of biometric, endocrinological and ultrasound analyses to examine and explain baseline steroid plasma levels of P4 and E2 in the two syntopic populations of terrestrial Galápagos iguanas *C. marthae* and *C. subcristatus*.

Figure 1. Galápagos Islands. The triangle indicates the volcano where *C. marthae* and *C. subcristatus* were studied.

2) Materials and methods

2.1) *Ethic statement*

Animal manipulation and blood sampling were performed according to a protocol that minimized animal stress, in accordance with the European Community guidelines and with the approval of the Galápagos National Park. Samples were exported and imported under the CITES permits 101/BG and IT/IM/2015/MCE/01711, respectively.

2.2) *Samples*

Samples of *C. marthae* and *C. subcristatus* females were collected in three different years: July 2010, June 2012 and 2014. Sample sizes and reproductive status considered in the present investigation are summarized in Table 1.
During all field sessions, approximately 2ml of blood were drawn from each iguana within 5 minutes from capture, using a 5 ml heparinized syringe. Blood was collected from the caudal vein and

kept on ice. A few hours later blood was centrifuged and plasma was separated. Plasma was stored at -10°C while in the field and at -40°C once back in the laboratory.
All captured iguanas were weighed and measured. The body condition index (BCI) was then estimated as the ratio of body mass/snout-vent length $(SVL)^3$ x 10^6 (the ratio was multiplied by 10^6 to reduce the number of decimals). Although simple, this index has been already used to describe the physical condition of marine (Laurie 1989; Wikelski and Trillmich 1997, Romero and Wikelski 2001) and land iguanas (Costantini et al. 2009).

2.3) *Ultrasound analysis*

For each female we determined the number of eggs, egg size, and the stage of development of follicles using a Sonosite portable ultrasound machine (FUJIFILM SonoSite, Inc.). Technical characteristic of the device and probe used, as well as the protocol applied, can be found in Gentile et al. (2016). Although abdomen palpation can be a possible method for diagnosing pregnancy, the use of ultrasound machine offers clear advantages. In fact, several studies identified it as the most reliable method to realize an accurate evaluation of reproductive conditions in reptiles (lizard: Gartrell et al. 2002; Gilman and Wolf 2007; tortoise: Robeck et al. 1990). The analysis allowed us to determine the reproductive status of each female, differentiating between development stages of eggs (Fig. 2): stage “a”, females showing follicles with eggs of homogenous, spherical and small dimensions; stage “b”, females with larger, yet not fully formed, unshelled eggs; stage “c”, females with large, fully formed, shelled eggs; stage “d”, non-reproductive females carrying no visible eggs inside follicles. An examination of the *corpus luteum* would prove useful to assess whether a female has laid a clutch of eggs in the recent past. However, while the *corpora lutea* persist in ovoviparous reptiles after egg-laying (Glasser and Bullock 2012), in oviparous reptiles as *Conolophus* they regress shortly after deposition (Yadav 2008). Additionally, along with its advantages, the ultrasound approach has the disadvantage that it does not allow the visualization of the *corpus luteum* (Norris and Lopez 2010).

Pregnancy status	**a**	**b**	**c**	**d**
2010 (July)				
C. marthae	0	0	4	14
C. subcristatus	0	0	0	9
2012 (June)				
C. marthae	0	0	2	16
C. subcristatus	0	0	9	5
2014 (June)				
C. marthae	2	1	0	17
C. subcristatus	1	1	8	10

Table 1. Pregnancy status of each species during three sampling seasons. (stage a) Females with only follicular eggs; (stage b) females with not fully-formed eggs with only shell membrane; (stage c) females with fully-formed eggs; (stage d) non-reproductive females when no follicles or eggs were visualised.

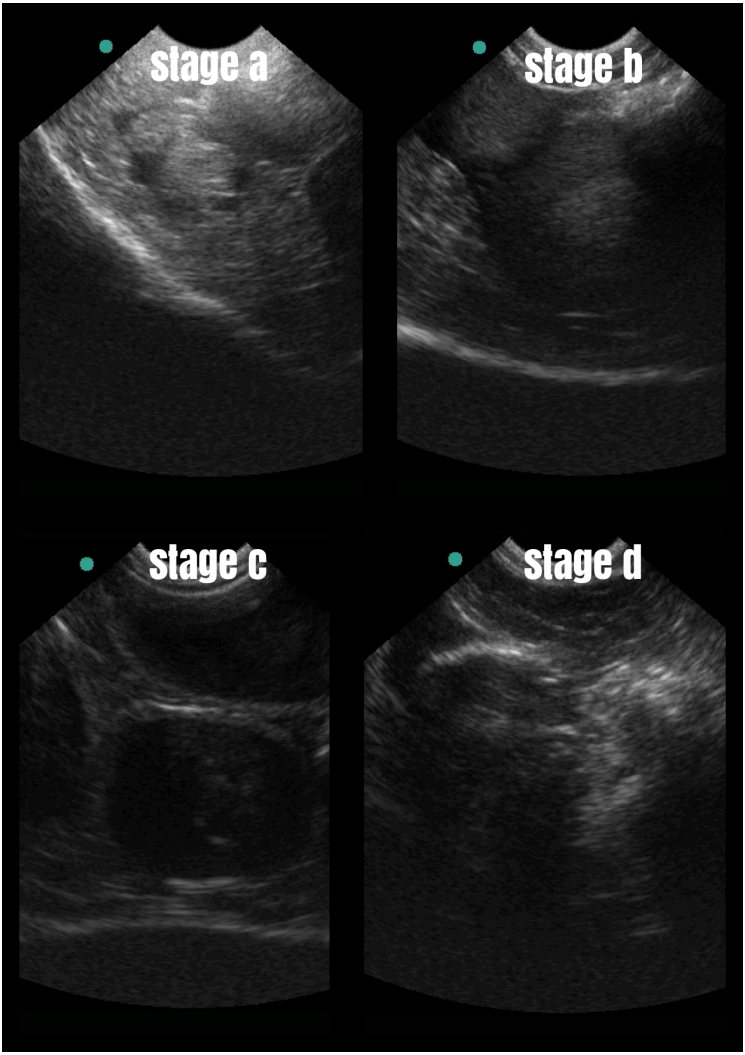

Figure 2. Original ultrasound images.
Stages: "a" follicles with eggs of homogenous, spherical and small dimensions; "b" large, yet not fully-formed, unshelled eggs; "c" large, fully-formed, shelled eggs; "d" no visible eggs.

2.4) *Hormone assays*

Plasma levels of sexual steroids hormones progesterone (P4) and 17β-estradiol (E2) were determined by using competitive enzyme-linked immunosorbent assays (ELISA).
Indeed, several studies have analysed steroid hormones in reptiles using radioimmunoassay (RIA) (turtles: Mahmoud et al. 1989; snakes: Highfill and Mead 1975; Taylor et al. 2004; lizards: Judd et al. 1976; Arslan et al. 1978; Amey and Whittier 2000; Husak et al. 2007). The only works on iguanine lizards regard *A. cristatus* and use RIA (Rubenstein and Wikelski 2005; Vitousek et al. 2010; Vitousek and Romero 2013).
Radioimmunoassay is a common method for quantifying the steroids hormones in vertebrates, however some problems associated to this method exist. The need of special facilities for handling radioactivity, the short stability time of the radiolabeled ligands and potential health risks are commonly associated to this methodology (Andoh 2006; Sink et al. 2008). On the contrary ELISA is generally faster and safer than RIA, it is less expensive and shows a greater stability of reagents. Overall, there is still lack of data on how these two methods are comparable. The little information available suggests that differences may be observed when comparing results from RIA and ELISA. Problems may especially reside in differences in protocols of analysis (Sink et al. 2008).
All ELISA immunoassays were performed at the Laboratory of Clinical Biochemistry (Tor Vergata University Hospital). Plasma samples were preserved at -40°C until assayed.
We used 50 µl of plasma for the determination of each hormone. Only for E2, plasma was diluted 1:2 with assay buffer (containing proteins and sodium azide) to remove matrix interference. All samples were assayed in duplicate and randomly distributed between plates.
We used the enzyme-linked immunosorbent assay kit (CEA459Ge) pre-coated with a monoclonal antibody for P4. The detection range of progesterone ELISA kits (CEA459Ge) was 1.23-100 ng/mL. The intra-assay variation was < 10%, the inter-assay variation < 12%.
We could not use ELISA kits of the same lot throughout the whole study, despite it was recommended (Sink et al. 2008). In fact, we used ELISA kits belonging to two different lots. To evaluate inter-lot variation, 5 individuals were analysed by using both lots. The power

curve $y = ax^b$, where $a = 0.059006$ and $b = 1.4786$, fitted data with a proportion of variance explained (R^2) equal to 0.995. We used such a curve to adjust readings from the second lot. In order to account for experimental error, a randomly generated number comprised within the minimum and maximum residual values of the regression was added to each predicted value.
For the 17β-estradiol (E2) we used the immunoassay KA2535 pre-coated with a polyclonal antibody. The detection limit of all estradiol ELISA kits was 14 pg/mL. The intra-assay variation was around 3%, the inter-assay variation 9%. Also for this hormone we used kits belonging to two different lots. As full correspondence in concentrations of retested animals was found, no adjustment procedure was applied in this case.
Both progesterone and 17β-estradiol assays were performed according to the instructions of manufacturers.

2.5) *Statistical analysis*

We used parametric and nonparametric test in order to analyse differences in hormonal plasma levels among years, between the two species, and between egg-carrying females (stages a, b, and c) and non-egg-carrying females (stage d), and to test the difference in clutch size between species. When data presented normal distribution we used Student's unpaired t-test and ANOVA, and when normality assumption was not achieved Mann-Whitney U-test and Kruskal-Wallis ANOVA were applied. Pearson correlation analyses were performed to test the relationship between clutch size and body metrics (BCI, SVL, and weight).
Statistical analyses were performed by using software Past (version 3.07 for MAC) with two tails and alpha set to 0.05.

3) Results

3.1) *Reproductive status*

Of the 18 *C. marthae* females sampled in 2010, 4 (22%) showed fully formed eggs (stage c). No eggs were present in any of the other 14 females (78%) (stage d). Two females (11%) sampled in 2012 carried

not fully formed eggs (stage c); the other 16 (89%) carried no eggs (stage d). Two females (10%) sampled in 2014 showed follicles with small, spherical eggs (stage a), one female (5%) carried not fully formed eggs (stage b) while the remaining 17 (85%) carried no eggs (stage d).
In 2010 all *C. subcristatus* females sampled carried no eggs (stage d). In 2012 we observed nine females (64%) with fully formed eggs (stage c), and five (36%) without eggs (stage d). In 2014, one female (5%) showed follicles with small, spherical eggs (stage a), one female (5%) carried not fully formed eggs (stage b), eight females carried fully formed eggs (stage c) (40%), and 10 females (50%) were found without eggs (stage d).
Considering both fully and not fully formed eggs, we estimated clutch sizes equal to 8.4 ± 3.4 and 5.4 ± 1.5 for *C. subcristatus* and *C. marthae*, respectively. The difference between sizes was statistically significant ($U = 20.5$, $P = 0.01$). For both species, we did not observe a significant linear relationship between clutch size and body measures (BCI, SVL, and Weigh) (for all tests $P > 0.05$).

3.2) *Progesterone (P4)*

Progesterone plasma levels in *C. marthae* and *C. subcristatus* are shown in Fig. 3. Overall, considering all sampled females, the *C. subcristatus* presented higher P4 concentration than *C. marthae* ($U = 764$, $P = 0.01$).
When sampling years were treated separately, *C. subcristatus* showed significantly higher levels than *C. marthae* in June 2014 ($U = 110$, $P = 0.042$).
In *C. marthae*, P4 plasma levels were higher in July 2010 than in June 2012 and 2014 (although not statistically supported), when instead *C. subcristatus* showed low values.
When analysing the difference between egg-carrying females (cumulating females at stages a, b, and c) and non-egg-carrying females (stage d), *C. marthae* presented higher concentration of P4 plasma levels in egg-carrying females ($U = 109$, $P = 0.042$). Moreover, we observed a significant difference in P4 concentration among females with eggs at three different stages (a, b, and c) ($H = 6.533$; $P = 0.036$, Tab. 2). Mann-Whitney post-hoc tests, after Bonferroni correction, showed a significant difference between females at stages

a and c (H = 2. 252; $P = 0.035$). The remaining pairwise tests resulted not significant, ($P_{a\ vs\ b} = 0.136$ and $P_{b\ vs\ c} = 0.081$). However, when we pooled females at stages a and b to increase the statistical power due to the small sample used, P4 levels resulted higher in females at stage c than in the pooled sample (U = 0, $P = 0.019$).

In *C. subcristatus* we did not observe a significant difference between egg-carrying and non-egg-carrying females (U = 157, $P = 0.18$), but we found different variances ($F = 3.11$, $P = 0.02$). Considering the June (2012+2014) and July (2010) sampling sessions separately, *F* test indicated a larger variance in June than in July ($F = 13.38$, $P = 0.001$). We also observed a statistically significant difference between variances when comparing females without eggs sampled in June (2012+2014) with females without eggs sampled in July (2010) ($F = 25.99$, $P = 0.0002$; Fig. 4). We observed a statistically significant difference between total egg-carrying females and non-egg-carrying females of 2010 (U = 27, $P = 0.018$), with egg-carrying females showing higher P4 levels. However, this significance disappeared when total egg-carrying females were compared with non-egg-carrying females of June (2012+2014) (U = 88; $P = 0.36$).

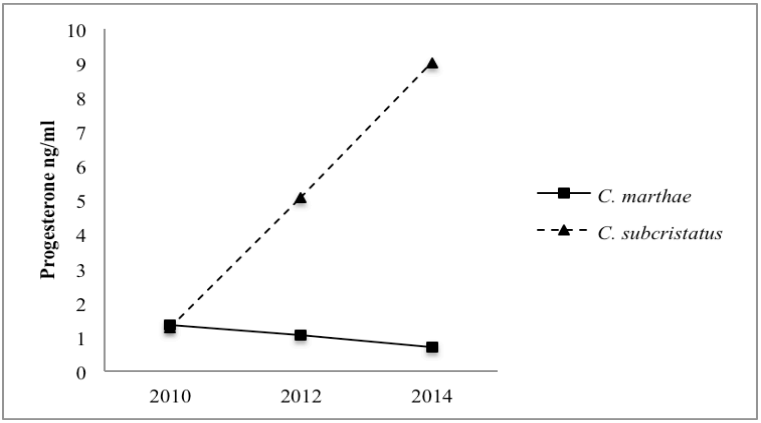

Figure 3. Progesterone patterns in *Conolophus marthae* and *C. subcristatus* during three sampling seasons. Concentrations are reported as median.

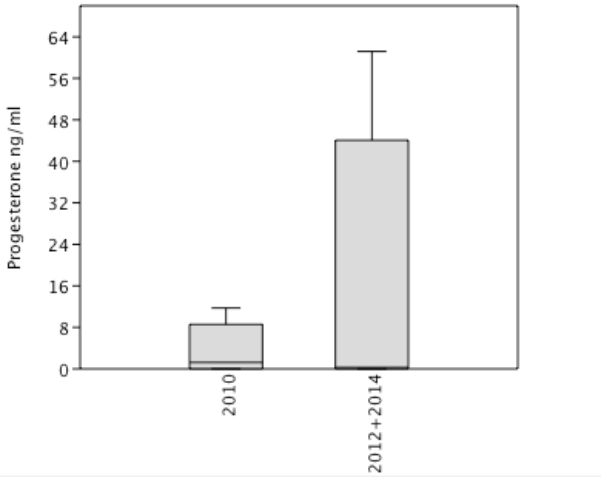

Figure 4. Progesterone plasma levels in non-pregnant females of July (2010) and June (2012+2014) in *C. subcristatus* (median ± SD).

	Progesterone concentration		
	Follicular eggs (a)	Not fully-formed eggs (b)	Fully-formed eggs (c)
N	2	2	5
Min	0.479	1.025	3.061
Max	0.503	2.358	19.045
Variance	0.0003	0.888	45.134
Stand. dev.	0.017	0.942	6.718
Median	0.491	1.691	5.059

Table 2. Variability in progesterone concentrations between three active reproductive states in *C. marthae.*

3.3) *17β-estradiol (E2)*

In *C. subcristatus* we found significant differences in 17β-estradiol plasma levels among years (H = 12.44, *P* = 0.002) (Fig. 5). Bonferroni post-hoc test indicated that estradiol plasma levels in 2010 were significantly lower than either 2012 (*P* = 0.004) or 2014 (*P* = 0.02). Instead, *C. marthae* did not show significant difference in estradiol levels among years (H = 2.21, *P* = 0.3).
Overall, we observed a higher concentration of estradiol in pink than in yellow iguanas (U = 273.5, *P* = 0.03).
In both species we did not observe a significant difference in estradiol plasma concentrations between females at reproductive stages a-c and stage d (*C. subcristatus P* = 0.4; *C. marthae P* = 0.5). However, in *C. marthae*, a statistically significant difference emerged when comparing females at stages b+c with females at stage a, these latter exhibiting higher estradiol concentration (*t* = - 2.9817, *P* = 0.02).
In *C. subcristatus*, females carrying no eggs showed estradiol levels and variances higher in June (2012+2014) than in July (2010) (U = 6, *P* = 0.001; *F* = 15.193, *P* = 0.001; Fig. 6).

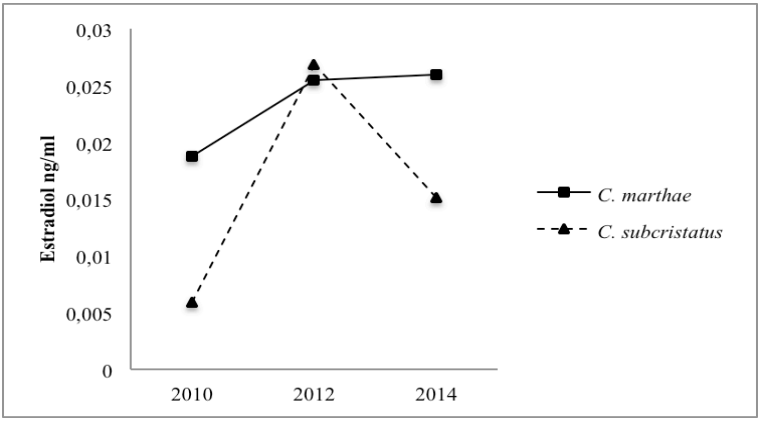

Figure 5. 17β-estradiol patterns in *Conolophus marthae* and *C. subcristatus* during three sampling seasons. Concentrations are reported as median.

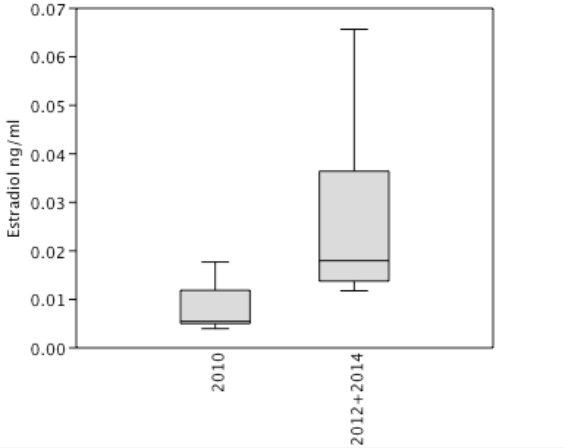

Figure 6. 17β-estradiol plasma levels in non-pregnant females of July (2010) and June (2012+2014) in *C. subcristatus* (median ± SD).

4) Discussion

One of the main findings of our study is represented by the observation of a contrasted pattern of progesterone plasma levels in the congeneric land iguanas *C. subcristatus* and *C. marthae*. As for *C. subcristatus*, despite difference in the analytical tools, the observed patterns and order of magnitude of hormone concentration levels are consistent with those found by Rubenstein and Wikelski (2005) in *A. cristatus*, predictably changing during the mating and nesting periods. In fact, in *A. cristatus* progesterone increases during the mating period as related to oviduct vascularity and pregnancy maintenance. Indeed, a role of progesterone in pregnancy maintenance has been documented in many reptiles (Highfill and Mead 1975; Arslan et al. 1978; Naulleau and Fleury 1990; Bonnet et al. 2001; Taylor et al. 2004). *Colonophus subcristatus* showed significant changes of progesterone and estradiol levels throughout the three reproductive seasons considered in our study. High plasma progesterone levels in June 2012 and 2014 contrasted the low progesterone levels observed in July 2010.

The ultrasound analyses indicated that in July 2010 no sampled females carried eggs, whereas those sampled in June 2012 and 2014 were at different reproductive stages, with many females carrying eggs at various stages of development (64% in 2012, 50% in 2014). The high number of females carrying eggs in 2012 and 2014 associated with high progesterone plasma levels, and the correspondent absence of egg-carrying females in July 2010, when we observed low progesterone levels, could be sufficient to assert that reproduction of *C. subcristatus* from V. Wolf is still ongoing in June and has ended by July. The difference in the variance of progesterone levels of females at stage d (2012+2014 versus 2010) is consistent with such a scenario. In fact, while the ultrasound analysis did not allow to discriminate if the lack of eggs would indicate mating phase or occurred deposition, hormonal profiles were informative especially when we pooled June 2012 and 2014 together and compared the pooled sample with July 2010. The higher variance in progesterone plasma levels exhibited by non-carrying-egg females in June could testify for the presence of females in different reproductive conditions: (i) females in which progesterone could have dropped after deposition (Taylor et al. 2004); (ii) females that did not reproduce, suffering the low hormonal levels typical of non-receptive females (Vitousek et al. 2010); (iii) females still in a mating phase, when hormone concentration is lower than in early nesting period but higher than in a post deposition condition (Rubenstein and Wikelski 2005). The presence in June of females still in a mating phase is also suggested by the lack of a difference in plasma P4 levels between total egg-carrying females and non-egg-carrying females of June. Such a difference emerged instead when total egg-carrying females and non-egg-carrying females of July were compared.

Further support to the described scenario is also provided by the analysis of estradiol. In fact, we know that in *A. cristatus* estradiol peaks during the mating period and strongly declines during all nesting phases (Rubenstein and Wikelski 2005). In *C. subcristatus* sampled in July, we did not observe egg-carrying females and both progesterone and estradiol levels were very low. Furthermore, estradiol concentration in non-egg-carrying females was lower in July than in June, when also higher variance was observed. This strongly suggests that in June many females had high levels of estradiol because still in a mating phase (probably in last copulation stage),

when E2 influences the vitellogenesis process (Edwards and Jones 2001; Guillette et al. 1997; Ott et al. 2000; Rubenstein and Wikelski 2005), receptivity, attractivity (Mason and Adkins 1976; Rhen and Crews 2000; Winkler and Wade 1998), and aggressive behaviours against males attempting to copulate again (Rhen et al. 1999; Woodley et al. 2000a; Woodley and Moore 1999b; Rubenstein and Wikelski 2005). In July, all females had concluded the breeding season and abandoned the nest sites, showing very low levels of estradiol. Other studies on reptiles showed that extremely low estradiol levels emerge especially in post-parturition phase (Jones and Guillette 1982; Taylor et al. 2004).

Of course, reproduction in *Conolophus* may vary between years and locations as influenced by environmental conditions and resource availability (Snell et al. 1984), as in many reptiles (Laurie 1990; Vitousek et al. 2010). Our data indicate that the laying season in *C. subcristatus* from V. Wolf may occur in June-July, as in Fernandina Island. Interestingly, these are the only two known sites where *C. subcristatus* reproduces in those months. Fernandina and V. Wolf are also among the westernmost volcanos in Galápagos. If this correlates with particular climatological and environmental conditions that may affect reproduction remains to be uncovered.

The pattern of progesterone plasma levels in *C. marthae*, opposed to that of *C. subcristatus*, and the presence of egg-carrying females in 2010 could suggest a slightly delayed reproduction in the pink species compared to the congeneric syntopic population.

Overall, in *C. marthae* we observed a significant increase of progesterone levels in egg-carrying females. Also in this case the role of P4 in pregnancy maintenance was clear. Furthermore, plasma progesterone concentration significantly varied with stage of gravidity; in fact, it increased when eggs reaching complete maturation (shell and yolk formation). This observation suggests a role of the *corpus luteum* and its primary product (progesterone) in the shell secretion as occur in many reptiles during pregnancy (Ferguson 1985; Guillette and Jones 1985; Guilette et al. 1989). Anyway, although in *C. marthae* we found a positive relationship between egg-development stages and hormone levels, we observed constantly low comparable levels of progesterone through the three sampled years (2010-2012-2014), limited number of egg-carrying females and reduced number of eggs, compared to *C. subcristatus*. The

concentration of estradiol was higher in *C. marthae* than in *C. subcristatus*, but no difference across years was observed in *C. marthae*. The association of progesterone with the pregnancy status was a signal of the ovarian system functioning. In oviparous reptiles, the *corpora lutea* persist during pregnancy producing progesterone (Norris and Lopez 2001) and, in *C. marthae* a higher production of progesterone during pregnancy appeared. Furthermore, in the pink species, estradiol level seemed higher at early stage of egg maturation; this is in agreement with the evidence that in reptiles estrogens are secreted by vitellogenic ovarian follicles to then decline as eggs remain in the uterus (McNicol and Crews 1979; Etches and Petitte 1990; Norris and Lopez 2010). However, although in *C. marthae*, hormonal profiles of progesterone and estradiol provide physiological evidence of a hormonal change at different reproductive stages, they did not allow the identification of a specific reproductive period.

Based on our data, for *C. marthae,* we could hypothesize the absence of a specific breeding season. The pink iguana could employ opportunistic reproductive strategies dependent on environmental conditions or interspecific interactions with *C. subcristatus*. Generally, reptiles tend to time egg incubation when a favourable season with minimum physiological stresses and maximum food resources is present. In pink iguana, we could hypothesize an individual opportunism as commonly occurs in other vertebrates (Milton et al. 2004) with females respond to stressful conditions varying reproductive period and clutch size. We could hypothesize that the lack of observed recruitment for this species (Gentile 2012) may be due to a limiting factor as for example predation on hatchlings and juveniles by hawks (*Buteo galapagoensis*) or feral cats (*Felis catus*). Indeed hawks and feral cats are constantly present on volcano and they are already described as cause of mortality in the marine iguana (Laurie and Brown 1990).

However, no *C. marthae* female resulted in reproductive conditions after a recent ultrasound surveys performed in November 2015 on a small number of healthy pink iguanas, or indirect evidence of reproduction activity (homospecific pairs, sperm at the cloaca of males and females) was found. This is in contrast with observation of such evidence in June/July (Gentile et al. 2016). Thus, for this reason, it is most likely that at present *C. marthae* may suffer from lack of

effective reproduction and the population results in attrition (Gentile 2012).

To conclude, our results suggest that hormonal profiles are fundamental to improve the knowledge on the reproductive biology of wild populations, especially when long-term observations are impossible. Our data indicate that *C. subcristatus* presents a specific and recognizable breeding season on Volcán Wolf. This season reaches its peak in June and concludes in July. In the same period, *C. marthae* shows reproductive activity, but the combination of hormonal profiles and ultrasound analysis demonstrated that such activity does not result in high numbers of reproductive females. Although opportunistic reproductive strategy cannot be completely ruled out for *C. marthae*, effective reproduction in this species seems hampered, determining attrition. In this regard, it is clear that further investigations are needed, especially aimed at uncovering the relationship between area of distribution, habitat characteristics and its usage, by tracking movements of individuals over time. This will help to locate nesting sites, currently unknown, and guide future conservation action for this critically endangered species.

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CHAPTER 3

Relationships between leukocyte profiles and *Hepatozoon* infection in Galápagos land iguanas, *Conolophus marthae* and *Conolophus subcristatus*

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1) Introduction

Parasites can directly impact the host physiology and behaviour by exerting important pressures on several aspects of host population dynamics as growth (Holmes 1982; Hudson et al. 1998) spatial distribution (Price 1980; van Riper et al. 1986) and reproductive success (Schall 1996; Pacejka et al. 1998; Amo et al. 2004). Haemoparasites frequently occur in ectothermic vertebrates and include protists, prokaryotes and viruses that inhabit the bloodstream with both intra-erythrocytic and extracellular forms. Blood parasites are very common in reptile species, with intracellular sporozoan haemogregarines being among the commonest (Telford 1984; Smith 1996). The genus *Hepatozoon* (Apicomplexa: Adeleorina), with more than 300 species reported infecting animals, represents the widest distributed haemoparasite group (Telford 1984; Smith 1996; Baneth et al. 2003; Vilcins et al. 2009; Tomé et al. 2012). The transmission of these intra-erythrocytic parasites typically occurs via the ingestion of an infected invertebrate containing sporocystis/sporozoites, followed by merogonic development in host internal organs (Telford 1984; Smith 1996). Very little data are available on the effects of haemogregarines on reptile hosts (Schall 1986; Manwell 1977; Sorci 1995), but they seem capable of provoking significant inflammatory responses (Wozniak and Telford 1991; Stacy et al. 2011) and diseases as hemolytic anemia (Telford 1984).

Commonly, culicine and anopheline mosquitoes, mites and ixodid ticks have all been shown to be potential vectors of transmission (Telford 1984). Ectoparasites as mites and ticks not only affect the host by transmitting blood parasites (Wozniak et al. 1996; Oppliger and Clobert 1997; Main and Bull 2000), but they may also directly affect both body condition and survivorship by causing lesions, blood loss, and anemia (Hair et al. 1992; Bull and Burzacott 1993; Goldberg and Holshuh 1993; Wikelski 1999; Fitze et al. 2004).

The immune system is the primary defence mechanism through which the organism protects itself from pathogen attacks, and leukocytes or white blood cells (WBC) are the crucial mediators of the immune response (Lobato et al. 2005; Davis et al. 2011). For the maintenance of an effective immune defence network, WBCs move continuously within tissues, through the bloodstream, destroying or neutralizing actively invading microorganisms (Dhabhar 2000).

Reptilian leukocytes can be classified as granulocytes (heterophils, eosinophils, basophils), and mononuclear cells (lymphocytes, monocytes, azurophils) (Stacy et al. 2011; Nardini et al. 2013). Azurophils are cell type unique to reptiles; however, most researchers group them with monocytes (Hawkey and Dennett 1989; LeBlanc et al. 2000; Davis et al. 2008).

Lymphocytes compose up to 80% of leukocyte types in many reptile species (Sypek et al. 1988; Stacy et al. 2011). They are highly specific immune cells involved in a variety of immunological functions such as synthesis and secretion of immunoglobulins and antigen elimination (Campbell 1996; Davis et al. 2008; Stacy et al. 2011). Heterophils (30% to 45% of leukocytes) are the primary immune phagocytosing cells entering the tissues during the inflammatory response (Jain 1993; Campbell 1995; Strik et al. 2007; Davis et al. 2008) by participating in destroying microorganisms with oxygen dependent or independent mechanisms (Thrall et al. 2012). The remaining WBCs are represented by: (i) eosinophils, associated with allergic inflammation (Thrall et al. 2012) and parasitism (Jain 1993; Strick et al. 2007); (ii) monocytes which are phagocytic cells associated with chronic infection often caused by parasites or bacteria (Gregory et al. 2004; Davis et al. 2008 Stacy et al. 2011); (iii) basophils, whose function is not well understood but seem to be involved in viral infections (Sypek et al. 1988; Strick et al. 2007; Stacy et al. 2011).

Overall, alterations in leukocytes number have been commonly described as a result of infections and diseases, and the characterization of leucocyte profiles (i.e. the relative numbers of different leukocyte types in the peripheral blood) is particularly useful in the field of conservation biology to describe an altered health status (Wakelin 1996; Davis et al. 2004; Davis et al. 2008). Although the response of leukocytes appears to be species- or genus-specific (Johnstone et al. 2012), general increase in heterophils and decrease in lymphocytes are observed in response to various stressors including parasitic infections (Maxwell and Robertson 1998; Ots et al. 1998; Davis et al. 2008; Müller et al. 2011). The magnitude of alteration of these WBC profiles depends on the intensity and persistence of the stressor (Averbeck 1992; Vleck et al. 2000). Since numbers of heterophils (H) and lymphocytes (L) are affected by the same stressors with opposite trends, the measurement of H/L ratio is commonly used

as diagnostic tool for assessing long-term stress in vertebrates (Gross and Siegel 1983; Maxwell 1993; Maxwell and Robinson 1998; Lobato et al. 2005; Davis et al. 2008; Xuereb et al. 2012; Lentfer et al. 2015).
Leukocyte H/L ratio have been not frequently used to demonstrate links between stress and parasites in reptilian wild populations. More often, haemoparasitic infections in reptiles were put in relationship with individual growth rate and body condition (Madsen et al. 2005; Ujivari and Madsen 2006; Curtis and Baird 2008). Some studies have clearly identified ecto-parasites as cause of mortality and in these cases, body mass and survivorship reduction have been observed (Sorci and Clobert 1995; Klukowski 2004). However, often the detrimental effects of ecto/endo-parasitic infections are not reported (Christian and Bedford 1995; Brown et al. 2006; Schlaepfer 2006; Sperry et al. 2009).
In the present study we analyse the impact of potential stressors of ecto- (*Amblystoma* spp.) and endo-parasites (*Hepatozoon* spp.) on health and haematologic parameters of two synthopic populations of Galápagos land iguanas present on Volcán Wolf (Isabela Island): *Conolophus marthae* (the pink iguana) (Critically Endangered, IUCN Red List), and *Conolophus subcristatus* (the yellow iguana) (Vulnerable, IUCN Red List). We are aware of the poor knowledge about patterns of natural variation of haematological parameters in reptile wild populations and their relationship with parasites. Specifically, we aim to test the impact of *Hepatozoon* spp. present in Galápagos Islands with more than one species (Bataille et al. 2012) and on Volcán Wolf with unique haplotypes (Gentile et al. in prep.) with respect to leukocyte profiles, haematocrit and body condition index. Moreover, we used a population of *C. subcristatus* occurring in a coastal area where ecto-parasites and haemoparasites are marginally present (Bahia Urbina), as "blank" condition for haematologic comparisons.
To our knowledge, this is the first time that such a diverse array of haematologic measures, during a study period of five years, have been used to estimate the impact of parasites on the heath of free-living iguanas species.

2) Materials and methods

2.1) *Study area and species*

The study was conducted in two different areas of Isabela Island: the Volcán Wolf (1.707 m.), the highest peak (1,707m) in the Galápagos archipelago located on north side of the island, and Bahia Urbina, a touristic costal area situated on the west side (Fig. 1).
Land iguanas were sampled from both areas. Instead, samples of *C. marthae* were obtained exclusively from the volcano, where it endemically occurs on the top and along the northwest slopes. The results of this study are based on samples collected on Volcán Wolf in five years, May 2006, 2009, July 2010, June 2012, 2014, and samples collected in Bahia Urbina only during June 2014.

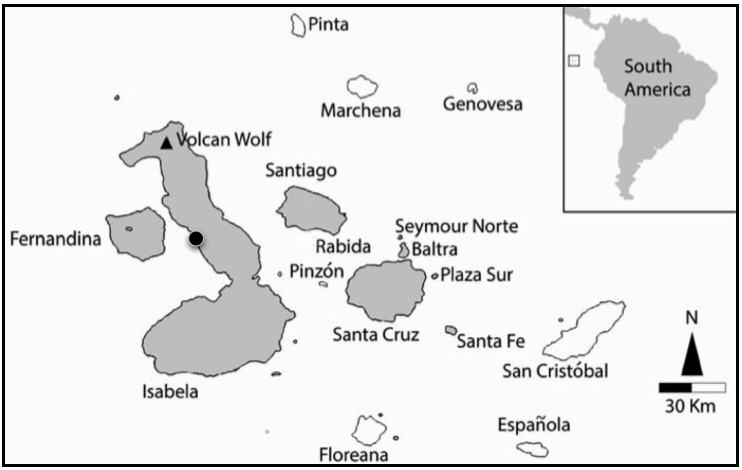

Figure 1. Galápagos Islands. The triangle indicates the volcano Wolf, the circle the coastal area Bahia Urbina where samples were collected.

2.2) *Sampling and blood collection*

We collected 2 mL from the caudal vein of each iguana using a heparinized syringe. We placed approximately 10 microliters of blood on the top of a slide and created a smear. Blood smears were air-dried, stored, and then stained following a modified Romanowky method (Work et al. 1998).
A portion of collected blood was placed in a microhaematocrit capillary tube and centrifuged for 2 minutes at 2000 rpm, to determine the proportion of red blood cells (haematocrit or PCV). The remaining blood was stored for additional studies.
After bleeding, each iguana was weighed and measured from the snout to the vent (snout-vent length, SVL). In order to explore the effects of parasites on nutritional state, we estimated an index of body condition (BCI) as body mass/ SVL^3 x 10^6 (Laurie 1989; Wikelski and Trillmich 1997, Romero and Wikelski 2001; Costantini et al. 2009).
Only in 2012 and 2014 we counted the number of ticks by scanning the armpits of each individual.
After all measurements and before they were released, iguanas were branded and PIT tags with a unique alphanumeric codes were implanted subcutaneously in each individual. Such marking allowed us to recognize individuals from year to year.

2.3) *Leukocyte formula and detection of parasites*

Each slide were returned to the laboratory and examined under oil immersion objective (x100). Data were collected from area of the smear where a uniform erythrocyte distribution and no cellular overlapping occurred. We described the leukocyte formula (proportion of different types of leucocytes) of each individual by examining a total of 100 leucocytes, as assessing the proportion of heterophils (H), eosinophils (E), basophils (B), lymphocytes (L), and monocytes (M). Azurophils were not considered. After the cell count, the H/L ratio was calculated for each iguana.
We analysed the parasitemia by recording the number of haemogregarine-infected erythrocytes observed in 20 minutes, time during which approximately 100 fields and 5 x 10^5 red blood cells were encountered (Valkiūnas et al. 2008). If no haemoparasites were detected after 20 minutes, the individual was classified as uninfected.

Such a method proved sufficiently efficient after testing parasitemia by a PCR-based approach (Fulvo 2010).
When the analysis of parasite load of each individual was completed, we estimated epidemiologic parameters as: (i) the intensity of infection as the percentage of infected red blood cells found in approximately 10000 cells (Godfrey et al. 1987); (ii) the prevalence as the percentage of individuals with infection; (iii) the incidence as the proportion of new infection cases within the study period, that is the percentage of individuals not infected in the past, but found infected after recapture.

2.4) *Statistical analysis*

We used the Shapiro-Wilks method to test normality. Where normality was not met, data were log-transformed.
Chi-squared contingency tests were used to compare parasite prevalence among years and species.
To determine whether endo-parasites affected leukocyte profile of *C. marthae* (*CM*) and *C. subcristatus* (*CS*) populations in Volcán Wolf, we performed general linear model (GLM) analises. Haematological parameters (H, L, E, M, H/L, parasitemia) were the response variables. We considered the following predictor variables (and their interaction): species, sex, and season (May: 2006+2009; June: 2012+2014; July: 2010). When a single white blood cell type was considered as a response variable the model included also parasitemia as covariate. GLMs were used to determine differences in haematological parameters (H, L, E, M, H/L, parasitemia) between the Volcán Wolf and Baia Urbina populations of *CS* by including the site as a further predictor factor. Since Bahia Urbina was sampled only in June 2014, the variable season was set as random factor nested within variable site. Least-Significant-Difference (LSD) post hoc pairwise comparisons were performed to test for between-group differences.
As information about ticks were collected only in two years, we used Pearson's correlation to evaluate possible relationship between ecto-parasitic and endo-parasitic loads, as well as to evaluate a possible relationship with leucocyte profile variations.
All analyses were performed by using Past (version 3.07 for MAC) and STATISTICA (StatSoft, version 8 for Windows) software with two tails and alpha set to 0.05.

3) Results

3.1) *Interspecific comparisons (Volcán Wolf)*

All sampled iguanas presented ticks on armpits, either at larval/nymphal or adult stages. We did not observe differences in the number of ticks between the study species (t = - 0.45, P = 0.6). No correlation between ticks and hemoparasites abundance was found or between tick load and haematological markers (for both species, $P >$ 0.05).
On Volcán Wolf, the prevalence of infection by *Hepatozoon* was very high in both species, ranging between 69.4% - 87.5% (Tab. 1; Fig. 2).

Year	*n*	% infected	Parasitemia		Ectoparasites	
			Mean	SE	Mean	SE
C. marthae						
2006	16	87.5	9.7	3.1	-	-
2009	79	87.3	16.2	3.6	-	-
2010	56	83.9	20.3	3.5	-	-
2012	82	76.8	29.3	6.4	40.9	2.3
2014	62	77.4	17.1	5.3	53.1	4.4
C. subcristatus (W)						
2006	25	72	160.4	133.5	-	-
2009	77	79.2	45.8	8.9	-	-
2010	20	85	168.4	97	-	-
2012	72	69.4	97.4	24.1	41.6	2.5
2014	53	75.4	30.6	8.5	53	2.9
C. subcristatus (BU)						
2014	31	9.7	8.6	7.6	0.19	0.08

Table 1. Prevalence, parasitemia and ticks load for *Conolophus marthae* and *Conolophus subcristatus* during the entire study period.

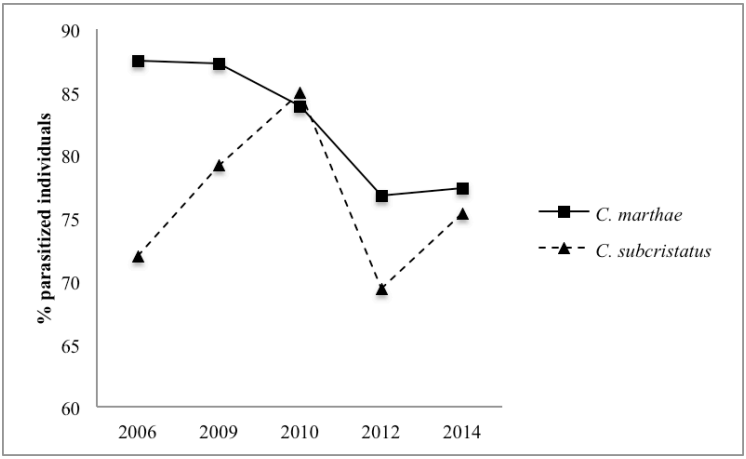

Figure 2. Percentage of individuals with infection by *Hepatozoon* (prevalence) during the study period.

Prevalence did not change over the study period (*C. marthae*: X^2 =3.9, df = 4, P = 0.4; *C. subcristatus*: X^2 =3.5, df = 4, P = 0.4) and between species (X^2 =3.44, df = 1, P = 0.080). In general, both species presented low values of infection intensity (*CM* 0.1%; *CS* 0.5%), with most iguanas showing low parasite loads (Fig. 3 - 4). For both species we observed high incidence of infection: 60% in *CM* (3 out of 5 recaptured individuals), 66% in *CS* (2 out of 3 recaptured individuals). We never observed the disappearance of *Hepatozoon* from an infected individual.

Parasitemia was higher in *CS* than *CM* ($F_{1,527}$ = 9.029, P = 0.003). It varied along the study period ($F_{2,527}$ = 4.553; P = 0.011), but no difference was found between species in the temporal pattern ($F_{2,527}$ = 1.925; P = 0.147). Both species presented a significant difference between the peak in July and the minimum in May (P = 0.044).

Lymphocytes (L) were the most numerous cells in the leukocyte differential count followed by H, M, E and B in both species (Table 2).

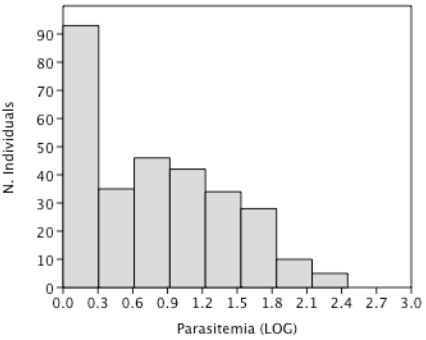

Figure 3. Distribution histogram of parasitemia in individuals of *Conolophus marthae.*

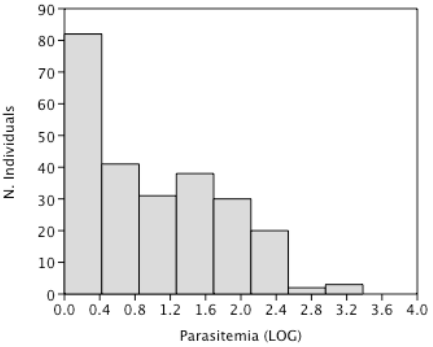

Figure 4. Distribution histogram of parasitemia in individuals of *Conolophus subcristatus.*

Infection status	Leukocyte parameters	*C. marthae*				
0		2006	2009	2010	2012	2014
	n	2	10	9	19	14
	Lymphocytes (%)	53 ± 6	53.7 ± 3.9	71.1 ± 4.8	62.4 ± 4.7	65.1 ± 3.9
	Heterophils (%)	35.5 ± 4.5	34.9 ± 3.4	21.4 ± 4.4	29.9 ± 4.2	23.5 ± 3.2
	Eosinophils (%)	4.5 ± 1.5	5.3 ± 0.9	0.5 ± 0.3	3.2 ± 0.6	3.1 ± 0.7
	Monocytes (%)	6.5 ± 3.5	5.5 ± 1.2	6.7 ± 1.1 0	4.4 ± 1	7.5 ± 0.9
	Basophils (%)	0.5 ± 0.5	0.6 ± 0.2	0	0	0.1 ± 0.1
	H/L ratio	1.3 ± 0.4	0.6 ± 0.1	0.3 ± 0.09	0.9 ± 0.4	0.4 ± 0.1
1	n	14	69	47	63	48
	Lymphocytes (%)	40.7 ± 5	53.3 ± 1.7	67.3 ± 2.2	51.8 ± 2.8	67.8 ± 2.2
	Heterophils (%)	42.7 ± 3.9	34.2 ± 1.5	24.6 ± 1.8	37.4 ± 2.3	21.8 ± 1.7
	Eosinophils (%)	12.4 ± 1.9	4.9 ± 0.4	2.1 ± 0.3	3.9 ± 0.5	3.1 ± 0.3
	Monocytes (%)	3.7 ± 0.6	7 ± 0.4	5.9 ± 0.5	6.7 ± 0.7	6.8 ± 0.5
	Basophils (%)	0.4 ± 0.2	0.5 ± 0.08	0.1 ± 0.04	0.03 ± 0.02	0.2 ± 0.05
	H/L ratio	1.5 ± 0.3	0.8 ± 0.06	0.4 ± 0.05	1.9 ± 0.7	0.4 ± 0.05
		C. subcristatus				
0		2006	2009	2010	2012	2014
	n	7	16	3	22	13
	Lymphocytes (%)	40.3 ± 4.1	52.3 ± 3.4	86.3 ± 0.3	47.9 ± 4.3	68.6 ± 4.3
	Heterophils (%)	43.3 ± 4	36.8 ± 2.9	9.6 ± 0.3	45.3 ± 4	21.8 ± 3.7
	Eosinophils (%)	9.7 ± 2.3	5.1 ± 0.7	0	1.7 ± 0.3	2.3 ± 0.7
	Monocytes (%)	6 ± 1.9	5.3 ± 0.9	4	5 ± 0.8	7.1 ± 1
	Basophils (%)	0.7 ± 0.3	0.4 ± 0.1	0	0.04 ± 0.04	0.07 ± 0.07
	H/L ratio	2.7 ± 1.1	0.8 ± 0.1	0.1	1.8 ± 0.6	0.4 ± 0.08
1	n	18	61	17	50	40
	Lymphocytes (%)	34.6 ± 2.5	49.7 ± 2	60.1 ± 2.9	47.2 ± 2.7	54.6 ± 3
	Heterophils (%)	47.5 ± 2.2	38.9 ± 1.5	30.6 ± 2.4	44.4 ± 2.3	32.1 ± 2.3
	Eosinophils (%)	13.2 ± 1.5	5.1 ± 0.4	2.8 ± 0.5	2.9 ± 0.7	4.8 ± 0.8
	Monocytes (%)	4.1 ± 0.7	5.9 ± 0.6	5 ± 1	5.4 ± 0.9	8 ± 0.6
	Basophils (%)	0.4 ± 0.2	0.4 ± 0.09	0.6 ± 0.2	0.08 ± 0.05	0.3 ± 0.09
	H/L ratio	1.5 ± 0.1	1 ± 0.1	0.5 ± 0.07	1.4 ± 0.2	0.9 ± 0.1

Table 2. Leukocyte profiles in *Conolophus marthae* and *Conolophus subcristatus* according to different degrees of *Hepatozoon* infection (0: uninfected; 1: infected). (*n*: number of smears analysed).

Conolophus subcristatus showed higher H ($F_{1,525} = 10.820$; $P = 0.001$) and H/L ratio ($F_{1,525} = 3.713$; $P = 0.053$) than *CM*. H, L and H/L ratio varied among seasons (H: $F_{2,525} = 19.345$; $P \simeq 0$; L: $F_{2,525} = 13.173$; $P \simeq 0$; H/L: $F_{2,525} = 12.697$; $P \simeq 0$), with no difference between species (species*season: $F_{1,525} = 0.467$; $P = 0.495$). Specifically, H and H/L ratio were significantly lower in July, while L showed the opposite pattern (for all post hoc tests $P \simeq 0$). With the only exception of E, all

white blood cells were influenced by parasitema, with contrasting trends: positive for H ($F_{2,525}$ = 4.160; P = 0.042) and H/L ratio ($F_{2,525}$ = 4.639; P = 0.032), and negative for L ($F_{2,525}$ = 3.960; P = 0.047) and M ($F_{2,525}$ = 4.035; P = 0.045).
In both species we did not observe either significant correlations between PCV and parasitemia (*CM*: P = 0.2; *CS*: P = 0.4) or differences of PCV between infected and non-infected individuals (*CM*: P = 0.2; *CS*: P = 0.2).
BCI did not differ between species but only between sexes, resulting higher in males of both species ($F_{1,535}$ = 16.216; P = 0.0001). Moreover, BCI did not differ between infected and uninfected individuals in both species and not correlate with parasitemia or tick load (for all tests, $P \geq 0.05$).

3.2) *Site comparisons (C. subcristatus)*

Overall, in Bahia Urbina we observed a lower number of ticks than in Wolf (t = 46, $P < 0.00001$) as well as a lower number of individuals infected by *Hepatozoon* (prevalence 9.7%; n = 31; Tab. 1).
Parasitemia was significantly higher in volcano than in Bahia Urbina by site ($F_{1,272}$ = 44.29, $P \simeq 0$) and season ($F_{1,272}$ = 3.774, P = 0.024). H, L and H/L ratio did not differ between sites (for all parameters $F_{1,272} \leq 1.138$, $P \geq 0.372$) but showed clear site-specific temporal patterns (season(site) - H: $F_{2,271}$ = 8.599, $P \simeq 0$; L: $F_{2,271}$ = 5.749, P = 0.017; H/L: $F_{2,271}$ = 10.028, $P \simeq 0$). Specifically, H and H/L ratio in Bahia Urbina (June) were lower than H and H/L ratio observed in V. Wolf in May (H: $P \simeq 0$; H/L: P = 0.00001) and June (H: P =0.0005; H/L: P = 0.0003), whereas no difference emerged between Bahia Urbina and V. Wolf (July) for both H and H/L ratio (H: P = 0.43; H/L: P = 0.97) (H/L: Fig. 5). L showed an opposite pattern with higher values being observed in V. Wolf in May (P = 0.00002) and June (P = 0.0003), while no difference was observed between B. Urbina and the July sample from V. Wolf (P = 0.99). BCI significantly differed between sites resulting higher in Bahia Urbina ($F_{2,272}$ = 66.153, $P \simeq 0$).

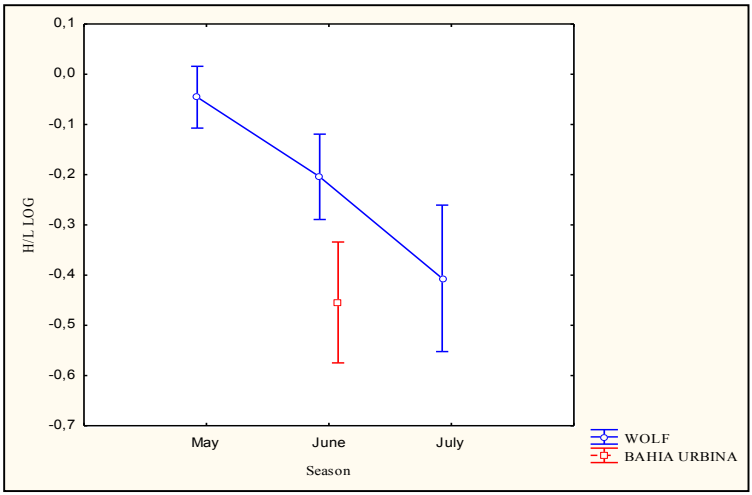

Figure 5. Pattern of H/L ratio in relation to season and site in *C. subcristatus.*

4) Discussion

Both populations of land iguanas displayed a high prevalence of infection by *Hepatozoon* with low levels of parasitemia during the whole study period. *Hepatozoon* never disappeared from infected individuals. On the contrary, many "new infection" cases occurred (incidence values were high for both species: *CS* 66%, *CM* 60%). Generally, once infected, free-living animals exhibit a relatively stable presence of haemoparasites over long periods (Smallridge and Bull 2000). For haemogregarines, elimination or decrease of parasitemia in reptiles has been observed in few studies (Smallridge and Bull 2000; Salkeld and Schwarzkopf 2005). Indeed, these haemoparasites, as many long-live protozoa parasites, persist throughout the host's life (De Biasi et al. 1989; De Vieira Santos et al. 2005). However, factors explaining long-term haemogregarine persistence in vertebrate hosts are poorly known. As already described for haemogregarine protozoans in snakes and turtles, we can hypothesize two scenarios: (i) cases of continuous re-infection with constant replenishment of

asexual multiplicative stages of parasite in host tissues, or (ii) liberation of dormant merozoites with consequential transformation into gametocytes (Jakes et al. 2003; Široký et al. 2004).

Some expected positive relationships could not be confirmed by our study. For example, we could not provide evidence of a positive relationship between the number of ticks and the number of specific types of leucocytes or in particular with H/L ratio, as observed in Lobato et al. (2005). We also found no effect of body size on H/L ratio, consistently with what observed in Mole Salamanders by Davis and Maerz (2008). However, we observed that even low levels of parasitemia, although they did not impact body condition index and haematocrit, they did affect leukocyte profiles, with heterophilic white blood cells showing the prevalent role in the immune response in *Conolophus*. Specifically, we observed a tendency of two most abundant white blood cells (H, L) to inversely respond to *Hepatozoon* infection as expected when immune system is activated against haemogregarines (Xuereb et al. 2012). Generally, in reptiles and birds, the proportions of heterophils and therefore H/L ratio show the most extreme changes under different levels of infection (Aguirre et al. 1995; Figuerola et al. 1999; Davis et al. 2004) and, in general, an increased proliferation and differentiation of these phagocytic leukocytes occur to enhance the response to infection (Thrall et al. 2012; Davis et al. 2008). The fact that *Conolophus* iguanas respond to parasites by showing a higher number of circulating heterophils (with the more parasitized *C. subcristatus* showing higher heterophils proportion than the less parasitized *C. marthae*) may reflect a high degree of activation of the innate immune system and a dependence of H proliferation with the magnitude of infection. Coherently, the opposite trend of lymphocytes could be interpreted as a stress-induced redistribution of L from the blood to lymphatic tissues or other organs as commonly described in vertebrates during a stress condition (Dhanhar et al. 1996; Dhabhar 2002). Nevertheless, we observed the minimum peak of H and H/L ratio in July when the parasitemia reached its maximum value. Thus, we hypothesize that endoparasites are not the only factor affecting this stress marker. The comparison between two populations of *C. subcristatus* allowed us to further explore this unexpected result.

On the volcano we observed a high H and H/L ratio in June when many females were reproductively active, and the minimum H/L ratio

during July, when *CS* apparently is completing the reproductive activity (Onorati et al. submitted). Our data could suggest that the stress experienced by iguanas during reproduction contribute together to parasites infection to the observed leukocytes' patterns. In fact, when we analysed *C. subcristatus* populations in July, we observed no differences in leukocyte profile between that from Bahia Urbina, considered the "blank condition" (almost no *Hepatozoon* and outside the breeding season), and that heavily parasitized from Volcán Wolf. This result could indicate that, even though *Hepatozoon* causes an activation of the immune system especially by augmenting phagocytic heterophils, the substantial difference in H/L between June and July could be due to the stressful-perceived reproductive phase. This result is consistent with studies of reproduction-induced stress in other ectotherms, where reproductive status determined increase in H/L ratio (Kilgas et al. 2006; Davis and Maerz 2008). In fact, we suggest that iguanas can have the physiological capability to modulate the activation of their immune system to different stressors: reproduction and endo-parasitic infection, in our case. Iguanas showed a strong activation of the immune system when they entered an energetically costly phase such as reproduction. The increased work of the immune system during breeding could directly result in a consecutive strong infection as soon as reproduction terminated and the immune response toned down, as occurred in July. Certainly, this issue deserves further attention and an in-depth investigation is in order to clarify if different intensity of contemporary acting stressors may result in a finely tuned immune response.

In conclusion, besides giving reference for future studies of iguanas in the wild, this study delivered background information important for the conservation of these iguana species and provided a substantial advancement in the knowledge of the impact of *Hepatozoon* in *Conolophus* species. The constancy of prevalence of infection across years suggests a stable host-parasite interaction which iguanas seem able to cope with. In fact, despite *Hepatozoon* has an impact in *Conolophus*, as indicated by WBC counts, the response of the immune system in relation to reproduction seems stronger.

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CHAPTER 4

Effects of parasitic infection and reproduction on corticosterone plasma levels in Galápagos land iguanas, *Conolophus marthae* and *Conolophus subcristatus*

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1) Introduction

In recent years, glucocorticoid levels have been increasingly used as physiological indices of individual and population health (Wingfield et al. 1997; Romero 2004; Walker et al. 2005; Wikelski and Cooke 2006; Bonier et al. 2009). Elevated baseline levels have been observed in animals facing both environmental (Foley et al. 2001) and anthropogenic disturbances (Creel 1997; Wingfield and Romero 2001). Generally, high levels of glucocorticoids are related to individuals or populations in worse health status (Bonier et al. 2009).

Glucocorticoids (GCs) are steroid hormones secreted in response to a multiplicity of stressors (Sapolski et al. 2000). When an internal and/or external environmental change occurs, the hypothalamic–pituitary–adrenal (HPA) axis stimulates the secretion of GCs by the adrenal glands to help organism in responding to stressful conditions (Wingfield et al. 1997; Wingfield and Ramenofsky 1999; Wingfield and Romero 2001; McEwen and Wingfield 2003; Wingfield and Sapolsky 2003; Wingfield 2013). Glucocorticoids are the final product of the HPA axis and participate in the control of homeostasis activating immediate life-saving processes (Romero et al. 2009). Normally, short-term glucocorticoid releases are helpful for individual survival because stimulate both physiological and behavioural emergency mechanisms exclusively oriented to overcome the perturbation (Wingfield and Romero 2001; Wingfield and Sapolsky 2003). However, long term activation of the stress response with chronically elevated GCs concentrations could be prejudicial. Prolonged elevated concentrations could expose the individual to a long-term overstimulation of survive mechanisms with consecutive inhibition of many fundamental functions including reproduction, growth, and immunocompetence (Sapolsky 1987; Wingfield et al. 1997; Dhabhar 2000; Sapolsky et al. 2000; Dallman and Bhatnagar 2001). Therefore, persistent high levels are usually detrimental to health, increasing the stress-related disease and pathology (Romero et al. 2009).

In reptiles, the corticosterone (CORT) is the primary adrenal glucocorticoid hormone produced to promote advantageous responses against stressful events (Greenberg and Wingfield 1987; Hanke and Kloas 1995). Many stressors have been observed to produce effects on CORT levels including physical factors (Lutterschmidt and Mason

2009; Telemeco and Addis 2014; Refsnider et al. 2015) and biotic stressors as predation (Thaker et al. 2009), social competition (Comendant et al. 2003) and parasitic infections (Dunlap and Schall 1995; Hanley and Stamps 2002; Sperry et al. 2009). However, the interpretation of stress response throughout CORT requires always great attention as its plasma levels change according to not only stressor-dependent factors (duration and intensity) but also individual-dependent factors (sex and reproductive status) that have to be considered when GCs are used as physiological indices of condition in wild populations (Breuner et al. 1999; Romero 2002; Moore and Jessop 2003). Because many factors play a role in defining the individual baseline corticosterone levels, to fully understand the consequences of specific stressors on endocrine activity, it is important to examine responses under different individual contexts (e.g. reproductive state), investigating concomitantly additional correlates as the immune system, whose connection with stress and glucocorticoids have been already well established (Dhabhar 2002; Martin 2009).

In this study we examine the corticosterone levels alterations that accompany haemoparasitic infections in two syntopic populations of Galápagos land iguanas living on Volcán Wolf (Isabela Island): *Conolophus marthae* (here the pink iguana) (Critically Endangered, IUCN Red List) and *Conolophus subcristatus* (here the yellow iguana) (Vulnerable, IUCN Red List). A high-density population of ticks (*Amblyomma* spp.) occurs in the area (Schatz 1991). Ticks are known to be potential vectors of haemoparasitic transmission (Telford 1984). Specifically, intracellular sporozoan haemoparasites, as haemogregarines of the genus *Hepatozoon*, are the most common reptilian haemoparasites transmitted by ticks.

The genus *Hepatozoon* (Apicomplexa: Adeleorina) is present in Galápagos Islands with different species (Bataille et al. 2012). Very little data are available on the effects of haemogregarines on reptile hosts (Schall 1986; Manwell 1977; Sorci 1995), but they seem able of provoking significant inflammatory responses (Wozniak and Telford 1991; Stacy et al. 2011) and diseases as hemolytic anemia (Telford 1984).

We also used a population of *C. subcristatus* occurring in a coastal area where notoriously the presence of parasites is missing (Bahia Urbina), as “blank” condition for endocrinological comparisons. Thus,

if glucocorticoids secretion is a function of infection, we expect the highest baseline corticosterone plasma levels in most parasitized population on the volcano. Moreover, considering the complex scenario relating to activity of adrenal gland, to better interpret the observed glucocorticoids patterns, we simultaneously analysed: (a) heterophyls/lymphocytes ratio commonly used as haematological marker of stress in several reptile species (Duggan 1981; Moberg 1985; Xuereb et al. 2012), (b) reproductive status of females through the sonographic identification of eggs, as CORT could increase during the egg-laying season (Wack et al. 2008), (c) testosterone plasma levels in males, which have been associated with increased parasite load because of its immunosuppressive activity (Folstad and Karter 1992; Saino et al. 1995; Salvador et al. 1996).

2) Materials and methods

2.1) *Ethic statement*

Animal manipulation and blood sampling were performed according to a protocol that minimized animal stress, in accordance with the European Community guidelines and with the approval of the Galápagos National Park. Samples were exported and imported under the CITES permits 101/BG and IT/IM/2015/MCE/01711, respectively.

2.2) *Field sites and sampling sessions*

The study was conducted in two different areas of Isabela Island: the Volcán Wolf, the highest peak (1,707m) in the Galápagos archipelago located on north side of the island, and Bahia Urbina, a touristic costal area situated on the west side (Fig. 1).
Iguana's blood samples were collected in Volcán Wolf (W) in July 2010 and June 2012, 2014; and in Bahia Urbina (BU) in June 2014.

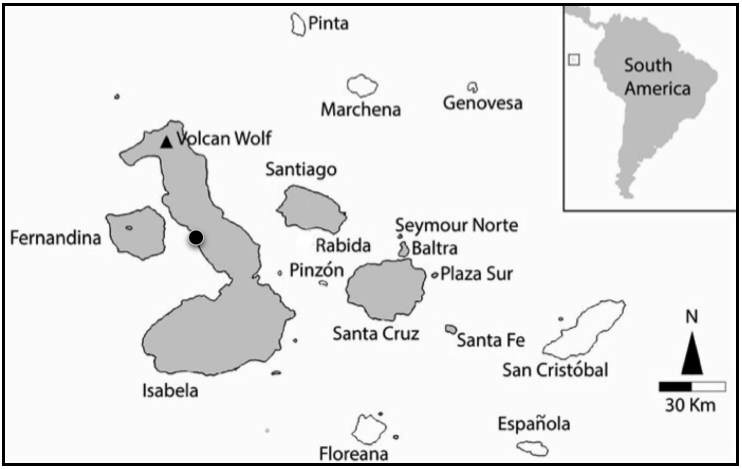

Figure 1. Galápagos Islands. The triangle indicates the volcano Wolf, the circle the coastal area Bahia Urbina where samples were collected.

2.3) *Field phase*

During all field sessions, 2 mL of blood was collected from the caudal vein of each individual using a 5 ml heparinized syringe. Blood samples were collected within 3-5 min from capture, under the assumption that this represent a sufficiently short time for corticosterone levels to represent baseline concentrations (Romero and Romero 2002). In fact, this time interval is sufficiently short to prevent that plasma levels of corticosterone be biased by capture stress (Wingfield et al. 1997; Sapolsky et al. 2000; Romero and Wikelski 2001; Romero 2004; Cash et al. 1997; Tyrrell and Cree 1998).
We placed approximately 10 microliters of blood on the top of a slide and created a smear. Blood smears were air-dried. Blood samples were placed on ice immediately after collection and later centrifuged for 2 minutes at 2000 rpm to separate the plasma. Each iguana was weighed and snout-vent length (SVL) was measured. The body condition index (BCI) was then estimated as the ratio of body mass/snout-vent length $(SVL)^3 \times 10^6$ (the ratio was multiplied by 10^6 to reduce the number of decimals). This index has been already used

for iguana species (Laurie 1989; Wikelski and Trillmich 1997, Romero and Wikelski 2001; Costantini et al. 2009). For each female we determined the number of eggs, egg size, and the stage of development of follicles using a Sonosite portable ultrasound machine (FUJIFILM SonoSite, Inc.) as in Onorati et al. (submitted). We determined the reproductive state of each female, differentiating between reproductive (egg-carrying) and non-reproductive (without eggs) females. We distinguished different reproductive stages: (stage a) females showing follicles with eggs of homogenous, spherical and small dimensions, (stage b) females showing larger, yet not fully-formed, unshelled eggs, (stage c) females showing large, fully-formed, shelled eggs; non reproductive females when no eggs were visible (stage d).

2.4) *Laboratory phase: haematological analysis*

Blood smears were stained following the Romanowsky method, with modifications (Work et al. 1998) to later count white blood cells (WBCs). We counted a total of 100 leukocytes. Cells were classified as heterophils, monocytes, basophils, eosinophils or lymphocytes. We calculated specifically the heterophil to lymphocyte ratio (H/L).
We determined the parasitemia recording the number of erythrocytes infected by *Hepatozoon* (so far the only known haemoparasite infesting *Conolophus* spp., Fulvo 2010) observed in 20 minutes. If no haemoparasites were observed after this time, the individual was classified as uninfected.

2.5) *Laboratory phase: hormonal analysis*

Despite radioimmunoassay (RIA) is a common method for quantifying the steroids hormones in vertebrates, we determined plasma levels of corticosterone and testosterone by competitive enzyme-linked immunosorbent assays (ELISA), for the reasons mentioned in Onorati et al. (submitted).
All ELISA immunoassays were performed at the Laboratory of Clinical Biochemistry (Tor Vergata University Hospital). Plasma samples were preserved at -40°C until assayed. For corticosterone we used five kits ELISA (KA0468) pre-coated with a polyclonal antibody. We used 10 µl of plasma diluted with 90 µl of assay buffer. The

detection limit was established to be 0.28 ng/mL. The intra-assay variation was 4.1% and the inter-assay variation 10.1%.
For testosterone we used four kits ELISA (KA0309) pre-coated with a monoclonal antibody. We used 50 µl of plasma for the detection of the hormone. The detection limit was established to be 5.67 pg/mL. The intra-assay variation was 10%, the inter-assay variation 11.3 %.
All samples were assayed in duplicate and randomly distributed between plates. All assays were performed according to the instructions of the kit manufacturers.

2.6) *Statistical analysis*

The analyses were performed by the STATISTICA 8 package for Windows, and Past version 3.07 for MAC.
Log-transformed values of all hormonal and haematological parameters were used to obtain normal distributions. We used one-way ANOVA with Tukey's HSD (Honest Significant Difference) post hoc pairwise comparisons in order to analyse differences in parasitemia and corticosterone plasma levels among years.
We tested for statistical differences of parasitemia, body condition index and H/L ratio between infected and uninfected and among sexes with unpaired Student's *t* test.
Generalized linear models (GLZs) with an identity-link function were performed to evaluate which factors better explained the variation of corticosterone and testosterone plasma levels. Females and males were analysed separately in GLZ models, as in vertebrates sex differences in adrenocortical activity have been described (Kirschbaum et al. 1992; Kudielka and Kirschbaum 2005). For corticosterone, for both sexes we built two different models, one pooling *C. marthae* (*CM*) and *C. subcristatus* (*CS*) living on the volcano and one pooling both populations of *CS* (W+BU). Testosterone was analysed only on males.
For males, all models included species (or site for model regarding only *CS* populations) as categorical factor and body condition index, parasitemia and H/L ratio as covariates. For females, all models included species (or site for model regarding only *CS* populations), reproductive state (yes or no) as categorical factor, and body condition index, parasitemia, H/L ratio as covariates. We tested also for the interaction between species and reproductive state.

3) Results

3.1) *Corticosterone and parasitemia*

Parasitemia of both species is shown for sex in Table 1.
Overall, *C. subcristatus* showed a higher value of parasitemia than *C. marthae* (t = - 4.3; P = 0.00005).
For both species, no difference in parasitemia was found among years, in males or females (for all $P > 0.05$). Not even a difference between sexes emerged (*CM*: t = 1.7, P = 0.08; *CS*: t = 0.9, P = 0.3). In both species we did not observe a significant difference in corticosterone plasma levels of infected and uninfected individuals when pooling together sexes and years (*CM:* t = 0.7, P = 0.5; *CS*: t = 1.2, P = 0.8). Parasitemia did not explain the variance of corticosterone plasma levels in both males (Wald = 0.005, df = 1, P = 0.94) and females (Wald = 0.74, df = 1, P = 0.39) on Wolf volcano and also considering only populations of *CS* (W+BU) (females: Wald = 0.02, df = 1, P = 0.88; males: Wald = 2.74, df = 1, P = 0.09). Only in *CS* of the volcano a positive correlation between H/L ratio and parasitemia emerged (r = 0.27; P = 0.04). In both species, BCI did not differ between infected and uninfected (*CM*: t = -0.7, P = 0.5; *CS*: t = 0.8, P = 0.4).

Sex	Species Year	N	Parasitemia		
			Mean	SE	Median
Females	*CM* (W)				
	2010	9	30	10.8	15
	2012	11	44.4	24.5	14
	2014	18	27.7	13.3	9
	CS (W)				
	2010	4	98.2	43.6	101.5
	2012	11	59.4	34.5	11
	2014	14	46.8	20.8	17
Males	*CM* (W)				
	2010	8	22.1	8.1	14
	2012	11	17.1	6.6	11
	2014	23	8.5	3.1	3
	CS (W)				
	2010	4	48	65.8	19.6
	2012	11	28	33.5	12
	2014	12	41	25	3.5
Females	*CS* (BU)				
	2014	15	1.6	1.6	0
Males	*CS* (BU)				
	2014	12	0.08	0.08	0

Table 1. Parasitemia of *C. subcristatus* (*CS*) and *C. marthae* (*CM*) from Volcán Wolf and *C. subcristatus* from Bahia Urbina.

3.2) *Corticosterone plasma levels in females*

The reproductive state of females is reported in Table 2.
In females of *C. marthae* corticosterone plasma levels ranged from 0.16 to 74.11 ng/ml while in *C. subcristatus* from 0.22 to 158 ng/ml (mean and medians are shown in Table 3).
For both species, we observed statistically significant differences among years (*CM*: $F = 6.3$; $P = 0.004$; *CS*: $F = 5.9$ $P = 0.007$). The pair-wise Tukey's HSD tests indicated respectively: in *CM* the highest CORT levels in 2012 ($P_{2012\text{-}2010} = 0.03$, $P_{2012\text{-}2014} = 0.007$), in *CS* a higher concentration in 2012 than in 2010 ($P_{2012\text{-}2010} = 0.009$). On the volcano, the variance of corticosterone levels was explained only by reproductive state (Wald = 7.89, $df = 1$, $P = 0.005$, Fig. 2) and by its interactive effect with species (Wald = 4.55, $df = 1$, $P = 0.03$, Fig. 3). Corticosterone variance was not statistically explained by H/L, although P value was borderline (Wald = 3.54, $df = 1$, $P = 0.059$).
No variable explained the variance of corticosterone levels in females of *CS* (W+BU) (all $P > 0.09$).
We did not observe a significant effect of body condition index in corticosterone variations (all $P > 0.2$).

Species	Year	Reproductive stage				Tot
		a	b	c	d	
CM	2010	0	0	3	6	9
	2012	0	1	0	10	11
	2014	3	0	1	14	18
CS	2010	0	0	0	4	4
	2012	0	0	10	1	11
	2014	1	1	8	5	15

Table 2. Reproductive states of females living on volcano.

Sex	Species	N	Reproductive state	Corticosterone ng/ml		
	Year		N	Mean	SE	Median
Females	*C. marthae*					
	2010	9	3	3.8	2.8	0.9
	2012	11	1	13.4	6.7	4
	2014	18	4	8.9	1.4	0.6
	C. subcristatus					
	2010	4	0	2.2	0.8	1.9
	2012	11	9	75.1	19.5	105
	2014	15	10	16	7.7	5.1
Males	*C. marthae*					
	2010	7	31.1	31.1	27.9	2.7
	2012	11	1.1	1.1	0.4	0.5
	2014	23	1.9	1.9	0.7	0.7
	C. subcristatus					
	2010	4	5.4	5.4	3.9	2.1
	2012	11	20.5	20.5	9.7	10.6
	2014	16	13.7	13.7	9.4	0.8

Table 3. Corticosterone plasma levels in C. *marthae* and *C. subcristatus* from Volcán Wolf.

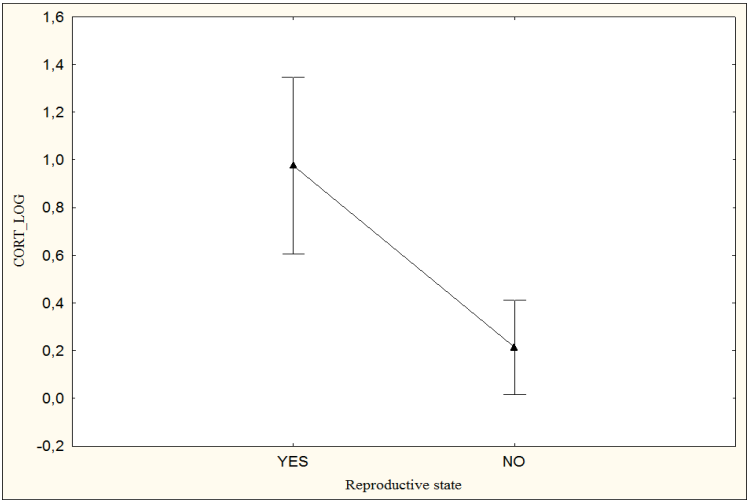

Figure 2. Corticosterone and reproductive state.

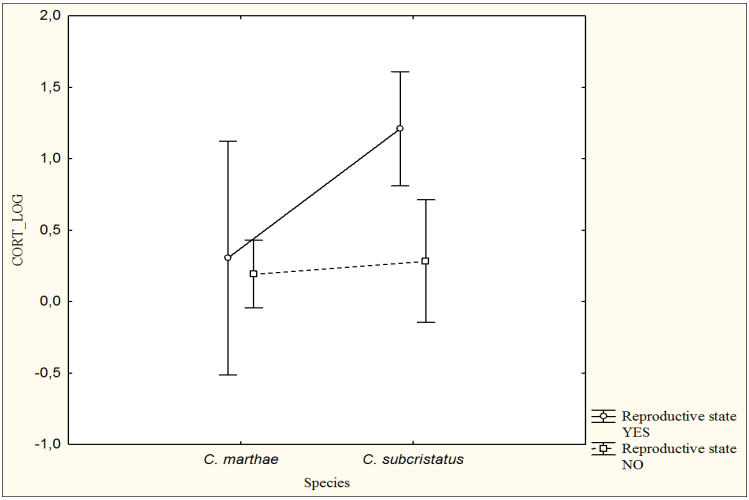

Figure 3. Corticosterone variation in relation to the interactive effect between species and reproductive state.

3.3) *Corticosterone and testosterone plasma levels in males*

In males of *C. marthae*, corticosterone plasma levels ranged from 0.21 to 13.9 ng/ml while in *C. subcristatus* it ranged from 0.19 to 153 ng/ml (mean and medians are shown in Table 3). For none of the two species we observed statistically significant differences among years (*CM*: $F = 1.5$, $P = 0.2$; *CS*: $F = 2.3$, $P = 0.1$). On Volcán Wolf, the variance of corticosterone levels was explained only by H/L ratio (Wald = 7.35, $df = 1$, $P = 0.007$). Site was the only explanatory variable of corticosterone variance for the model with pooled populations of *C. subcristatus* (Wald = 10.35, $df = 1$, $P = 0.001$). Males from Bahia Urbina showed higher corticosterone plasma levels than those from the volcano (Fig. 4). As for females, corticosterone variance in males was not statistically explained by H/L, although *P* value was borderline (Wald = 3.64, $df = 1$, $P = 0.056$).

We did not observe a statistically significant correlation between plasma levels of corticosterone and testosterone (for both populations on the volcano $P > 0.05$). In the GLZ, species was the only explanatory variable of variance in testosterone levels on the volcano (Wald = 4.62, df = 1, $P = 0.03$); in particular males *CM* showed higher testosterone concentrations than males *CS*. Parasitemia and H/L ratio did not explain variation in testosterone plasma levels on the volcano (Parasitemia: Wald = 0.05, $P = 0.82$; H/L ratio: Wald = 0.03, $P = 0.86$).

We did not observe a significant effect of any of the considered factors on testosterone levels for the model with pooled populations of *C. subcristatus* (all $P > 0.3$).

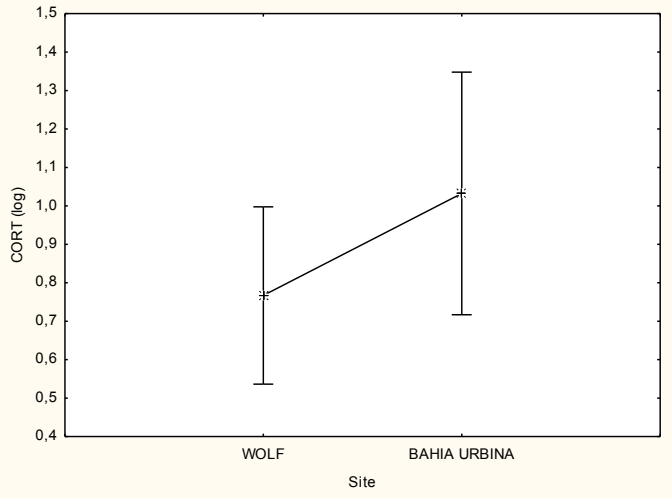

Figure 4. Corticosterone variation in *Conolophus subcristatus* in relation to site.

4) Discussion

In this study we used corticosterone plasma levels to investigate possible impacts of *Hepatozoon* on the stress physiology of Galápagos land iguanas; however, we did not find any evidence in support of a relationship between the level of haemoparasite infection and glucocorticoid plasma concentration in *C. marthae* and *C. subcristatus* on Volcán Wolf. In fact, in both species, we did not observe significant differences in baseline corticosterone levels or body condition index between infected and uninfected individuals. Only in males we observed a difference between sites, but unexpectedly, the higher corticosterone concentration was found in the population of Bahia Urbina, almost not impacted by *Hepatozoon*. These results indicate that the intensity of haemoparasite infection is not reflected in the glucocorticoid levels of Galápagos land iguanas.

However, the unexpected difference in stress hormone levels between V. Wolf and Bahia Urbina calls for attention. Bahia Urbina is a tourist coastal area regularly visited by humans; in contrast V. Wolf is a

restricted area where no tourism is allowed. Tourism could be a hypothetical factor that could explain the different corticosterone levels at the two sites. However, we are aware that the two sites may also differ for other characteristics. Thus, we cannot distinguish whether the differences that we observed are due to population-dependent factors, such as different social status and/or habituation capacity (Romero 2004), site-dependent factors as tourism (Romero and Wikelski 2002), and environmental parameters (Smith et al. 1994; Romero and Wikelski 2001) that could differ between sites. For this reason, we need to further investigate this aspect. Anyway, this comparison between populations allows us to exclude a chronic stress condition due to haemoparasites. We could hypothesize that the level of haemoparasitism that we observed might not meet the required threshold to activate endocrine responses. We know that these parasites could be well adapted to their natural hosts and sometimes are considered not to cause stress and disease (Nardini et al. 2013). However, despite elevated corticosterone plasma concentration with parasitemia was not detected, in *C. subcristatus* an increase in H/L ratio was found. Thus, in the yellow iguana, an activation of immune system especially in phagocytic cells emerged, as reported for many parasitized vertebrates (Davis et al. 2004; Lobato et al. 2005).

Most studies on stress physiology, including the present investigation, were based on correlative data. In reptiles, as in other vertebrates, a number of factors affect the adrenocortical activity (Wingfield et al. 1992; Romero 2002; Moore and Jessop 2003). In this study, plasma level of corticosterone positively correlated with reproductive condition in females. Corticosterone levels appeared elevated in females carrying eggs (small, not fully-formed or fully-formed) in both land iguana species. Also in marine iguanas, CORT was mostly elevated during the gestation and nesting period before eggs were laid, to decline significantly immediately after egg-laying (Rubenstein and Wikelski 2005). However, the connection between reproductive condition and plasma glucocorticoid levels is not fully elucidated even for the better-studied groups as fish and birds (Lattin et al. 2016). In our study, the observed increase of CORT in reproductive females could reflect the energetic demands of reproduction (Wingfield 1988). This would be consistent with the energy mobilization hypothesis of Wingfield and Ramenofsky (1999), according to which corticosterone concentrations are highest during periods that require energy supply.

However, CORT does not appear as a simple mediator of energy reserve mobilization for reproduction. An increase of CORT seems to have a role in the eggs production process. This hypothesis is supported by the observation that, while all non-reproductive females showed similar corticosterone baseline levels without a difference between species, in reproductive females we observed a difference in favour of the yellow iguana, in which most reproductive females (90%) showed mature eggs. This CORT increase in reproductive females with fully-formed eggs could be the direct consequence of a metabolic change required for egg development, as already described in other reptiles (Wilson and Wingfield 1992). Indeed, generally, a positive association between reproductive state and glucocorticoids level has been observed for many egg-laying vertebrates (Silverin and Wingfield 1982; Wilson and Wingfield 1992; Wack et al. 2008). Many studies demonstrated glucocorticoid involvement in processes related directly to egg development (vitellogenesis, oocyte maturation, ovulation) (Grassman and Crews 1989; Moore and Jessop 2003; Taylor et al. 2004). This is the first study where a significant relationship between reproductive condition and baseline plasma corticosterone levels is described for *Conolophus* species.

Another issue we investigated concerned the testosterone plasma levels in males as opposed to corticosterone levels. Several studies of non-mammalian vertebrates have shown that exogenous corticosterone can reduce plasma testosterone levels to varying degrees (Moore and Zoeller 1985; Wingfield and Silverin 1986; Tokarz 1987). However, plasma levels of testosterone and corticosterone can also rise simultaneously (Orchinik et al. 1988). These opposing results underline that the interpretation of relationship between adrenal and gonadal axis is not simple. In males of pink iguana we observed higher testosterone levels than yellow iguanas. However, a difference in corticosterone levels never emerged between two species. The lack of correlation between individual corticosterone and testosterone levels indicates that the highest testosterone levels we observed in *C. marthae* are partially independent from stress hormones. In most vertebrates, full expression of aggressive behaviour requires elevated basal levels of gonadal steroids (Book et al. 2001). Increases in testosterone are associated with more sexual and aggressive behaviour (Miles et al. 2007). In our study, the higher testosterone levels in *C. marthae* are in agreement with observations

in the field, where males appeared more aggressive and challenging after being released than the congeneric yellow males. Testosterone can be involved in aggression associated with establishment and maintenance of a breeding territory (Wingfield and Marler 1988; Wingfield et al. 1990). This is conceivable to occur in *Conolophus* as well, even though the lack of a relationship between parasitemia, H/L ratio and testosterone variation would not support the immunosuppressive action generally associated to high level of such a hormone.

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CHAPTER 5

Conclusions

This study had an important place in field research since it was the first investigating the endocrine and physiologic aspects of Galápagos land threatened iguanas.
Along the first part of PhD project, the analysis of sex steroid hormones progesterone (P4) and 17β-estradiol (E2) allowed the description of a specific breeding season on Volcán Wolf for *Conolophus subcristatus*, while for *C. marthae*, effective reproduction in this species seems hampered, determining attrition.
The gain of knowledge in reproductive biology permitted also the interpretation of stress responses of both immune and endocrine system. In fact, the identification of breeding season permitted controlling a possible confounding factor, as reproduction, in the study of stress dynamics.
The second step of the project regarded the stress physiology. This was the first study addressing this issue, with different stress markers, in *Conolophus* iguanas. Thanks to haematologic profiles, it was described how *Hepatozoon* caused an activation of immune system especially of phagocytic heterophils forms; nevertheless it was also clarified that both reproduction and parasites infection contributed to the observed leukocytes' patterns. In this study, the physiological capability of iguanas to modulate the activity of the immune system in response to different stressors (reproduction and endo-parasitic infection) emerged explicitly for the first time in reptiles. Another aspect that emerged in these iguanas' species during the stress physiology analysis was that H/L ratio appeared to be a more persistent indicator of stress by parasites than the more sensitive corticosterone levels. The stress hormone levels rose exclusively as a response to a natural and life-threatening situation as reproduction. These results confirmed that the role of glucocorticoids during reproduction is complex and that the interaction between adrenal and gonadal systems can be important for reproductive efficiency, dispelling the assumption that adrenal activation is necessarily deleterious. Overall, I proved that baseline corticosterone and H/L ratio cannot be used interchangeably as indicators of stress by parasites, but together can provide a comprehensive picture about the

stress status of iguana species in *in-situ* studies.
The final additional result of this study was of methodological implication. In fact, while the specific goals of the project varied between two different parts of thesis (reproduction and stress physiology), both had in common the methodological approach that was the use of ELISA (enzyme-linked immunosorbent assay) for analysing plasma steroid hormone levels. This study successfully validated ELISA method in land iguana species. This procedure appeared to be a good candidate for an alternative method of more expensive and dangerous RIA (radioimmunoassay), and this will be useful in future for endocrine studies concerning steroid hormones in other iguana species.
In conclusion this study improved the knowledge of the reproductive biology of *Conolophus* species. The gained information will be the basis for further researches aimed to reveal possible reproductive difficulties especially for the new and threatened species *C. marthae*. Additionally, this study provided a comprehensive assessment of stress physiology related to parasites, providing sensible data for the implementation of future conservation actions.

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