Environmental and neuroendocrine control of seasonal phenotypes and song behavior of an equatorial songbird

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Author contributions

Here I present my doctoral research that was performed at the Max Planck Institute for Ornithology, Seewiesen. The whole study was carried out from May 2010 until February 2016. For the first three studies (**Chapters 1-3**), I performed most of the field work, data collection, laboratory work, data analysis and writing of the manuscripts under guidance of my supervisor Prof. Dr. Manfred Gahr, who also contributed to conceive the study, field work, scientific discussion and writing. Dr. Frédéric Sèbe contributed to the field work and data collection (**Chapters 1-3**). Dr. Maria Luisa da Silva contributed to field work and helped with acquisition of permits necessary to perform field work in Brazil (**Chapters 1-3**). Particularly in **Chapter 1**, Dr. Elizabeth Yohannes contributed making the analysis of stable isotopes and interpreting the results. Dr. J. Marcelo Brazão-Protázio contributed with the statistical analysis of dawn-song timing. Finally, **Chapter 4** was performed in collaboration with Dr. Wolfgang Goymann. I executed most of the experiment, data collection and writing of the manuscript, Dr. Wolfgang Goymann contributed with conceiving the study, data collection and writing. Monika Trappschuh contributed with the development of experimental techniques and hormone analysis. Prof. Dr. Manfred Gahr contributed with the manuscript writing. All authors approved the final manuscripts written and published.

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Summary

At higher latitudes, as a result of the predictable and ubiquitous nature of the annual photoperiod, most temperate-zone bird species have converged in using day length as a primary environmental cue to time seasonal processes. Instead, in equatorial biomes the environmental fluctuations have usually a more heterogeneous degree of seasonality. Hence, the neural and endocrine mechanisms that mediate the occurrence of seasonal behaviors and life history stages might operate in diverse manners among equatorial songbirds. In the present thesis I investigated the regulatory mechanisms underlying the annual cycle and song behavior of the equatorial silver-beaked tanager, an endemic Amazon songbird with an entirely tropical phylogenetic background. Thereby, I sought to study proximate regulatory mechanisms that have likely evolved with small photoperiodic cycles. I show that males inhabiting the equatorial zone of the eastern Amazon, a highly productive region that surrounds the Amazon estuary, have marked seasonal schedules of molt and dawn-song behavior. This region imposes singular environmental conditions for birds, including unconstrained food availability over the year. Accordingly, male silver-beaked tanagers exhibited uniform annual levels of baseline corticosterone (**Chapter 1**). However, individual baseline corticosterone and testosterone levels are clearly associated with the molting status of males. On the other hand, males exhibited important seasonal changes in testis size and gonadal testosterone production, which denote a strong seasonal breeding pattern (**Chapters 1 & 2**). Particularly interesting is the neuroendocrine regulation of seasonal dawn-song, a behavior directly involved in the breeding territoriality of males, whose seasonal activation appears as decoupled from the seasonal peak of testosterone levels in plasma that occurs later in the breeding (**Chapter 2**). Further, although at equatorial latitudes environmental photic cues have a very small magnitude of variation over time, they do seem to influence the occurrence of dawn-song in male silver-beaked tanagers on a daily basis, as well as at a seasonal level. Besides this, males also integrate the occurrence of rainfall cycles as an environmental signal for the timing of seasonal phenotypes and dawn-song. Finally, in **Chapter 4** I complemented my PhD research by proposing a new method of steroid hormone implantation to be used for the study of wild birds. Overall, the present thesis demonstrates a concerted orchestration of seasonal events in equatorial males. This phenology of silver-beaked tanagers is mediated by conserved neuroendocrine regulatory networks that function with a different temporal and relational pattern when compared with widely studied temperate-zone bird species

General introduction

“Species must be defined as ranges of irreducible variation”

Stephen Jay Gould - The Flamingo’s Smile (1985)

Throughout their lives organisms undergo changes in structure, physiology and behavior, which occur in interaction with a changing environment. From a biological perspective, a living organism cannot be regarded as independent from its surrounding environment, because the environment and the organism actively codetermine each other (Lewontin, 1983). Therefore, organism-environment interaction is a fundamental topic of biology that unifies different research fields (Wingfield et al., 2011). A foundational aspect in this topic is to identify causal correspondences between the changes we observe over time in both organism and environment (Mpodozis et al., 1995). In this respect, the comparative research on neuroendocrinology offers a unique scenario, because the neural and endocrine systems mechanistically engage phenotypic changes in animals (i.e. in morphology and behavior) with external perturbations of the environment.

Animals in nature are exposed to seasonal fluctuations of the environment caused by geophysical cycles of the Earth. Inhabiting a seasonal world makes it necessary to time and integrate systemic processes such as reproduction, molting, migration or hibernation with the changing seasons (Wingfield, 2008). At temperate latitudes, where seasons are well differentiated, the predictable annual change in day length (photoperiod) is the primary cue used by animals to time seasonal phenotypes (Dawson et al., 2001; Gwinner, 1989). Besides, supplementary environmental cues such as temperature, food availability, precipitation and/or photic cues can be used to fine-tune the annual timing of seasonal traits (Dawson, 2008). In vertebrates this process involves perception of the seasonal environment through sensory mechanisms, neural transduction within the central nervous system, and neuroendocrine and endocrine cascades to regulate morphological, physiological and behavioral responses (Wingfield, 2012). Laboratory research on captive animals has greatly advanced our knowledge of how neuroendocrine networks regulate the expression of seasonal phenotypes. However, a more integrative understanding of how these systems mediate the influences of the environment must rely on studies of animals in nature. Most research on mechanisms underlying seasonal phenotypes in wild animlas has been carried out at the temperate regions, of northern hemisphere. This bias limits our knowledge about neuroendocrine functions, and impedes comparisons across species, latitudes, biomes and life histories.

Although neural and endocrine regulatory mechanisms are highly conserved across vertebrates, there must be diverse ways in which these systems can be adjusted to customize responses to different environmental conditions. How the neural and endocrine systems vary in regulating seasonal phenotypes under different environmental conditions is still an open question, particularly in tropical and equatorial habitat.

The overall purpose of my PhD thesis is to investigate environmental factors and neuroendocrine mechanisms underlying the occurrence of seasonal phenotypes, particularly seasonal song, of an equatorial songbird species, the silver-beaked tanager (*Ramphocelus carbo).*

Significance of birds as a study model

Most bird species are diurnal, conspicuous and rely on ornate visual and auditory signals to facilitate social interactions. In general, the taxonomic lineages and geographical distributions have been well determined for the majority of bird species around the planet. There are avian species inhabiting all regions and continents, in very diverse types of habitats. All these aspects have contributed to making birds a unique research model for biological research (Konishi et al., 1989). The well-documented natural history of birds has allowed scientists to address key questions about behavioral mechanisms. In the neuroendocrinology field, birds have a particularly rich history as research subjects (for reviews see Goodson et al., 2005; Schmidt, 2010; Wingfield, 2005a). Avian brain regions are important scientific references for the understanding of neural function's effects on behavior. Well known examples are the neural regions regulating sexual behaviors and the avian song control system of songbirds (Gahr, 2000; Nottebohm et al., 1976; Panzica et al., 1996). Some key findings include the demonstration of neurogenesis in the adult brains of songbirds (Nottebohm, 1989), the identification of a remarkable degree of hormone-related neuroplasticity (Balthazart et al., 2010; Gahr, 2004), and the localization of hormone receptors in structures of the avian brain that coordinate behavior (Bentley and Ball, 2000;

Bernard et al., 1999; Gahr, 2000). Especially important are the effects that deploy steroid hormones throughout the whole ontogeny of birds, acting on the sexual differentiation of brain and behavior and modulation of the adult phenotype (Gahr, 2004).

In this thesis I study an oscine songbird species. Almost every landmass on the biosphere, with the exception of Antarctica, is inhabited by a diversity of songbird species, Passeriformes of the oscine suborder. A unique attribute of oscine songbirds is their ability to produce songs that are usually learned from adult conspecifics. They possess a specialized neural circuit and vocal organs that allow them to produce diverse and elaborate song (Ball et al., 2004; Gahr, 2014).

Annual cycle of birds

Throughout the year, birds express suites of morphological, physiological and behavioral traits related to specific systemic processes, e.g. reproduction, molting and migration. These assemblages of correlated phenotypic traits are called “life history stages”. Each life history stage typically occurs at a particular time of the year in a recurrent way, comprising the annual cycle (Wingfield, 2008). Across bird species, the temporal organization of the annual cycle varies, depending on several factors such as the phylogenetic background, habitat characteristics and/or social system. However, a common aspect among these is the tight relationship between the annual cycle organization and the seasonality of the environment the birds inhabit (Wingfield et al., 2011). The pionering study of (Rowan, 1925) demonstrated for the first time that yearly variations in day length triggered onset of gonadal development in birds and regulated their migratory behavior. Later on, Aschoff (1955) implemented the concept of Zeitgeber (i.e., synchronizing cues) to propose that environmental stimuli can act as synchronizers of cyclic internal processes (Gwinner, 2003). For instance, next to day length, supplementary cues such as food availability, temperature, and rainfall, can also be used as a Zeitgeber to fine-tune the timing of life history stages with the local environment (Dawson, 2008; Wingfield, 2012).

The neuroendocrine control of the annual cycle of birds involves diurnal and seasonal rhythms entrained by environmental cues, as well as neural stimulation and inhibition of hormone secretion by experiences and behavior. Once released hormones feedback on the adult brain to influence neural activity and reversibly modulate gene expression (McEwen, 2012). “Environmental endocrinology” is a derived research area that emerged in an attempt to place the internal hormonal dynamics of animals in a broader context, by including natural environmental changes as a constituent part (Bradshaw, 2007). In recent years, this research area has gained important advances because of the development of assay techniques that permit hormones to be measured in small blood samples (Wingfield and Farner, 1975). Thus, many experimental studies in wild birds have demonstrated that natural annual variations of steroid hormone levels in the blood exert an important influence on the expression of life history stages. For instance, methods to manipulate hormones, like increasing the natural hormone level via artificial hormone implants, have been adopted to test the mechanisms of steroid hormones (Ketterson et al., 1996).

Although research in wild birds has greatly advanced our knowledge as to how steroid hormones mediate the expression of seasonal phenotypes, relatively little work has been done to understand these mechanisms in tropical or true equatorial birds. By studying these “non-traditional” models, we can expand our knowledge of the environmental and hormonal mediators of seasonal phenotypes, and gain insights concerning the diversification of avian life histories of birds.

Steroid hormones

Hormones are molecules synthetized and secreted by endocrine glands (or other specialized organs) and function as biochemical signals. A common feature of all vertebrates is the utilization of steroid hormones (SHs). Not only is the chemical structure of the steroid hormones conserved among taxa, but also their systemic functions (Baker, 2003). Early in development SHs exert enduring effects on the global organization of organisms by specifying processes such as sexual differentiation and behavioral ontogeny (Gahr, 2004). These persisting effects are frequently called organizational actions. As organisms mature, variations in steroid hormone signals can trigger detailed phenotypic transitions and influence regulatory processes such as metabolism, homeostasis or reproduction (Balthazart et al., 2010; Wingfield, 2005b). In doing so, SHs activate seasonal physiological and/or behavioral conditions. These transient effects of the SHs are termed activational actions.

The parent compound from which all SHs are derived is cholesterol. The synthesis of SHs is usually described in two broad stages: (1) cholesterol transport into the mitochondria and formation of pregnenolone and (2) pregnenolone metabolism by tissue-specific steroidogenic enzymes (Martinez-Arguelles and Papadopoulos, 2010). These processes are controlled by anterior pituitary trophic hormones, which stimulate the influx of cyclic AMP (cAMP) and calcium (Ca^{2+}) into the steroidogenic cells (Gallo-Payet and Payet, 2003; Garren et al., 1971; Issop et al., 2013; Jefcoate, 2002). This rise in intracellular cAMP and calcium results in the mobilization of free cholesterol. The SHs synthesis is initiated at the inner mitochondrial membrane where the conversion of cholesterol to pregnenolone is catalyzed by the enzyme P450scc (Cholesterol side-chain cleavage enzyme) (Miller, 2013; Stocco and Clark, 1996). Pregnenolone can then be converted to tissue-specific steroid hormones through steroidogenic enzymes (Issop et al., 2013). Pregnenolone is the precursor of five major classes of SHs In vertebrates: progestins, estrogens, androgens, glucocorticoids and mineralocorticoids. These SHs are grouped into categories according to organ source and/or physiological functions. The sex steroids (also called gonadal steroids) are androgens, estrogens, and progestogens. The adrenal steroids (synthesized in the adrenal gland) are the glucocorticoids and mineralocorticoids.

Seasonal rhythms in the systemic production of SHs have been shown in many bird species, having a strong impact in the control of the annual cycle and the seasonality of behavior. For instance, breeding events require recrudescence of the hypothalamic–pituitary–gonadal (HPG) axis, during which the hypothalamus increases secretion of gonadotropin-releasing hormone (GnRH) that stimulates the release of luteinizing hormone and follicle stimulating hormone from the anterior pituitary, resulting in gonadal activation and increased sexual steroid hormones production. It is well known that seasonal variations in androgens and estrogens activate courtship, copulatory and aggressive behaviors in males, including singing (Balthazart et al., 2010; DuVal and Goymann, 2011; Fusani and Gahr, 2006; Lehrman and Wortis, 1960; Lehrman, 1955). Similarly, the production of glucocorticoid hormones is controlled by the hypothalamic–pituitary–adrenal (HPA) axis, in which the hypothalamus release corticotropin-releasing hormone that stimulates the secretion of adrenocorticotropic hormone from the pituitary, influencing the glucorticoid production in the adrenal cortex. Glucocorticoids are involved in cyclic rhythms of energy mobilization, osmoregulation, and stress responsiveness (Cherel et al., 1992; Goldstein, 1993; Sapolsky et al., 2000; Wingfield et al., 1994), and in the regulation of behaviors such as migration, food and water intake, locomotor activity and fleeing (Cornelius et al., 2013; Fleming et al., 2004; Jenni et al., 2000; Romero, 2002).

Experimental steroid hormone manipulation

Some of the most-conclusive evidence about the actions of steroid hormones in regulating life history traits comes from experiments that have manipulated their levels in the plasma of individuals. Classical studies typically involved the removal of the natural source of the hormone (i.e. the gland) to investigate the effects on the behavior, physiology and morphology. The pioneering work of Berthold (1849) on the castration of the rooster is an iconic example of this approach. However, there are reasons for which such an approach might be inappropriate for field studies. For instance, a gonadectomy is a complicated procedure to perform in the field. In addition, it is an invasive operation that requires a recovery period for birds, which might interfere with the observations and conclusions. Thus, unless it is strictly necessary for research purposes, other experimental methods might be preferable. An alternative approach commonly used in free-ranging birds is to insert implants containing a particular steroid hormone and observe the effect on the trait in question (Edler et al., 2011; Ketterson et al., 1996; Wingfield, 1984). The most used methods to implant steroid hormone are silastic tubing (silicone tubing) or time-release pellets, which gradually release the hormone from a self-disintegrating matrix (Edler et al., 2011; Fusani, 2008). However, often the steroid hormone dosages used in such studies have been in a pharmacological range (especially during the first days after implantation), far beyond what an animal would experience under naturally relevant circumstances (Fusani, 2008). This drastic hormone manipulation is well justified within a purely mechanistic perspective, but when investigating hormone dependency of traits under more natural conditions, this approach may provide misleading results. Thus, no matter what method is chosen for the treatment, a measurement of the circulating concentrations of the hormone soon after implantation is recommended. The rationale of keeping the concentrations within physiological levels is particularly important when studying natural occurring traits.

An alternative approach to studying short-term effects of increased circulating testosterone within a physiological range is the use of GnRH injections. The GnRH is the hypothalamic-releasing hormone that causes the pituitary to secrete luteinizing hormone into the blood circulation, which then induces the testes to produce testosterone (Lacombe et al., 1990; Wingfield et al., 1979). A big advantage of this approach is that it serves as an indicator of gonadal activity in wild birds, since it induces short-term increase in plasma testosterone concentrations that reflect each individual's gonadal capacity to produce this hormone (from baseline to the potential maximum) during a particular life-history stage (Goymann et al., 2015).

Steroid hormone receptors in the avian brain

Comparative studies of SH actions on the adult avian brain has generated effective contributions to probe biological functions associated with brain plasticity and seasonal expression of behaviors (Balthazart et al., 2010; Gahr, 2014).

Through activational actions, SHs can influence changes in the cytoarchitectural, cytochemical and connectivity patterns among different brain nuclei. This brain plasticity can have diverse implications in the expression of cyclic behaviors (Balthazart et al., 2010; Gahr, 2014). One mode of action for SHs (the genomic mechanism) is the alteration of gene expression by binding to intracellular-located nuclear steroid receptors that function as ligand-activated transcription factors (Fig.1) (Carson-Jurica et al., 1990). Thus, SHs regulate expression of various genes in a network-like manner, which are involved in different systemic processes. In addition to functioning as ligand-activated transcription factors, SH receptors also have been shown to mediate rapid activation of non-genomic signaling pathways independent of their transcriptional activity (Falkenstein et al., 2000). The receptor mechanisms mediating these rapid effects are still not well understood. Some studies postulate the existence of membrane-bound receptors for the rapid action of SHs (Boonyaratanakornkit and Edwards, 2007).

Structure of steroid hormone receptors

The nuclear hormone receptors constitute a large, evolutionary-related family of proteins that are ligand-activated transcription factors. This family includes the receptors for steroid hormones (and also for thyroid hormones, vitamins A and D-derived hormones, retinoids and other fatty acids; (Kumar and Thompson, 1999). In general, SH receptors are characterized by their modular structure containing three regions: 1) the well-recognized and evolutionary-related DNA-binding domain, which anchors the receptor to its target

DNA; 2) The less closely related ligand-binding domains, which form the C-terminal part of the protein containing the second activation region that is essential for the ligand-dependent activation function; and 3) the quite diverse and poorly conserved amino-terminal domains, which are associated with a ligand-independent activation function (Mangelsdorf et al., 1995). Each of these domains has been shown to be capable of complex interactions with other proteins.

Mechanisms of action

The unliganded SH receptor is an oligomeric complex associated with several heat shock proteins functioning as chaperone proteins (McEwen, 1988; Thompson and Kumar, 2003). These interactions are requisite for proper protein folding and the stabilization of the unbound receptor, avoiding the aggregation and nonspecific binding of the receptor to DNA (Jensen, 1996). The specific binding of the hormone to its receptor induces the release of chaperone proteins, leading to a conformational change and the hyperphosphorylation of the receptor (Weigel and Moore, 2007). Consequently the receptor dimerizes and binds to a specific DNA sequence, known as the “Hormone Response Element”(Beato et al., 1987) . Binding of receptors to DNA increases or decreases gene transcription by altering the rate of recruitment of general transcription factors and influencing the recruitment of RNA polymerase II to the initiation site (Kininis et al., 2007). Thus, it is thought that the regulation of transcription occurs by a mixture of mechanisms, involving direct interactions of each receptor with specific DNA sequences, and with a variety of proteins. These include other sequence-specific DNA-binding transcription factors, the basal transcription factors, integrators of transcription, and various coactivators/corepressors (Charlier, 2009; Charlier and Balthazart, 2005). These do not seem to bind DNA directly, but they associate with activated DNA-bound nuclear receptors and participate in signal transduction of the basal transcriptional machinery (Bulynko and O’Malley, 2011; Perissi and Rosenfeld, 2005). So, hormones such as steroids, can act on the nervous system via their respective receptors to alter neuronal gene transcription, resulting in changes in physiology, morphology and behavior.

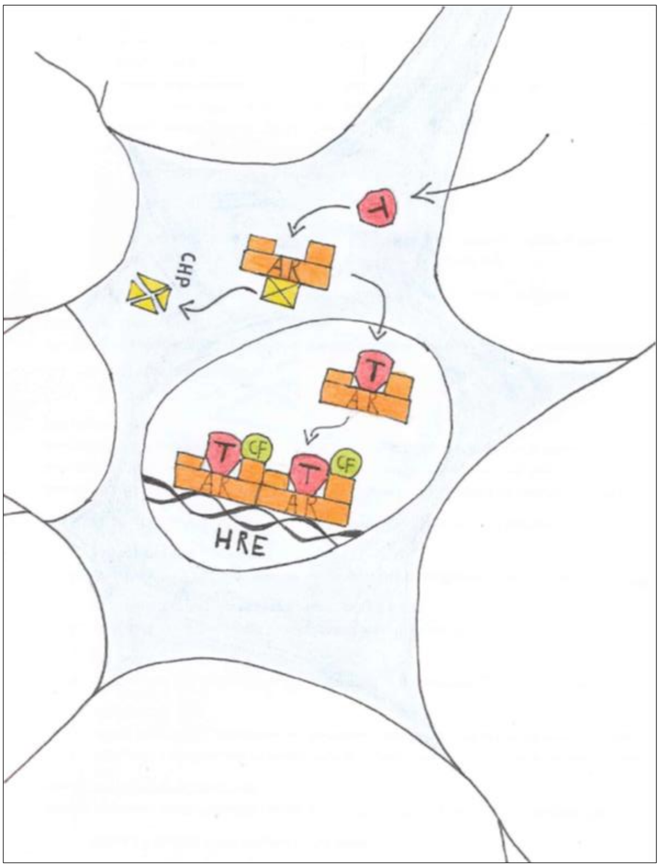

Figure 1. Representation of the genomic mechanism of action of androgen receptors in the neuron. Testosterone (T), or its metabolites, enters the cell and binds the modular androgen receptor (AR). Upon binding, the AR undergoes a conformational change and releases chaperone proteins (CHP) (e.g. heat-shock proteins). Phosphorylation occurs, and the AR translocates to the nucleus where it dimerizes. The AR dimer binds to a hormone response element (HRE) in the DNA, and the recruitment of cofactors (CF) occurs. Target genes are transcribed and translated into proteins.

Distribution in the avian brain

Comparative studies of the distribution of SH receptor expressing-cells in the avian brain have shown that brain regions that contain such cells are phylogenetically conserved (Gahr, 2001, 2000; Matsunaga et al., 2011; Metzdorf et al., 1999). A conserved pattern of SH receptor distribution in the brains of birds implies the existence of a preserved organizational signaling, operating early in the ontogeny of all bird species' embryos (Metzdorf et al., 1999). After development of receptors in a brain area, the expression might become constitutive and continue throughout life (Gahr, 2001). However, the level of expression of SH receptors in the avian brain undergoes cyclical and seasonal variations in a region-specific manner (Apfelbeck et al., 2013; Belle et al., 2003; Breuner and Orchinik, 2001; Canoine et al., 2007; Fusani et al., 2000; Gahr and Metzdorf, 1997). This fact entails that the SH actions on behavior can be determined not only by the concentration they reach in target brain areas, but also by the sensitivity of those brain cells.

In particular oscine songbirds possess androgen-sensitive brain regions that are a part of a network of forebrain nuclei involved in the production and learning of song (Balthazart et al., 1998; Bernard et al., 1999; Fusani et al., 2000; Gahr and Metzdorf, 1997; Metzdorf et al., 1999; Nastiuk and Clayton, 1995; Soma et al., 1999).

Songbirds and song behavior

Songbirds are a large taxonomic group with great diversity in the extent that song-related behavior depends on gonadal hormones, especially testosterone and its metabolites (Frankl-Vilches et al., 2015).

At the neural level, song behavior of songbirds is controlled by the song control system, a network of interconnected brain nuclei involved in song production, perception, and learning (Nottebohm et al., 1976). In all songbird species, the nuclei of this brain circuit including the sensory-motor integration area HVC (used as a proper name) are directly sensitive to testosterone by expressing androgen receptors in its neurons (Gahr, 2014). Further, in the song control system estrogen receptors alpha are only expressed in HVC nucleus (Gahr et al., 1993). Thereby, testosterone (and its metabolites) is directly involved in both the developmental acquisition and the seasonal production of songs.

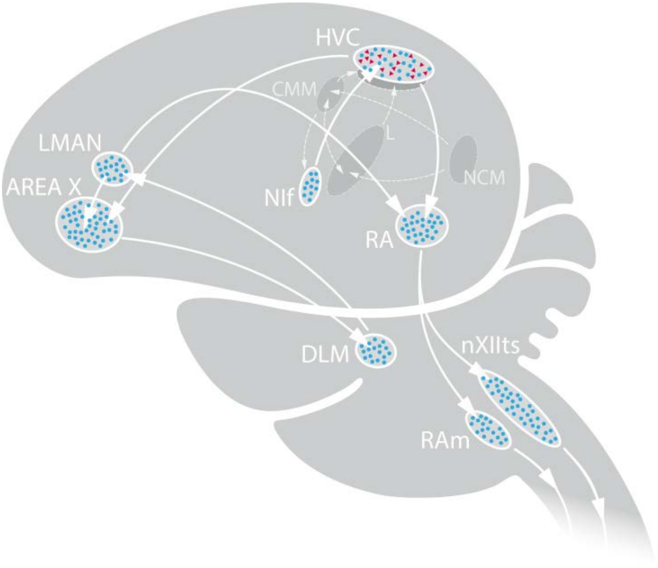

Figure 2. The song control system of oscine songbirds. The nuclei of this interconnected neural network express androgen receptors (blue dots). The HVC nucleus is a sensory-motor integration area, which also expresses estrogen receptors (red dots). *(picture kindly provided by Susi Seltmann)*

In general, male song is often produced as part of a sequence of courtship and territorial behaviors. In nature, these types of behaviors are expressed in cyclical way, usually linked to the occurrence of reproduction. The classical mechanism of seasonal activation of song in oscine songbirds is associated with the increase of day length as a primary environmental cue. For instance, longer day lengths in the spring trigger activation of the GnRH axis that stimulates the production of gonadal testosterone, which in turn exerts activational effects on seasonal song through binding androgen receptors on the song control system nuclei (Smith et al., 1997). Besides, testosterone influences the motivation to sing by acting directly in androgen-sensitive hypothalamic brain areas of songbirds that are associated with activation of appetitive sexual behaviors (Alward et al., 2013). Thus, song behavior is activated by testosterone, expressed primarily throughout the breeding stage, and plays a central role in the reproduction of songbirds.

In several species, seasonal changes in song behavior parallel neurochemical and morphological changes in song control nuclei (induced by circulating testosterone) (Nottebohm, 1981). These include changes in nuclei sizes, cells volume, cells turnover, dendritic arborizations and/or neurotransmitter content (Gahr, 2014; Tramontin and Brenowitz, 2000). Although it is not a generalized phenomenon, within the song control system the correlation between anatomical morphology and singing behavior has been reported mainly for the HVC, a brain region that integrates sensorimotor information and projects to all major pathways implicated in birdsong production. Neuroplasticity of HVC is thought to be primarily regulated by changes in circulating levels of testosterone (Balthazart et al., 2010; Gahr, 2014, 2004).

The song and the song control system of songbirds have been an excellent model for studying the mechanisms and functional significance of seasonal plasticity in the brain and behavior. However, most of research about song behavior mechanisms have been investigated temperate zone songbirds (Small and Moore, 2009). Thus, the great diversity represented within the songbird lineage offers a unique opportunity, still unexploited, for comparative studies to address the interaction of SHs, neural systems and the environment in the context of a naturally-occurring behavior.

Tropical and equatorial oscine songbirds

In mid- to high-latitude species, the predictable change in photoperiod is the initial predictive cue used to provide the annual template for timing phenology (Dawson et al., 2001; Gwinner, 1989; MacDougall-Shackleton et al., 2009). However, there is a large number and variety of songbird species (the largest vertebrate order) living in tropical and equatorial regions with small or near absent photoperiod cycles. The extent of seasonality and predictability of the environment differs enormously among tropical biomes, and in many cases tropical birds are faced with comparatively fewer environmental constraints in their habitat (Goymann and Helm, 2014). Life history traits of tropical birds are diverse and quite different from temperate zone models upon which most empirical studies are based. For instance, tropical birds lay smaller clutches and care for their young longer than temperate species (McNamara and Houston, 2008; Stutchbury and Morton, 2008). Tropical birds also have slower growth rates and generally lower metabolisms (Wiersma et al., 2007). Besides, birds near the equator tend to be residents year-round and have more extended breeding seasons than their temperate counterparts.

Songbird species from tropical lineages have evolved along with small photoperiodic cycles. The expression of seasonally appropriate behaviors, such as the song and associated territoriality, are typically mediated by seasonal changes in the neuroendocrine system. However seasonal regulatory mechanisms in tropical and true equatorial songbirds have not been as well studied as in temperate- zone species. It is not known how equatorial songbirds detect and translate local environmental cues into neuroendocrine signals that govern reproduction. Because seasonally breeding equatorial songbirds may not have the opportunity to rely on changes in photoperiod to time sexual behaviors such as singing, they must rely on other cues, such as food availability and/or rainfall. This could lead to local phenologies that vary on a much finer geographic scale than is observed at higher latitudes.

Thesis outline and aims

The thesis is focused on five main topics: a) the relationship between the annual cycle coordination of males and the equatorial characteristics of the habitat in the eastern lowland Amazon (**Chapter 1**), b) the production of steroid hormones in equatorial males, such as corticosterone and testosterone, in relation to the expression of seasonal traits

(Chapter 1 and 2), c) the neuroendocrine regulation of dawn-song behavior in equatorial males associated with gonadal testosterone **(Chapter 2)**, c) the influence of rainfall over the HPG axis regulation of gonadal activity in males **(Chapter 3)**, d) the development of a new implantation method to experimentally manipulate SH titers of birds within a natural physiological range (**Chapter 4**).

The main study subject of my thesis is an equatorial songbird species, the silver-beaked tanager (*Ramphocelus carbo).* This species is non-migratory, sexually dimorphic, and endemic from the Amazon region of South America (Valente, 2000). Further, among songbirds, the genus *Ramphocelus* consists entirely of species distributed throughout the tropics (Burns and Racicot, 2009) that have historically evolved along with small photoperiodic cycles. Silver-beaked tanagers belong to the family Thraupidae (tanagers) that constitutes the second largest avian family, which emerged exclusively from a Neotropical radiation (Burns et al., 2014).

Figure 3. Male silver-beaked tanager (Ramphocelus carbo)
Photo by barlovento /CC BY-NC-ND 2.0

The study site was located approximately 60 km northeast of the city of Belém, Brazil, very close to the equator line (1°12’07”S 48°18’07”W), and 30 m above sea level, in the Amazon River basin. The annual variation in day length is ~9 minutes (on-line day length calculator; United States Naval Observatory, Astronomical Applications Department). The zone has a tropical rainforest climate, typically hot and wet throughout the whole year (according to Köppen climate classification; (Peel et al., 2007). Although it rains almost every day (i.e. no real dry season) in this region, there is a distinct rainy season with very high daily rainfall (INMET; weatherbase.com) that spans on average from December through May, while the drier season lasts from June until November (Liebmann and Marengo, 2001; Moraes et al., 2005). The silver-beaked tanagers inhabit forest margins around the Amazon estuary. Relatedly, the inflow of both sea and fresh water to estuaries provides a large amount of nutrients in the sediment and soil, making estuaries among the most productive ecosystems in the biosphere (Kennish, 2002; Mann et al., 2005).

In **Chapter 1**, I studied the environmental and endocrine regulatory mechanism underlying the occurrence of seasonal phenotypes in equatorial silver-beaked tanagers. During the course of one year, I tracked testosterone and corticosterone levels of males in relation to their molting stage and expression of dawn-song, a behavior directly involved in breeding territoriality. Additionally, I examined the daily and seasonal timing of dawn-song over two years of recordings. Moreover, I investigated stable isotope signatures in blood and feathers of males to assess the degree of seasonality in the diet sources and habitat characteristics. All results obtained are presented in the context of the data regarding environmental abiotic conditions of the study site.

In **Chapter 2**, I studied the interplay between gonadal testosterone production and testosterone sensitivity of HVC in the activation of dawn-song behavior of male silver-beaked tanagers. During the course of one year, I examined the neuroanatomical characteristics and androgen receptor expression of HVC in relation to plasma testosterone levels, testis size, and occurrence of dawn-song behavior in males.

In **Chapter 3**, I investigated the effect that rainfall exerts on the HPG axis regulation and testis activity of males under semi-natural conditions. To do so, I experimentally exposed two different groups of males to contrasting simulated rainfall conditions (wet and dry) inside separate open-air aviaries. Then, we estimated the gonadal activity of males by measuring gonadal testosterone production before and after the injection of exogenous GnRH. The GnRH treatment allowed for assessing maximum physiological capacity of testis to produce testosterone.

Finally in **Chapter 4**, I compared the temporal dynamics of the release of testosterone from both silastic tubing and time-release pellets over time. These two implanting devices have been traditionally used in comparative endocrinology studies. In addition, I searched for alternative biodegradable matrices that would allow for a more constant release of testosterone. I comparatively show a new method for implanting birds using beeswax, which allows for increasing the plasma testosterone levels within a natural physiological range.

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Chapter 1

Timing in constant days: Phenology and dawn-song regulatory mechanisms of equatorial silver-beaked tanagers

Abstract

In most birds studied, the predictable annual change in day length is the primary environmental cue used to time seasonal processes. Besides, due to their pleiotropic actions and responsiveness to the environment, steroid hormones are major internal mediators for the expression of seasonal phenotypes, including molting and song behavior of songbirds. However, a large diversity of bird species lives at equatorial habitat where the annual variation in day length is nearly absent, but regulatory mechanisms used to time phenology remain elusive in true equatorial birds. Here, we studied equatorial silver-beaked tanagers, an endemic Amazonian songbird that inhabits a highly productive ecosystem, under frequent rain and uniform conditions of temperature throughout the year. First, we investigated timing and steroid hormone control of molting and dawn-song behavior in males. In parallel, we analyzed isotopes signatures of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ in the blood and feathers of males, in order to assess potential seasonal variations in habitat and dietary characteristics. Despite the equatorial latitude, we show that the silver-beaked tanager is a highly seasonal songbird, with marked cycles of molt and dawn-song. Individuals underwent no major changes of diet over the year, but photic cues and rainfall cycles appear as important environmental pacemakers. Distinctive aspects in the testosterone and corticosterone regulation of molting and dawn-song of males are discussed in relation to environmental factors. Our study not only contributes to expand the knowledge about diversification of life histories, but also to identify regulatory mechanism of phenology in equatorial species.

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Introduction

Animals typically experience seasonal changes in morphology, physiology and behaviour according to cyclical fluctuations of the environment. This temporal coupling between seasonal phenotypes and the changing environment is known as phenology (Forrest and Miller-Rushing, 2010; Helm et al., 2013). Since the seminal studies of Rowan (1925, 1926), a large amount of research, particularly in birds, has demonstrated that the annual changes in day length (photoperiod) operate as a proximate cue to time seasonal phenotypes (Wingfield, 2008, 2005). Annual photoperiodic cycles are ubiquitous and highly predictable in temperate regions, but supplementary environmental cues (e.g. in temperature and food availability) are used to fine-tune the phenology in birds at the local scale (Dawson, 2008; Wingfield, 2012).

Due to their responsiveness to environmental changes and their pleiotropic actions, steroid hormones such as testosterone and corticosterone are important physiological mediators in the expression of seasonal phenotypes (Wingfield, 2005). These hormones are highly conserved across taxa in their mechanisms of action and function (Fan and Papadopoulos, 2013; Kawata, 1995). For instance, changes in plasma levels of corticosterone (the main glucocorticoid in birds) are linked to systemic adjustments in energetic metabolism and homeostasis, while testosterone levels are associated with territory establishment and sexual functions of males. In general, the mechanisms underlying phenology have been better studied in birds than in other animals. However, research continues to be biased toward birds breeding at higher latitudes. This bias limits our understanding of phenology in different habitats, and impedes physiological comparative analysis across species with different evolutionary histories.

Among songbirds, the genus *Ramphocelus* consists entirely of species distributed throughout the tropics (Burns and Racicot, 2009) that have likely evolved along with small photoperiodic cycles. Therefore, within the tropics a good model to investigate regulatory mechanisms underlying seasonal phenotypes is the silver-beaked tanager *(Ramphocelus carbo)*. In a previous study, we reported that equatorial males living in a habitat of low seasonality expressed seasonal gonadal production of testosterone in correlation with seasonal changes of testis size. Besides, these equatorial males display a dawn-song behavior in a seasonal manner associated to the settlement of breeding territories (Quispe et al., 2016). Interestingly, the activation of dawn-song was dissociated from the seasonal peak of plasma testosterone levels. In fact, the rise of testosterone levels occurred several weeks after males started to produce dawn-songs.

The present work aims to broaden the study in equatorial silver-beaked tanagers. The topic is particularly interesting because these birds are exposed to uniform environmental conditions over the year, including factors that are traditionally considered important for the annual cycle timing. The region has a tropical rainforest climate, typically hot and wet throughout the whole year (according to the Köppen climate classification; Peel et al., 2007), although there is a distinct rainy season (Liebmann and Marengo, 2001; Moraes et al., 2005). Silver-beaked tanagers inhabit forest margins around the Amazon estuary. The inflow of sea and fresh water of estuaries provides a large amount of nutrients in the sediment and soil, making estuaries among the most productive ecosystems in the biosphere (Kennish, 2002). In this scenario, we investigated the production of testosterone and corticosterone in male silver beaked tanagers throughout the year in relation with the occurrence of molting. In addition, during two consecutive years we examined the relationship between daily and seasonal timing of dawn-song behavior (a territorial breeding behavior), and its regulation by testosterone and environmental cues. Further, in order to identify potential environmental cues involving the use of dietary sources, we examined isotopes tracers of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ in the blood and feathers of males throughout the year. Given the photoperiod and temperature annual conditions, and their vicinity to the estuary, we hypothesized that silver-beaked tanagers have a very stable diet all year-round. In relation to this, if silver-beaked tanagers are not exposed to seasonal limiting factors, we predicted that males might also maintain invariable levels of baseline corticosterone throughout the year. On the other hand, we expected to find a seasonal increase of testosterone levels in plasma associated with breeding, which might constrain or at least affect the occurrence of molting. Our study seeks to integrate physiology, behavior and the environment to understand regulatory mechanisms of phenology in an equatorial songbird.

Methods

Subject, study site and field procedures

The silver-beaked tanager (*Ramphocelus carbo*) is a non-migratory, sexually dimorphic species (Carvalho, 1957; Sanaiotti and Cintra, 2001; Valente, 2000). It belongs to the passerine family Thraupidae, which comprises 12% of the Neotropical avifauna (Burns et al., 2014), while the genus *Ramphocelus* consists entirely of species distributed throughout the tropics. Male silver-beaked tanagers typically display dawn-song that is directly involved in the establishment of breeding territories (Quispe et al., 2016). After the breeding season, the silver-beaked tanager undergoes a prebasic molt (Valente, 2000), as typically occurs in most passerines.

The study site was located approximately 60 km to the northeast of the city of Belém in Brazil (1°12′07″S 48°18′07″W, 30 m above sea level) in the Amazon River basin. The annual variation in day length is ~9 minutes (on-line day length calculator; United States Naval Observatory, Astronomical Applications Department). According to the Köppen climate classification, the region has an equatorial rainforest climate, which presents annual average temperatures of 26 ± 4 C°. Although there is no real "dry" season because it rains almost every day, there is a distinct rainy season with very high daily rainfall between December and May while the drier season lasts from June until November (Liebmann and Marengo, 2001; Moraes et al., 2005).

Only individual males were caught using mist nests and playback of conspecific songs. The sampling schedule for steroid hormones and molt registers was divided in five monthly periods: April, July, and November of 2011, January and February of 2012. Immediately upon capture, a blood sample from the wing vein was obtained to determine baseline corticosterone (within 3 minutes of capture); approximately 2 minutes later a second blood sample was obtained from the same individual to determine testosterone concentrations. Overall, 57 males were sampled for baseline corticosterone during the whole period, and 109 males were sampled for plasma testosterone concentrations. Blood samples were collected into heparinized capillaries and stored on ice until we returned to the field station to centrifuge them, then the plasma was separated and frozen (- 40 °C). After the blood sample was taken, males were weighed and checked for molting and released back to nature. We classified individuals as molting when at least one of the flight feathers (tail and wings) were symmetrically molting.

For the stable isotope analyses in blood cells, we collected blood samples of different males during four monthly periods: April, July, and October of 2011 and January 2012. Seven blood samples were obtained during each period (28 in total). Once collected, blood samples were kept frozen (- 40 °C) until analysis. In addition, for the stable isotope analyses of feathers, we collected one of the central rectrices (or tail feathers) that seemed recently molted, in a good shape and with smooth edges. We obtained 15 feathers from non-molting adult male individuals on July 2011 and 14 feathers from molting males, but with already grown central rectrices, in February 2012. Stable isotope signatures obtained from blood cells provide short- to medium-term information (about 2-3 weeks before sampling time), while feathers reflect the diet at the time when they have grown (Bauchinger and McWilliams, 2009; Bearhop et al., 2002; Cherel et al., 2005)

Hormone analysis

Testosterone and corticosterone concentrations were determined by radioimmunoassay following the procedures described in Goymann et al. (2006). Samples were assayed in duplicate and distributed randomly between two assays. The extraction recovery for testosterone was 88.0% ± 2.8% (mean ± sd). Hormone concentrations were calculated with Immunofit 3.0 (Beckmann Inc., Fullerton, CA, USA). The lower detection limits of assays were 0.37 pg/ml and 0.40 pg/ml, respectively, and all samples were above the detection limit. The intra-assay coefficients of variation were 9.1 % and 4.2 %, respectively; the intra-extraction coefficients of variation of a chicken plasma pool were 3.3 % and 6.0 %, respectively. The inter-assay coefficient of variation between the two assays was 9 % and the inter-extraction coefficient of variation between the two assays was 15.9 %. For corticosterone, samples were assayed in duplicate in one assay. The extraction recovery was

79 % ± 3.9 % (mean ± sd), the lower detection limit of the assay was 4.14 pg/ml, and all samples were above the detection limit. The intra-assay coefficient of variation was 4.8 %, and the intra-extraction coefficient of variation of a chicken plasma pool was 9.0 %.

Stable isotopes analysis

We measured stable isotope ratios for carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), and sulfur ($\delta^{34}S$) in blood cells and feathers. Feather samples were rinsed with methanol and air-dried in a fume hood. The stable isotope analysis was conducted on sub-samples of approximately 0.7 mg cellular blood (plasma-free), and 0.5 mg feathers were weighed into small tin cups to the nearest 0.001 mg, using a micro-analytical balance. Dried, powdered samples were loaded into tin capsules and combusted in a Vario Micro cube elemental analyzer (Elementar, Analysensysteme, Germany). We fed the resulting gases via gas chromatography into the inlet of an IsoPrime isotope ratio mass spectrometer (Micromass, Manchester, UK). Measurements are reported in δ-notation in parts per thousand deviation (‰) relative to international standards for carbon (Pee Dee Belemnite, PDB) and nitrogen (atmospheric N2, AIR, S02), according to the equation δ (‰) = 1000 x (Rsample/Rstandard-1).

Two sulfanilamide (Iso-prime internal standards), and two Casein were used as laboratory standards for every 10 unknowns in sequence. The reference material used for sulphur isotope analysis was sulfanilamide-calibrated and traceable to NBS-127 (barium sulphate, $\delta^{34}S$ = +20.3 ‰). Replicate assays of internal laboratory standards indicated measurement errors (SD) of ± 0.05‰, 0.15‰ and 0.05‰ for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, respectively.

For blood cells, 28 males were sampled during four sampling periods (April, July, October of 2011 and January of 2012), with seven individuals sampled in each period. For the analysis of feathers, we sampled 29 males and only used complete developed feathers.

Song recordings

To identify the timing of dawn-song behavior at a local scale, we recorded the overall activity over two years using two passive audio recorders (SM1: Wildlife Acoustics) that were 500 m apart from each other. Each recording device was attached to trees 1.5 m above the ground and surrounded by at least three territories of ringed males. The recorders were programmed to record continuously once per week from 5:00 am until 7:00. We obtained weekly recordings from April 2011 until October 2012. Hence, the seasonal and daily onset of dawn-song behavior was registered during a period of 19 months. Due to technical problems, during some of the weeks we only obtained recordings from one of the recorders, and there were no recordings from August of 2011. So, from a total number of 81 weeks recorded, 47 weeks were recorded with both recorders (188 hours recorded) and 38 weeks only with one (76 hours recorded).

Statistical analysis

R version 3.2.0 (R Development Core Team 2015) was used for all statistical analyses. Hormone levels in plasma and stable isotope signatures in blood were analyzed using linear models. Changes in circulating levels of testosterone were tested at five time points (April, July, November of 2011, and January and February of 2012) with body mass and molting condition as covariates; testosterone data were log-transformed to meet normality assumptions. Additionally, changes in plasma corticosterone (April, July, November of 2011, and January and February of 2012) were tested with body mass and molting condition; corticosterone data were log-transformed. Further, two correlation tests between 1) corticosterone and testosterone levels, and 2) corticosterone levels and hour of sampling were performed using Pearson analysis.

In order to estimate the influence of environmental factors on the occurrence of dawn-song over time (seasonal occurrence), we used a generalized linear model with logit link function and binomial error distribution. We tested whether the probability of dawn-song to occur was determined by rainfall rate (monthly amount of rain), duration of the day, and twilight daytime.

For the analysis of stable isotope signatures, we used linear models. We checked whether stable isotope ($\delta^{15}N$, $\delta^{13}C$, $\delta^{34}S$) values in blood changed over time with months of the year as the explanatory variable (April, July, November of 2011 and January of 2012). Similarly, changes in the isotopic values of feathers were analyses over two sampling periods (July 2011 and February 2012).

Results

Hormonal profiles

Month of sampling had a significant effect on plasma testosterone. Testosterone levels were significantly higher in November, January 2011, and February 2012 compared to April 2011 ($F_{4,97} = 9.9$, $p < 0.0001$) (Fig 1). Furthermore, molt and testosterone were significantly related ($F_{1,97} = 18.7$, $p < 0.0001$): males with higher concentrations of circulating testosterone were not undergoing molt (Fig 2). Body mass and testosterone were not related ($F_{1,97} = 0.2$, $p = 0.66$).

We found no effect of month of sampling on baseline corticosterone concentrations (Suppl 1) ($F_{4,49}$ = 0.4, p = 0.76). However, molt was significantly related to baseline corticosterone ($F_{1,49}$ = 7.4, p = 0.008), with molting males expressing lower levels of corticosterone than non-molting males (Fig 2). There was no effect of body mass on baseline.

Testosterone and corticosterone were weakly correlated (r = 0.27, p = 0.049). Baseline corticosterone significantly decreased over time from morning to evening (r = - 0.35, p = 0.009) (Suppl 2).

Annual timing of dawn-song

The occurrence of dawn-song over the year was statistically affected by the monthly rainfall rate and day length (GLM = both $p < 0.0001$) (Fig 5). No significant effect of twilight time was found.

Stable isotopes signatures

We found no seasonal changes of δ^{15}N in blood cells (Fig 3) ($F_{3,23}$ = 0.6, p = 0.6), and no seasonality as well of δ^{15}N in feathers (Fig 4) ($F_{1,27}$ = 0.009, p = 0.9). Similarly, no seasonal changes were found in δ^{13}C of blood cells (Fig 3) ($F_{3,23}$ = 1.24, p = 0.32), and there were no changes of δ^{13}C in feathers over seasons (Fig 4) ($F_{1,27}$ = 0.55, p = 0.46).

There was a significant difference in δ^{34}S of blood cells over seasons. δ^{34}S signatures in blood cells were statistically higher in January 2012 in comparison to April 2011 (Fig 3) ($F_{3,23}$ = 3.8, p = 0.02). In addition, the values of δ^{34}S in feathers also changed significantly throughout the seasons, where δ^{34}S was significantly higher in February 2012 than in July 2011 (Fig 4) ($F_{1,27}$ = 14.8, p = 0.0006).

Discussion

The habitat of silver-beaked tanagers has very low seasonality, including in food availability. Nevertheless, males use important seasonal schedules in relation to the molt and reproductive functions (dawn-song territoriality and gonadal testosterone production). The regulatory mechanisms underlying these seasonal phenotypes are inter-dependent, in which steroid hormones are important mediators.

The levels of testosterone in plasma peaked during October – November (Fig 1). These results agree with a previous study (Quispe et al., 2016) where the seasonal levels in circulating testosterone have been shown to correlate with testis size. This outcome confirms that equatorial silver-beaked tanagers breed seasonally at the end of the dryer season. The peak in testosterone coincides with the period of increasing day length at this equatorial latitude (+5 min). Photoperiodic changes of 17 minutes have been shown to stimulate gonadal growth in a tropical bird (at 9°N latitude) (Hau et al., 1998), but so far there is no evidence that a bird could sense a time difference of 5-6 minutes. Alternatively, other environmental cues might be used by silver-beaked tanagers to stimulate gonadal production of testosterone, such as the predictable changes of rainfall or seasonal changes in cloud cover and light intensity (Gwinner and Scheuerlein, 1998; Hau et al., 2008, 2004).

No seasonality was found in plasma levels of baseline corticosterone (Suppl. 1). Glucocorticoids play an important role in the ability of the organism to respond to energetic constraints. For instance, seasonal changes of environmental temperatures and food supplies can affect baseline corticosterone of birds (Ouyang et al., 2012; Romero, 2002; Wingfield and Sapolsky, 2003). Thus, the unconstrained habitat conditions of silver-beaked tanagers might explain the invariant baseline levels of corticosterone over the year. Indeed, similar results have been reported for other songbird species living in environmentally stable habitat (Gonzalez-Gomez et al., 2013). On the other hand, we found a correlation between corticosterone levels in plasma and day time. Individuals sampled early in the morning tended to have higher levels of circulating corticosterone (Suppl. 2). In relation, diel rhythms of baseline levels of glucocorticoids have been reported to mediate the initiation of daily activity in birds and foraging onset (Breuner et al., 1999). The results suggest that, as the day progresses, corticosterone may influence physiological and behavioral responses of equatorial silver-beaked tanagers.

We found marked seasonality in the molting of males. Yet, there were at least a few males molting during each sampling period of the year, which indicate some degree of heterogeneity of molt phenology. Indeed, this condition is often observed in tropical birds. The great majority of individuals molted during the rainy season, suggesting an influence of rain on molting (Suppl. 3). On the other hand, at the individual level, molting males had comparatively lower concentrations of circulating testosterone than non-molting males (Fig 2). Hence, it is likely that the timing of molt in silver-beaked tanagers is more related to the end of the reproductive cycle than to environmental cues per se. Relatedly, the results suggest the existence of a physiological trade-off between reproduction and molt. In most species studied, a temporal overlap of breeding and molt tends to be minimized or avoided, as both stages require large mobilization of energy and nutrients (Romero et al., 2005). Similarly, individuals that were molting tended to have comparatively lower levels of baseline corticosterone (Fig 2). This is a pattern commonly observed in temperate zone passerines (Romero, 2002). There are a number of trade-off hypotheses that have addressed this issue, which include energetic, behavioral, and physiological constraints

(Cornelius et al., 2011). One well-known action of glucocorticoids is the proteolytic effect (gluconeogenic), which might limit the availability of critical amino acids required for feather growth (Cornelius et al., 2011; Romero et al., 2005). Overall, our results suggest that silver-beaked tanagers might adjust baseline corticosterone in relation to the individual state, such as molting.

Dawn-song was performed seasonally by male silver-beaked tanagers. The seasonal onset of dawn-song was dissociated from the peak in testosterone levels within approximately two months (Fig. 1). These results confirm a previous study (Quispe et al., 2016), where it is reported that the peak of testosterone correlates with the increment in the dawn-song rate that occurs later in the breeding season. Further, the seasonal onset of dawn-song coincided with the annual decline of rainfall (the beginning of the drier season) and with the period of a small increase in day length (9 minutes increase over 6 months). There is a silent period during the rainy season, in which no dawn-song occurs, coinciding with a day length decrease (Fig. 5). Besides this seasonal regulation, there was a precise daily timing of the dawn-song onset in which males mirrored the civil twilight time (Fig. 5). Thus, we demonstrate that equatorial males adjust their daily activity according to small variations in civil twilight. From a statistical perspective, the seasonal timing of dawn-song is dependent upon the seasonal changes of rainfall and the subtle fluctuations of day length. Instead, the annual variation of civil twilight time had no significance on the seasonal daily dawn-song, but instead on the daily timing. From a biological point of view, it has been shown that equatorial songbirds are equipped with strong endogenous circannual rhythms that can be synchronized with environmental factors, including photic cues. However, photoperiodic synchronization at equatorial scale has never been proved before in any vertebrate. On the other hand, It demonstrated that seasonal rainfall exerts important influences on the seasonal schedules of equatorial songbirds (Gwinner and Dittami, 1990; Moore et al., 2005), including seasonal song (Moore et al., 2004). Our observations suggest that seasonal dawn-song of male silver-beaked tanagers is controlled by an integrated perception of the rain and photoperiodic cycles. In addition, despite the small day to day variation, civil twilight might control the daily dawn-song onset of equatorial males.

As predicted, our analysis showed no seasonal differences of $\delta^{13}C$ and $\delta^{15}N$ in feathers and blood cells. The higher $\delta^{15}N$ values obtained for feathers in comparison to blood (Figs 3, 4) are normal considering the different fractions of constituent protein (Bearhop et al., 2002; McCutchan et al., 2003). The $\delta^{13}C$ ratio is an indicator of diets based on plants with distinct photosynthetic pathways (e.g., C3 vs. C4) (Inger and Bearhop, 2008; Kelly, 2000). The $\delta^{15}N$ ratio estimates changes in trophic levels, since the $\delta^{15}N$ of a consumer is typically enriched relative to its prey (Post, 2002). Thus, the results suggest that the diet of silver-beaked tanagers remains seasonally uniform, with no changes in trophic level. The near absent annual fluctuations in temperature and photoperiod, the daily occurrence of rain (even during the dry season), and the high nutrient deposition of the estuary might keep the availability of food supplies relatively constant for silver-beaked tanagers.

Surprisingly, there was strong seasonality of $\delta^{34}S$ ratios. The values obtained from blood cells at four different sampling periods showed that $\delta^{34}S$ signatures were significantly lower in April 2011 compared to January 2012 (Fig. 3). In addition, the analysis of feathers over two sampling periods indicated that $\delta^{34}S$ values obtained in July 2011 were significantly lower than the values of February 2012 (Fig. 4). Since oceanic-derived sulfates are generally more enriched in $\delta^{34}S$ than terrestrial sources, $\delta^{34}S$ signatures are used as an indicator of marine influences on diet (Florin et al., 2011). It should be noted that δ^{34}S values do not necessarily reflect direct consumption of marine sources, but also the proximity of soil and food sources to the sea (Haché et al., 2014). The Amazon estuary is a wide transition zone subject to marine and fluvial influences. Thus, δ^{34}S signatures of silver beaked tanagers might indicate seasonal changes in the composition of mixed water. The period of greatest freshwater discharge in the Amazon estuary is between April and June (after the rainy period), while the lowest discharge occurs between October and December (Nittrouer and DeMaster, 1996; Rockwell Geyer et al., 1996). Accordingly, the highest δ^{34}S values obtained in blood cells (January) correlates with a period of lower freshwater discharge, whereas the lowest δ^{34}S values (April) coincide with the period of greater fluvial discharge (Fig. 3). Similar results were obtained with feathers, were the highest δ^{34}S values were obtained in feathers presumably grown in December – January, whereas the lowest values were obtained from feathers presumably grown in April-May (Fig. 4). These results open a new exciting question, in relation to the seasonal dynamics of estuaries as environmental cues to time seasonal processes in passerines species. For instance, changes in estuary characteristics can potentially function as a supplementary cue for silver-beaked tanagers associated with changes in rainfall.

Alternatively, δ^{34}S ratios could also denote changes: a) the trophic position (McCutchan et al., 2003) or 2) the quality of the dietary protein in relation to the intake of sulfur amino acids (Arneson and MacAvoy, 2005; Florin et al., 2011). For the first case, a trophic dietary shift in silver-beaked tanagers is not supported by δ^{15}N, which remains constant throughout the year. However, it could be that the δ^{34}S changes observed indicate a dietary shift towards particular food items rich in sulfur amino acids (methionine, cystine, cysteine and taurine). Further studies that examine the precise food sources of silver-beaked tanagers throughout the year are necessary to prove this possibility.

In summary, the results presented here provide important information about the environmental and endocrine regulatory mechanisms underlying the annual cycle of an equatorial songbird. We found an evident interplay between the seasonal regulation of molting and reproduction, in which testosterone and corticosterone dynamics are involved. The habitat of these birds has an annual rainfall pattern that divides the year into two main seasons, the rainy and the drier seasons. This environmental condition influences the timing of dawn-song and breeding territoriality, gonadal activity, and molting of equatorial silver-beaked tanagers. Furthermore, isotope analysis suggests seasonal changes in water composition of the Amazon estuary may potentially function as a supplementary environmental cue. Particularly interesting is the timing of the dawn-song behavior in males, which seems to be environmentally regulated at an annual and daily scale by a complex interaction of rain with photic cues. The research of the mechanisms of phenology under the absence of photoperiodic changes is essential to generate a broad knowledge about the diversification of life histories (Forrest and Miller-Rushing, 2010; Helm et al., 2013). Moreover, understanding the timing of biological processes is particularly important nowadays as we are confronted by an accelerated rate of global climate change.

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Figures

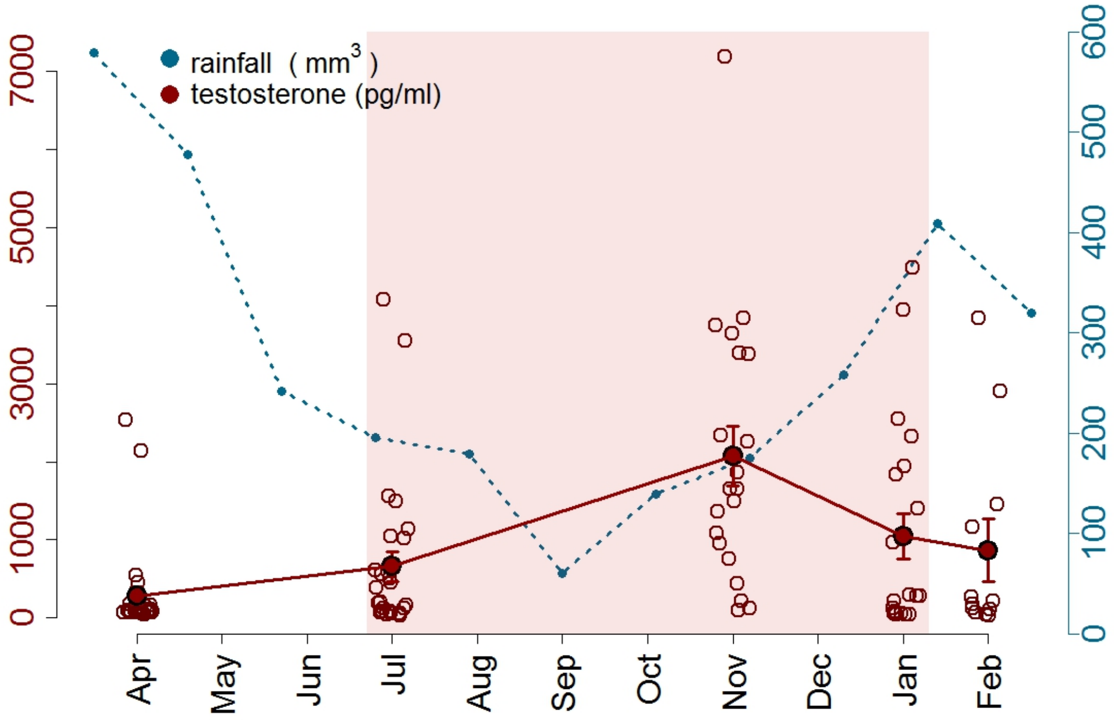


Figure 1. Testosterone levels in plasma throughout the year. The red line and filled circles shows means (+/- SE) of circulating testosterone levels of males over the five sampling periods (April, July, October 2011, and January, February 2012). Red open circles indicate male individual testosterone levels. The blue line indicates the rainfall rate represented as the monthly values from April 2011 until February 2012. The red shaded area indicates the period during which dawn-song occurred.

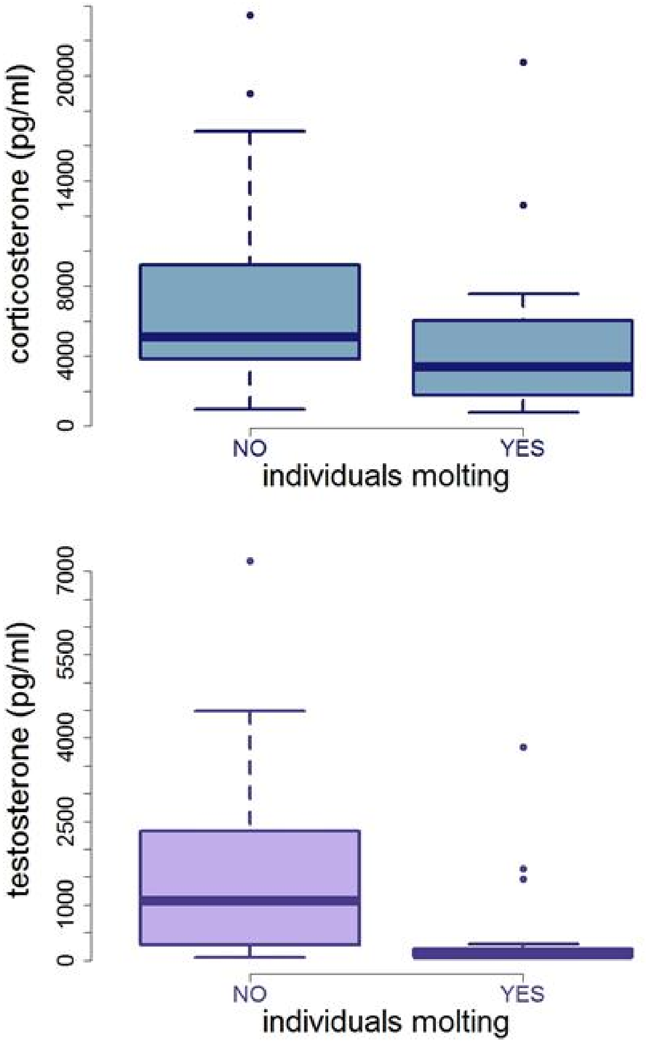


Figure 2. Plasma levels of steroid hormones in relation to the molting status. The upper panel shows plasma baseline levels of corticosterone relative to the individual molt conditions of males. The lower panel shows testosterone levels in plasma in relation to individual molt conditions of males. Box plots indicate medians, 10th, 25th, 75th, and 90th percentiles, dots indicate individual outlier values. Hormonal values were obtained over five different time points throughout the year.

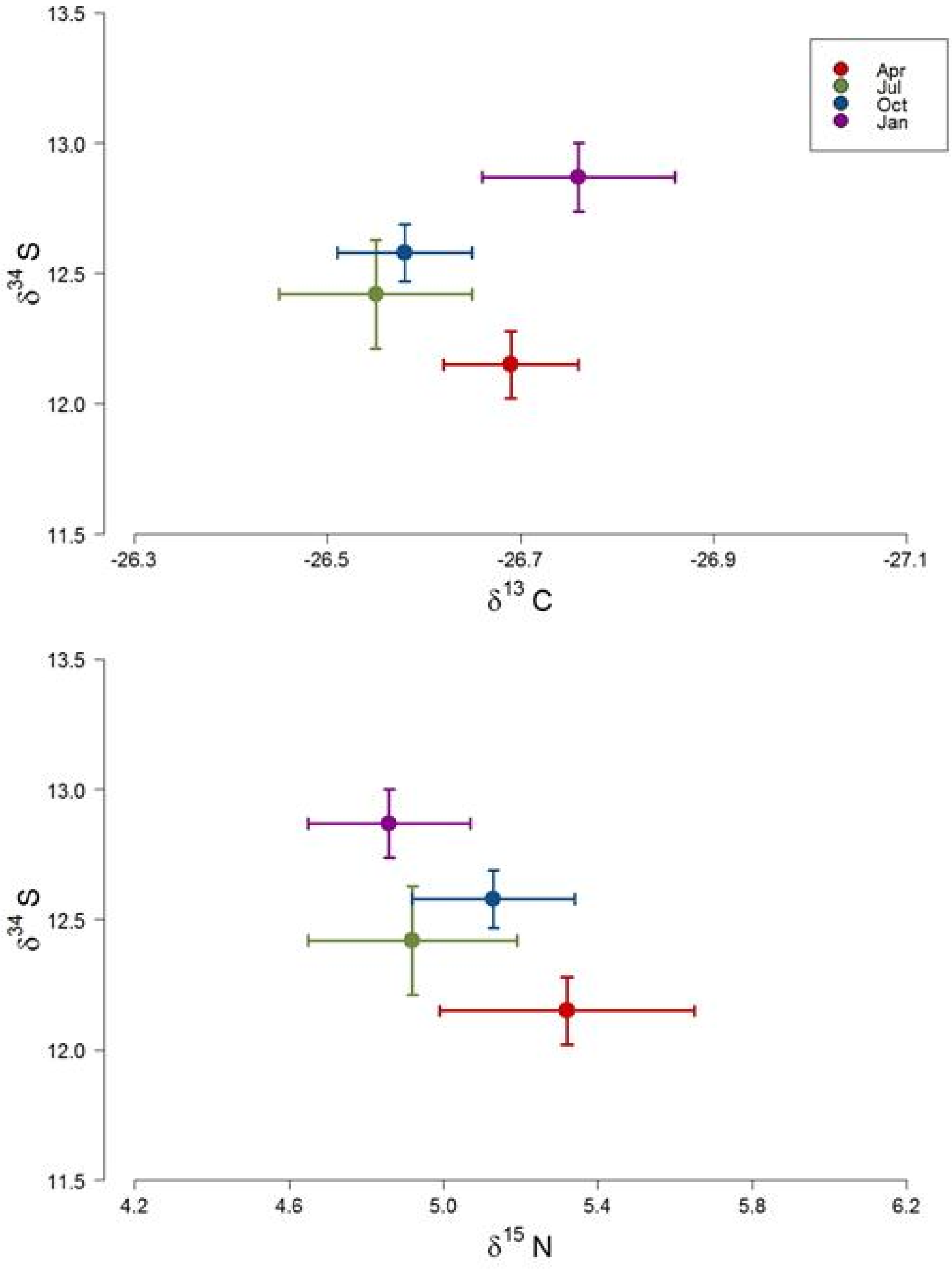


Figure 3. Stable carbon, nitrogen and sulfur isotope values for blood cells. The upper panel shows the δ^{34}S ratios in relation to δ^{13}C ratios. The lower panel shows the δ^{34}S ratios in relation to the δ^{15}N ratios. The symbols represent the mean values (+/- SE) over four sampling periods (April, July, October 2011 and January 2012).

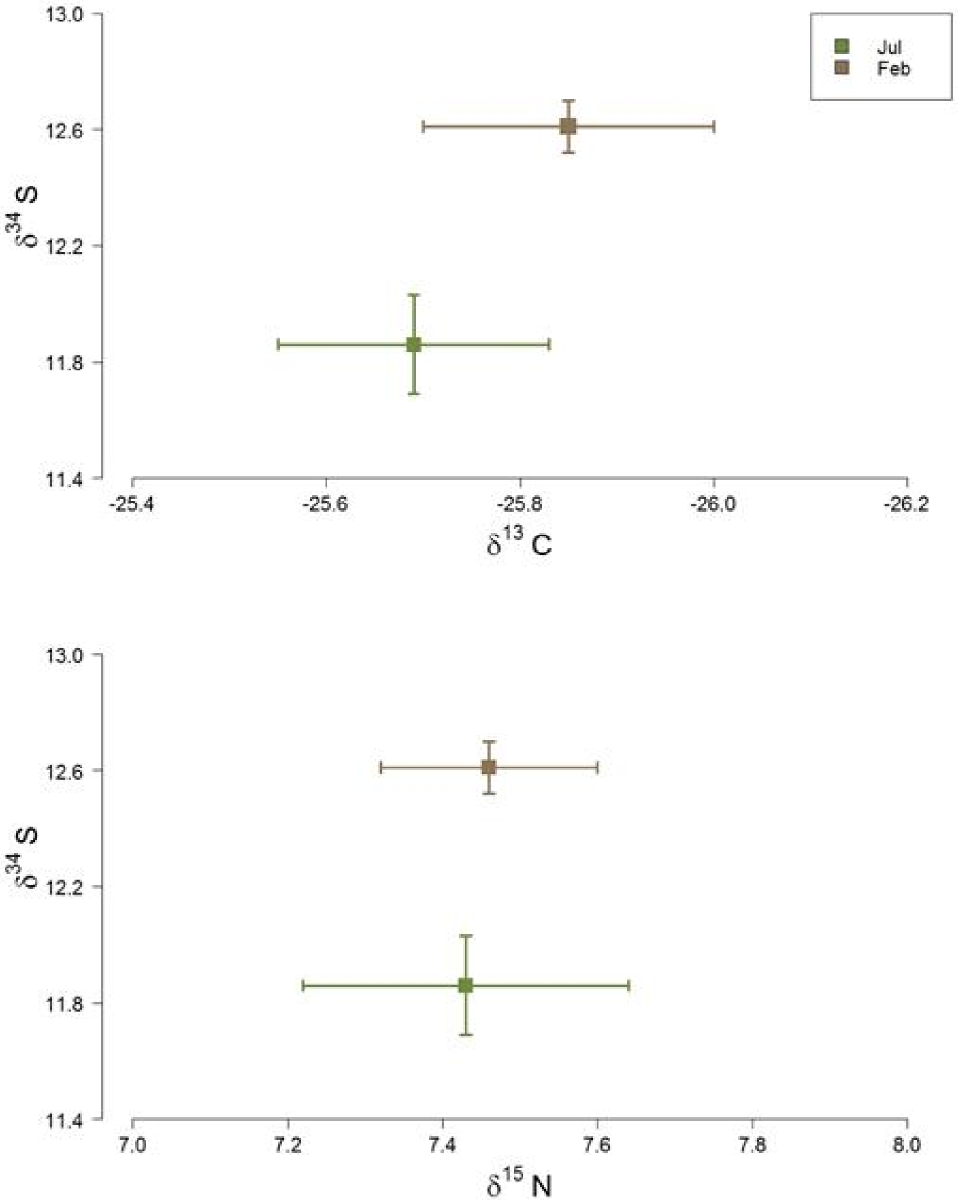


Figure 4. Stable carbon, nitrogen and sulfur isotope values for feathers. The upper panel shows the $\delta^{34}S$ ratios in relation to $\delta^{13}C$ ratios. The lower panel shows the $\delta^{34}S$ ratios in relation to the $\delta^{15}N$ ratios. The symbols represent the mean values (+/- SE) obtained from rectrice feathers over two sampling periods (July 2011 and February 2012).

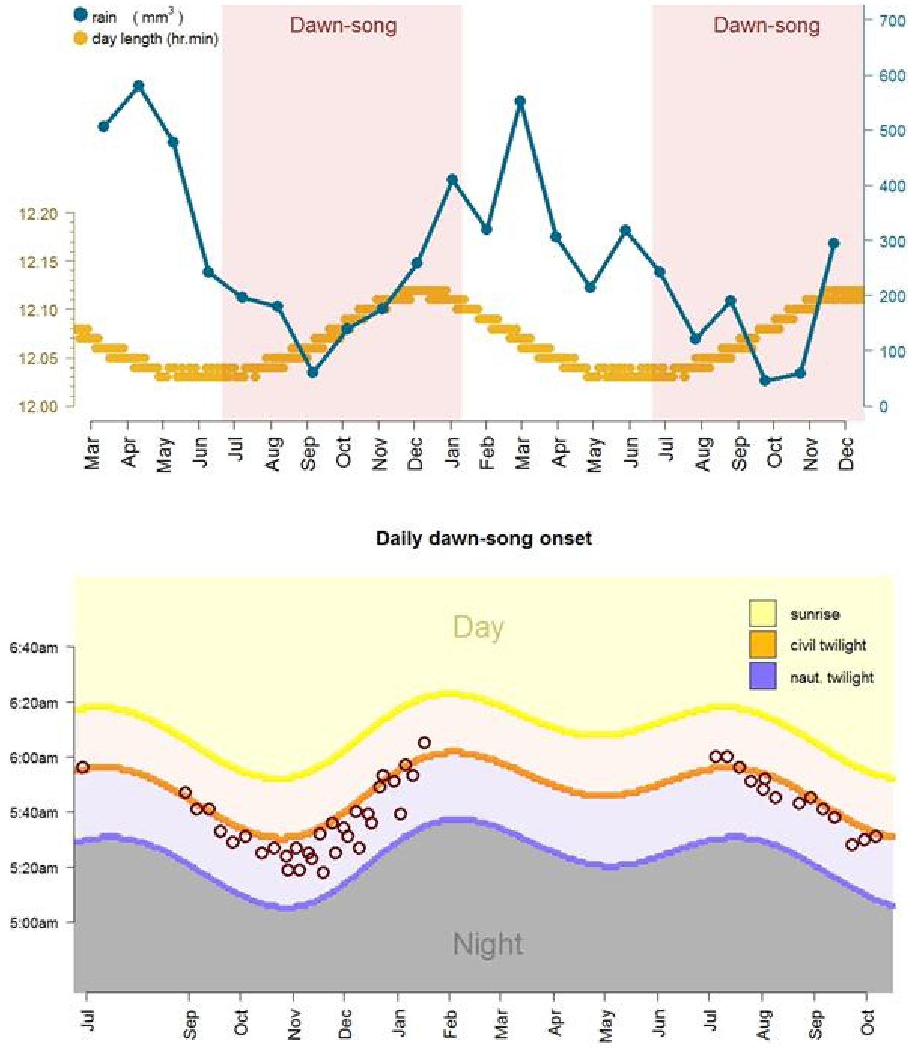
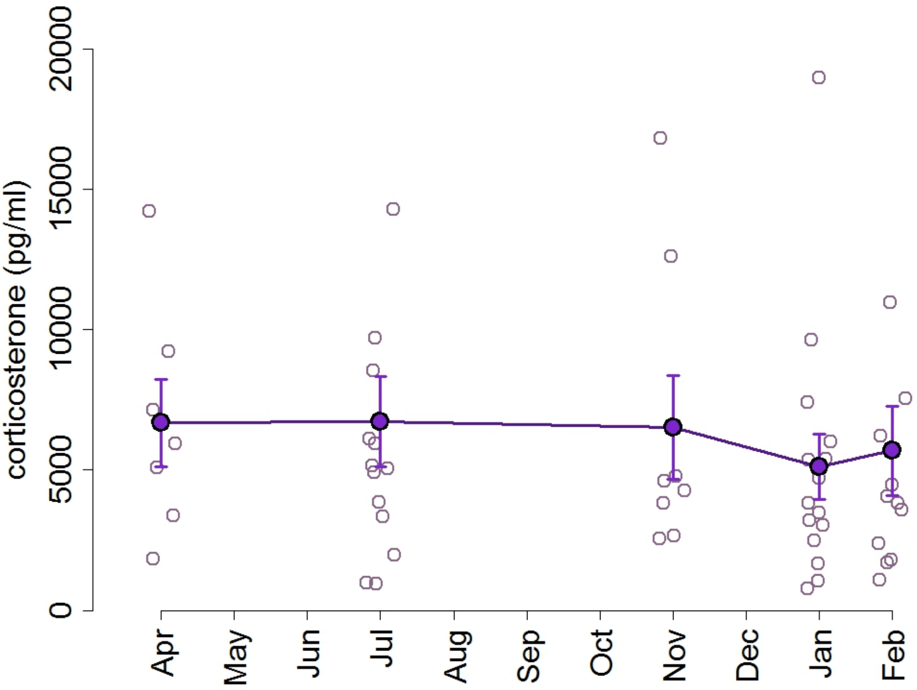
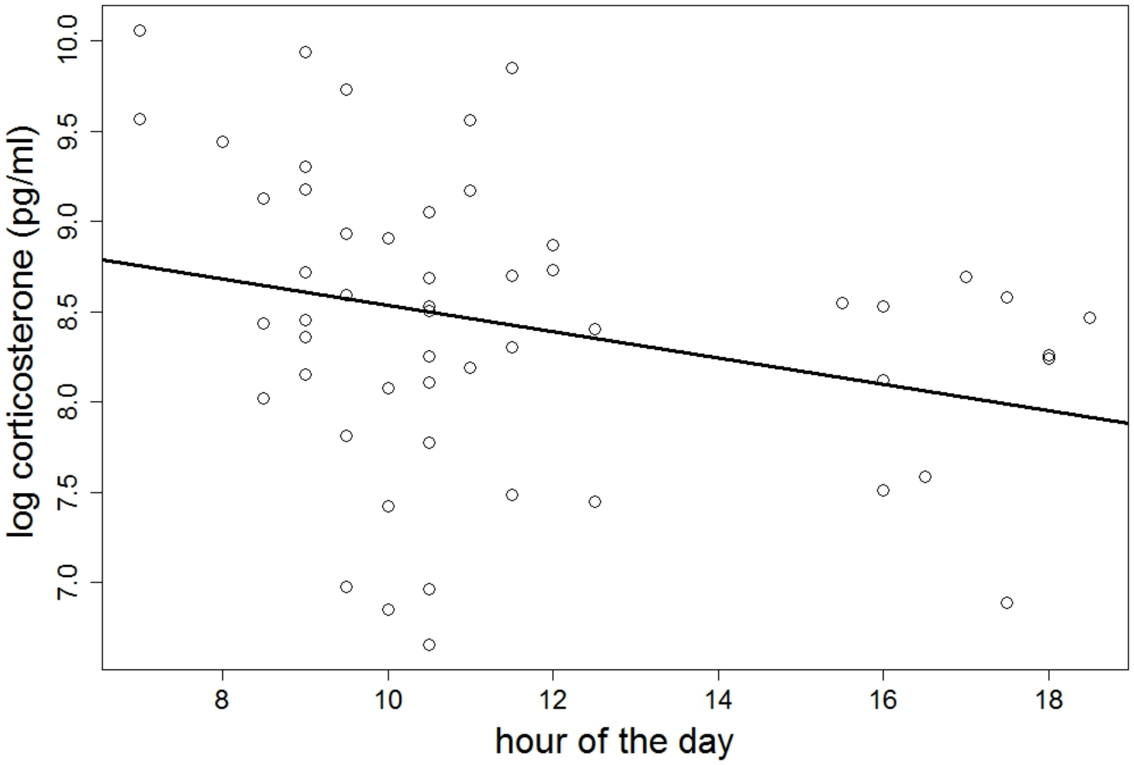


Figure 5. Seasonal and daily timing of dawn-song behavior. In the upper panel, the red shading indicates the period of the year when dawn-song occurred. The blue line shows the amount of rainfall in mm^3 and the yellow dots indicates the duration of days (from sunrise to sunset) in hours, from March 2011 until December 2012. The lower panel shows in yellow the daily hour of the sunrise, in orange the daily hour of the civil twilight, and in purple the daily hour of the nautical twilight from July 2011 until October 2012. The red open circles represent the first dawn-song that occurred in the day. Due to technical difficulties, no recordings were available from August 2011.

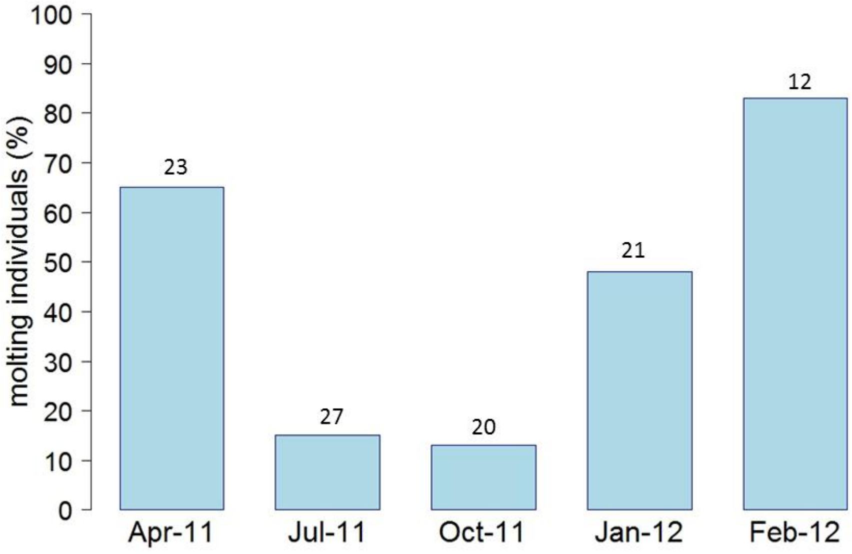
Supplementary material



Supplement 1. Corticosterone levels in plasma throughout the year. The purple line and filled circles shows means (+/- SE) of the baseline levels of corticosterone in males over the five sampling periods (April, July, October 2011, and January and February2012). Purple open circles indicate male individuals’ baseline levels.



Supplement 2. Correlation between corticosterone and daytime. The scatter plot shows a negative correlation between log-transformed levels of baseline corticosterone and the hour of the day when the sample was taken (*r* = - 0.35, *p* = 0.009). The samples were collected over the course of five sampling periods throughout the year.



Supplement 3. Rate of individuals molting throughout the year. The bar plot shows the percentage of individuals that were found molting out of the total number of individuals caught during each sampling period. Sample sizes are given above bars

Chapter 2

Dawn-song onset coincides with increased HVC androgen receptor expression but is decoupled from high circulating testosterone in an equatorial songbird

Abstract

The song of songbirds is a testosterone-sensitive behavior that is controlled by brain regions expressing androgen receptors. At higher latitudes, seasonal singing is stimulated by increasing day-length and elevated circulating testosterone. However, a large number of songbird species inhabit equatorial regions under a nearly constant photoperiod, and the neuroendocrine mechanisms of seasonal song in these species have rarely been investigated. We studied males from an equatorial population of the silver-beaked tanager (*Ramphocelus carbo*), an Amazonian songbird. We found seasonality in dawn-song behavior, which was displayed continuously for more than half a year throughout an extended breeding territoriality stage. The seasonal activation of dawn-song was correlated with an increased area of androgen receptor expression in HVC, a major brain area of song control. However, testosterone levels remained low for several weeks after activation of dawn-song. Circulating levels of testosterone were elevated only later in the breeding season, coinciding with a higher dawn-song output and with the mating period. Our results suggest that the seasonal activation of dawn-song and territoriality involves an increase of androgen target cells in HVC. This mechanism could potentially function to circumvent adverse effects of high testosterone levels in a species with an extended breeding season.

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Dawn-song onset coincides with increased HVC androgen receptor expression but is decoupled from high circulating testosterone in an equatorial songbird

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HIGHLIGHTS

- The study site undergoes minimal changes of photoperiod throughout the year.
- The seasonal rainfall patterns favor an extended breeding territoriality.
- Males show marked seasonality in dawn-song behavior associated with territoriality.
- Seasonal initiation of dawn-song is dissociated from high testosterone levels.
- Males circumvent adverse effects of a long period of high testosterone levels.

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ABSTRACT

The song of songbirds is a testosterone-sensitive behavior that is controlled by brain regions expressing androgen receptors. At higher latitudes, seasonal singing is stimulated by increasing day-length and elevated circulating testosterone. However, a large number of songbird species inhabit equatorial regions under a nearly constant photoperiod, and the neuroendocrine mechanisms of seasonal song in these species have rarely been investigated. We studied males from an equatorial population of the silver-beaked tanager (*Ramphocelus carbo*), an Amazonian songbird. We found seasonality in dawn-song behavior, which was displayed continuously for more than half a year throughout an extended breeding territoriality stage. The seasonal activation of dawn-song was correlated with an increased area of androgen receptor expression in HVC, a major brain area of song control. However, testosterone levels remained low for several weeks after activation of dawn-song. Circulating levels of testosterone were elevated only later in the breeding season, coinciding with a higher dawn-song output and with the mating period. Our results suggest that the seasonal activation of dawn-song and territoriality involves an increase of androgen target cells in HVC. This mechanism could potentially function to circumvent adverse effects of high testosterone levels in a species with an extended breeding season.

1. Introduction

In temperate-zone songbirds, long spring days stimulate the production of gonadal testosterone, which in turn activates seasonal reproductive behaviors [1,2] such as song, a key component of courtship and territoriality in birds [3]. In the brain, song behavior is controlled by a network of interconnected neural nuclei collectively called the song control system. In all songbirds, the nuclei of this brain circuit, including the sensory-motor integration area HVC (used as proper name), express androgen receptors (AR) and thus are directly sensitive to testosterone [4]. In many species, males undergo seasonal changes in song behavior accompanied by changes in song nuclei morphology, which is regulated to a high degree by seasonal variations in testosterone levels [5]. Further, exogenous testosterone treatments can increase song activity, modify song nuclei anatomy, and increase the expression of AR in adult non-reproductive males [6–8].

The majority of songbird species (which make up the biggest radiation of birds today) are found in tropical regions. Most tropical songbirds also breed and sing on a seasonal basis although they experience only small annual changes in day-length. Generally speaking, tropical species exhibit long breeding periods, and have lower levels of testosterone than either temperate-zone species or other tropical species with short well-defined breeding periods [9–12]. Testosterone-

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dependent regulation of song behavior has been well studied at an individual level in temperate zone songbirds, but research on tropical species is scant [13]. Moreover, within the tropics studies of songbirds from truly equatorial regions that undergo minimal photoperiodic changes are nearly absent, probably due to inaccessible habitat. Therefore, neuroendocrine mechanisms underlying seasonal singing in equatorial songbirds remain largely unexplored.

The dawn-song is a period of intensive song that occurs at the first hour of light, before sunrise. This behavior is typically expressed in males, and associated with the courtship and territorial settlement [14]. Here, we investigated the interplay between gonadal testosterone production and testosterone sensitivity of HVC in the dawn-song behavior of male silver-beaked tanagers (*Ramphocelus carbo*) of an equatorial population from the eastern Amazonia (Brazil). This geographic region undergoes very small annual changes in photoperiod, but exhibits two main predictable seasons: the rainy and the drier season. This seasonal rainfall pattern creates conditions that favor an extended dawn-song period associated to the establishment of the breeding territories during the drier period. Thus, in absence of photoperiodic changes dawn-song was seasonally expressed by silver-beaked tanagers, during a period that extends up to 6–7 months. We found that the seasonal initiation of dawn-song behavior correlated with low plasma levels of testosterone, and was accompanied by an increase of the area of the HVC defined through AR-mRNA expression. Testosterone was found in higher levels later in the breeding season, probably associated with the mating stage, during a period of increased dawn-song output. Given that maintaining high levels of testosterone for a prolonged period impose physiological constraints, our results suggest that equatorial silver-beaked tanagers circumvent those adverse effects through the initiation of seasonal dawn-song with lower levels of testosterone in plasma.

2. Methods

2.1. Subjects and study site

The silver-beaked tanager is a sexually dimorphic, non-migratory seasonal breeder [15–17] very common in the east of Brazilian Amazonia [18]. Silver-beaked tanagers, belong to the songbird family Thraupidae, the second-largest family of birds, which comprises 12% of the Neotropical avifauna [19]. Further, the genus *Ramphocelus* consists entirely of species distributed throughout the tropics [20].

Our study site was located in the Amazon River basin, approximately 60 km to the northeast of the city of Belém in Brazil, very close to the equator (1°12′07″S 48°18′07″W, 30 m above sea level). The annual variation in day-length is ~9 minutes (on-line day-length calculator; United States Naval Observatory, Astronomical Applications Department). The region has an equatorial rainforest climate with little variation in average temperatures throughout the year (26 ± 4 °C). Although there is no real "dry" season (it rains almost every day), there is a distinct rainy season with very high daily rainfall that spans on average December through May; the drier season (here named as the dry season) lasts from June until November [21,22]. We delimited our study to a local zone with a radius of 5 km along the edge of primary Amazon rainforest. The information about precipitation levels were obtained daily from the INMET website (Instituto Nacional de Metereologia).

2.2. Field procedures, recordings and tissue collection

We conducted field work over the course of 1 year between 2011 and 2012. For neuroanatomical and endocrine studies, 20 males were caught throughout four monthly periods: April, July, and October of 2011 and January of 2012 (five males in each sampling period). All males were caught using mist nests and playback of conspecific calls and songs. Immediately upon capture (<5 min), a blood sample was taken from the wing vein, collected into heparinized capillaries and stored on ice until return to the field station. The blood samples were then centrifuged, and the plasma was separated and frozen at −40 °C. After the blood sample was taken, males were weighed and transported to the field station; brains were removed and frozen on dry ice 10 min after euthanasia by decapitation (approximately 15 min after capture). The brains were stored at −40 °C in a portable deep freezer. After brain extraction, the size of both testis was measured using calipers, and the volume was calculated as the volume of oval bodies $(4/3 * \pi * (\text{width}/2)^2 * (\text{length}/2))$.

The occurrence of dawn-songs within the study site was daily assessed by personal observations throughout the four sampling periods described above (April, July, and October of 2011 and January of 2012). Besides, we counted active nests discovered at the study site during different sampling periods. In July, once the dawn-song was seasonally initiated, 10 dawn-singing males were caught and ringed with a unique combination of colors for later identification of dawn-songs displays. We noted that individual males regularly displayed dawn-songs in exclusive areas with an approximate radius of 10 m, which define each male's territory. Male silver-beaked tanagers sang primary (and intensively) at dawn, and occasionally during the rest of the day. During the periods when no dawn-song occurred, no further song activity was observed during the rest of the day.

In addition to the daily personal observation, the overall dawn-song output was recorded with two passive recording devices (SM1: Wildlife Acoustics) attached to a tree at 1.5 m above the ground. These recording devices were placed in trees surrounded by at least three territories of ringed males. Each recording session was carried out from 5:00 am until 7:00 am. One recording was obtained in July and two in September, October, November December and January. We stopped to record during March, since there was no presence of dawn-song during February. Due to technical reasons, we could not analyze the recordings during the month of August 2011. To estimate the overall dawn song output, we summed the amount of time during which dawn-song occurred within 30 min after onset of the first dawn song detected in the recordings.

All males sacrificed for neuroanatomical and hormonal analysis were captured in territories located at least 2 km away from the territories used for dawn-song recordings and observation. Males caught during July, October and January, were observed singing before they were sacrificed. No dawn-song was observed in males sacrificed in April.

2.3. Brain data analysis

Frozen brains were cut into 20 μm parasagittal sections on a cryostat (Leica Microsystems GmbH, Wetzlar, Germany) and collected on Superfrost slides (Menzel GmbH, Braunschweig, Germany) in 10 parallel series. The first series was selected for Nissl staining; the second adjacent series was used for in situ hybridization of AR-mRNA. The in-situ hybridization methodology is described in next sub-section. Brightfield photomicrographs of the Nissl-stained brain sections were made with a Leica DM6000B digital microscope (Leica Microsystems), equipped with a Leica DFC420 5 MP CCD digital color camera. Autoradiograms were scanned with an Epson scanner using SilverFast Ai software as 16-bit gray values and with a resolution of 2400 dpi for later analysis in ImageJ. HVC volumes were estimated based on the Nissl-staining and AR-mRNA expression. For each Nissl stained and AR-labeled brain section we delineated HVC, summed the area measured, and multiplied by 200 μm (interval between sections) to obtain the total HVC volume. For each individual, the HVC was delineated and calculated twice with Nissl-stained and twice with AR-mRNA labeled sections and the final volumes were the average of these measurements. All measurements were carried out by one person, blind to the identity of the sections.

Optical densities of AR-mRNA expression levels were measured in a square (0.2 mm × 0.2 mm) that was positioned in the middle of the HVC area of every second section that included this nucleus. Optical density measures were averaged across all values of the HVC sections. To control for background staining the optical density in a control area just adjacent to the HVC measured was subtracted from the value obtained in HVC.

Cell density in HVC was estimated from Nissl-stained sections under high magnification. At the lateral, central and medial levels of each HVC, three counting frames of 120 μm^2 were analyzed. We counted all profiles that contained one or two nucleoli throughout the entire depth (20 μm) of the section using the optical dissector technique [23]. Density measurements are presented as 10^4 cells per mm^3.

2.4. In-situ hybridization

Riboprobes were synthesized from cDNA previously cloned from zebra finch androgen mRNA [24]. Antisense and sense ^{35}S-CTP-labeled probes were transcribed from the T7 and SP6 promoter region of a pGEM7Zf + vector using the Riboprobe System (Promega, Madison, WI). Brain sections were fixed in 4% formaldehyde in phosphate-buffered saline (PBS; 0.01 M; pH 7.4) for 5 min, washed in DEPC-treated PBS, and incubated in 0.25% acetic anhydride in ethanolamine (TEA; 0.1 M; pH 8.0) for 10 min to reduce nonspecific binding. After a washing step in 2× standard saline citrate (SSC), sections were dehydrated in serially increasing percentages of ethanol, and left to dry at room temperature. Sections were hybridized under a cover slide with ^{35}S-CTP-labeled sense or antisense riboprobes (0.4×10^6 cpm/slide) in hybridization buffer with 50% formamide and 10% dextran sulfate overnight at 55 °C. After hybridization, slides were immersed in 2× SSC at room temperature to remove the cover slides and incubated in RNase A (20 μg/ml) for 30 min at room temperature. Sections were then consecutively washed for 30 min in 2× SSC at 50 °C, 0.2× SSC at 55 °C, and 0.2× SSC at 60 °C, dehydrated in ethanol containing 0.3 M ammonium acetate, and dried for 1 h at room temperature. All the slides used with brain sections were processed in two separate groups. Finally, slides were exposed to Kodak BioMax MR film (Sigma-Aldrich Co., St. Louis, MO) in lightproof boxes for 3 weeks at room temperature, developed in Kodak D-19 developer, washed in tap water, and fixed with Kodak fixer.

2.5. Hormone analysis

Testosterone concentrations were determined by radioimmunoassay as previously described [25]. Samples were assayed in duplicate and distributed randomly between two assays. The extraction recovery was 88.0% ± 2.8% (mean ± sd). Hormone concentrations were calculated with Immunofit 3.0 (Beckmann Inc., Fullerton, CA, USA). The lower detection limits of assays were 0.37 pg/ml and 0.40 pg/ml tube, respectively, and all samples were above the detection limit. The intra-assay coefficients of variation were 9.1% and 4.2%, respectively; the intra-extraction coefficients of variation of a chicken plasma pool were 3.3% and 6.0%, respectively. The inter-assay coefficient of variation between the two assays was 9% and the inter-extraction coefficient of variation between the two assays was 15.9%.

2.6. Statistical analysis

R version 3.2.0 (R Development Core Team 2015) was used for all statistical analyses. Correlation between log-transformed testosterone levels and testis sizes were performed using Pearson analysis. Hormonal and brain data were analyzed using parametric linear models. As we found no effect of body mass in none of the models, we removed this variable from all our analysis. Thereby, changes in circulating levels of testosterone were analyzed across seasons, and testosterone data were log transformed to meet assumptions of normality. Seasonal changes in HVC size estimated with Nissl-staining were analyzed with cell density and testosterone levels as predictors. Seasonal changes in HVC size delineated by the AR-mRNA expression were analyzed with optical AR-mRNA density and testosterone levels as predictor variables. Changes in the AR-mRNA optical density of HVC were analyzed across seasons with testosterone levels and cell density as predictors; AR-mRNA optical density data were log transformed to meet the assumptions of normality. Finally, we tested whether cell density changes across seasons in response to testosterone.

3. Results

3.1. Dawn song and territories

Ringed males were only observed displaying dawn-songs within their own territories. We found seasonality in dawn-singing and territory occupancy (Figs. 1, 3). The seasonal activation of the dawn-song behavior occurred in July, which is the period with the shortest day-length of the year for that equatorial region (Fig. 3). Although at this latitude, the annual variation in day-length is only of 9 minutes. Dawn-songs were continuously displayed by males from July 2011 until January 2012, all along the dry period till the beginning of the next rainy season. The overall dawn-song output of the population over the non-rainy season showed a similar pattern of increase as the plasma testosterone levels in individual males (Fig. 1). Dawn-song did not occur in February 2012 and during April 2011. We recorded the presence of four nests in October 2011 and of one nest in January 2011, while no nests were found in April and July 2011 (Fig. 3).

3.2. Testosterone profiles and testicle sizes

The circulating testosterone levels were higher in October and January compared to April (intercept) ($F_{4,15} = 4.24$, $p = 0.02$) (Figs. 1, 3). We found a significant positive correlation between testosterone levels and testes size of males ($r = 0.69$, $p = 0.0007$).

3.3. HVC size, androgen receptor density, and cell density

We found that the size of HVC determined by AR-mRNA expression was significantly increased in July and October ($F_{3,16} = 18.8$, $p = 0.0001$) (Figs. 1, 2), with no effect of circulating testosterone levels ($F_{1,16} = 3.50$, $p = 0.08$), and no effect of AR-mRNA optical density ($F_{1,16} = 1.8$, $p = 0.19$). There was no seasonal changes in HVC size delineated through Nissl-staining ($F_{3,16} = 1.25$, $p = 0.32$). The AR optical density HVC did not significantly differ among the four periods studied ($F_{3,16} = 0.19$, $p = 0.89$). In the same way, no seasonal changes were found in cell density of HVC ($F_{3,16} = 2.22$, $p = 0.12$).

4. Discussion

Male silver-beaked tanagers from an equatorial population showed a strong seasonal pattern in dawn-song behavior. The seasonal onset of dawn-song production occurred during the period of the shortest day-length of the equatorial region and was accompanied by low circulating levels of testosterone (Figs. 1, 3). This is in contrast to patterns seen in most temperate-zone songbirds, where seasonal activation of singing is achieved by the increase of gonadal testosterone production in concordance with increasing photoperiod [26–28].

Generally, the dawn-song behavior functions principally in the mediation of social relationships, for instance through communication between male territorial neighbors [14,29]. Accordingly, individuals male silver-beaked tanagers displayed dawn-song within their territories throughout the whole dry season (during 6–7 months), until the beginning of the next rainy season (Fig. 3). Thus, the dry season might generate an annual window that favors the extended period of dawn-song behavior associated with the establishment of breeding territories. Numerous studies in birds have shown that plasma titers of testosterone are correlated with the expression of territorial behaviors,

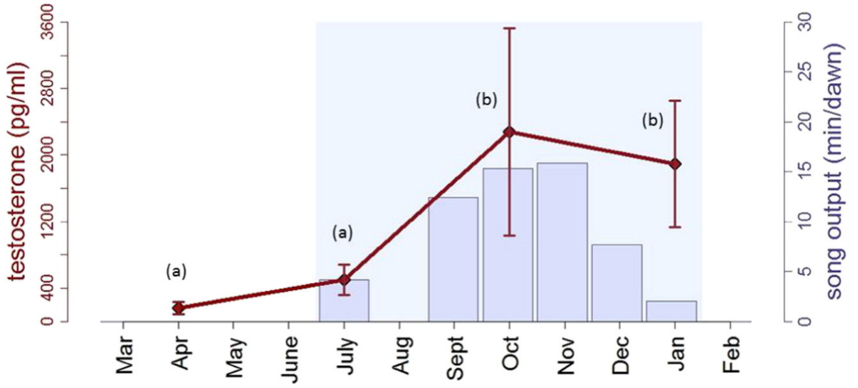

Fig. 1. Plasma testosterone levels of males in relation to dawn-song output. The red line shows means (+/− standard error) of circulating testosterone levels over the four sampling periods (April, July, October and January). Different letters represent significant differences ($p < 0.05$). The bars show the dawn-song output represented as the mean number of minutes of dawn-songs per day in the month (except in July when one recording was obtained). There is no data available from August. The blue shading indicates the period during which dawn-song occurred. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

including song [30–32]. Tropical bird species with higher levels of testosterone often have short and well-defined breeding seasons, and typically establish territories for short periods [9]. On the other hand, It has been suggested that tropical birds with extended periods of territoriality may be more responsive to low circulating concentrations of testosterone [33,34]. Long periods with high circulating levels of testosterone imply physiological impairments for animals. These adverse actions may include high metabolic activity, reduced immunocompetence, and/or elevated mortality [32]. Our data indicate that the seasonal activation of dawn-song in silver-beaked tanager occur with lower levels of circulating testosterone. This mechanism may allow circumventing the effects of a protracted period of high testosterone. One possibility is that the occurrence of small or short-term increases in circulating testosterone could activate seasonal dawn-song in males during the territory establishment in July. In relation, there is an apparent subtle increase of testosterone levels in males caught in July compared to males caught in April (Fig. 1.), although there is no statistical significance. Further, the overall dawn-song output increased later in the breeding season along with the increase of testosterone (Fig. 1). This association suggests that higher levels of testosterone in plasma might be necessary at later parts of the breeding stage (i.e. for consummatory sexual behaviors such as mating [35]), so testosterone may influence the dawn-song activity of males in a context-dependent manner [36,37].

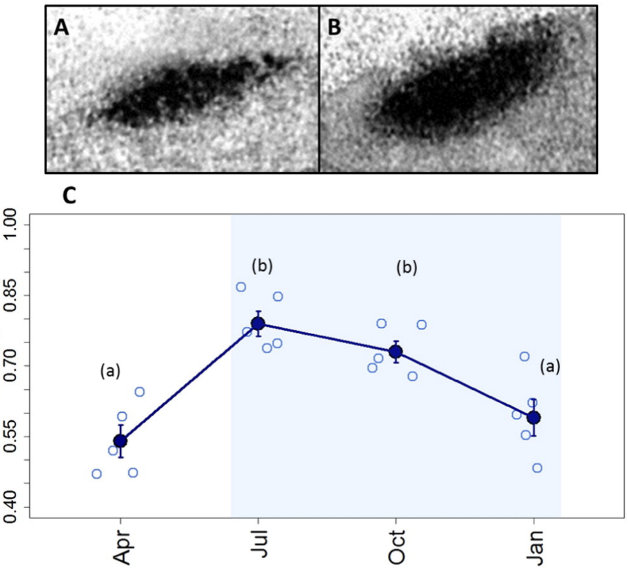

Fig. 2. Seasonal change in the volume of HVC defined by AR mRNA expression. Autoradiogram of in-situ hybridization of AR-mRNA expression in HVC from two different males, one obtained in April 2011 (A), and the other in July 2011 (B); note the difference in size of two equivalent HVC sections. (C) HVC volume delineated by AR-mRNA expression (blue line). Solid circles show the mean of the month (+/− standard error), open circles represent individual measures ($n = 5$ per period). Different letters indicates significant differences. The blue shading indicates the period during which dawn-song occurred. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Previous studies conducted in highland equatorial rufous-collared sparrows (*Zonotrichia capensis*) have shown a positive relationship between high levels of circulating testosterone and the activation of song in free-ranging males, with a relatively short and well-defined breeding period [11]. This pattern is the one more typically described for non-tropical songbirds. Given that the rufous-collared sparrow is situated within a clade of Nearctic songbirds [38,39], it is likely that the species has conserved a testosterone-dependent mechanism of singing that evolved at higher latitudes, prior to expanding into the tropics. The results found in the rufous-collared sparrows contrast with our results in silver-beaked tanagers, where high plasma levels of testosterone were decoupled from the seasonal activation of dawn-song during the onset of an extended breeding territoriality.

The seasonal onset of dawn-song in equatorial silver-beaked tanagers temporally coincided with an increased HVC area, as delineated by the AR-mRNA borders (Figs. 2, 3). This correlation indicates that seasonal changes in the AR-mRNA expression in HVC cells might be involved in the seasonal activation of dawn-song. As we found no changes in the total cell density and AR-mRNA optical density of HVC (Table 1), the results suggest a seasonal increase in the number of HVC androgen target cells. Although, AR mRNA levels does not always predict its AR protein expression [40]. Visual comparison of adjacent Nissl stained and AR mRNA labeled HVC sections indicated that the AR mRNA delineated HVC extended mainly beyond the medial and caudo-ventral border of the Nissl-defined HVC during July and October. From our data we cannot infer the nature and mechanism underlying this difference, however, based on previous studies [41–43] we hypothesize that transient changes in the number of androgen target cells among RA-projecting neurons of HVC might occur. Given the characteristics and functional significance of RA-projecting neurons in HVC [41,42], we speculate that such changes could play an important role in the activation of singing in a species-specific manner. Further investigation is necessary to clarify this assumption. On the other hand, an increase of androgen target cells in HVC can potentially function as a mechanism to allow low levels of testosterone to activate seasonal dawn-song. As demonstrated in spotted antbirds (*Hylophylax n. naevioides*) a Neotropical suboscine, territorial behavior can be elicited under low levels of circulating testosterone and is facilitated by the increased sensitivity to testosterone in a regulatory brain related area [33]. Alternatively, dehydroepiandrosterone (DHEA), a non-gonadal androgen precursor,

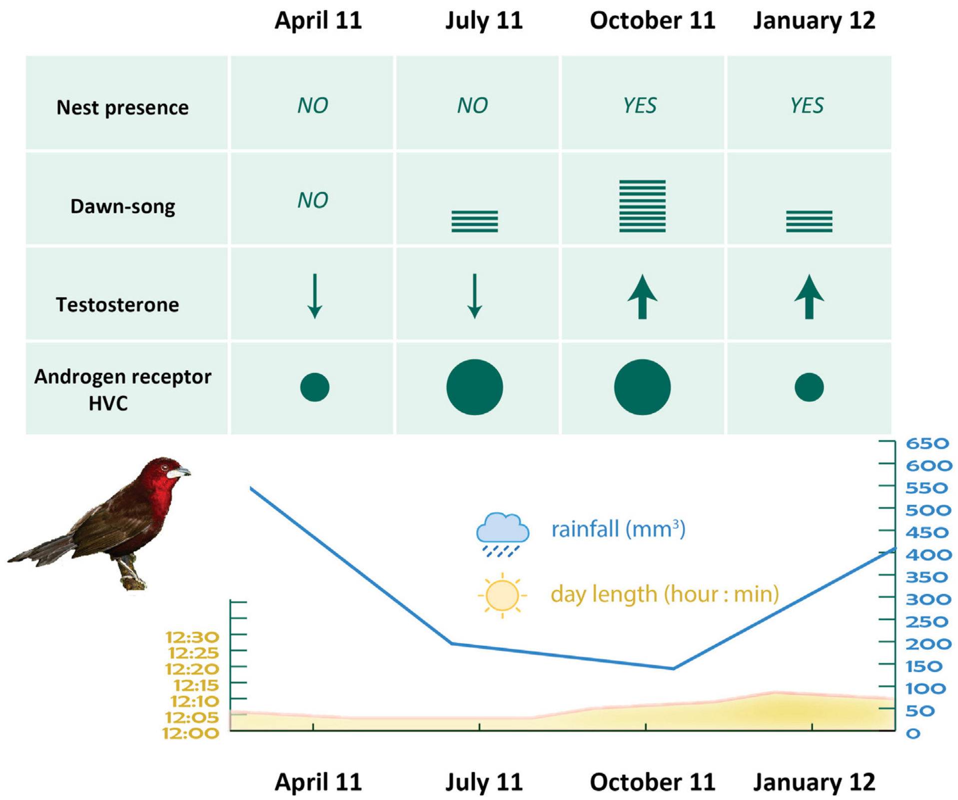

Fig. 3. Seasonal parameters of equatorial male silver-beaked tanagers in relation to environmental fluctuations. The gridded box above illustrates seasonal changes of four life history parameters over the year: 1) active nest found, 2) occurrence of dawn-song, 3) testosterone levels in plasma, 4) and volume of HVC estimated by its mRNA AR expression. Differences in symbol sizes represent seasonal differences. The graph below shows in yellow the variation in day length and in blue the variation in rainfall over the seasons (silver-beaked tanager image obtained from original picture [45]). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

could potentially provide a substrate for local brain synthesis of testosterone during seasonal activation of dawn-song in silver-beaked tanagers. Several studies suggest that DHEA can regulate territorial song [44] and HVC plasticity of songbirds when circulating levels of testosterone are low [45–47].

The seasonal increase in circulating testosterone temporally corresponded with a larger number of breeding nests observed in the field and with the breeding period reported previously for silver-beaked tanagers in this geographic area [17]. Further, individual levels of testosterone were highly correlated with testis size. We also have evidence that the testosterone levels change seasonally in a quite synchronous way among males within our study site (Quispe et al. in prep), which suggests a highly synchronized breeding of equatorial silver-beaked tanagers. Previous studies on Neotropical birds inhabiting tropical rainforests indicate that day-length, rainfall and food availability can contribute to cueing breeding schedules. [11,48–50]. Thus, in silver-beaked tanagers the seasonal increase of gonadal testosterone may be triggered by an increase of 4–5 min in the photoperiod, or alternatively by seasonal changes in rainfall (Fig. 3). Photoperiodic changes of 17 min have been demonstrated to stimulate reproduction in a tropical bird (at 9°N latitude) [48], however, there is no evidence of an equatorial bird able to sense 4–5 min of annual change in day-length. Yet, changes in cloud cover can produce predictable changes in light intensity at equatorial regions, a cue which could also be used by birds to time seasonal behaviors [51], such as singing. On the other hand, strong endogenous circannual rhythms can be particularly important for birds living at the equator [52]. Experiments on captive equatorial Stonechats (*Saxicola torquata*) provided evidence that these songbirds synchronize their strong circannual rhythmicity with the seasonal rainfall [53].

Undoubtedly, the ecological bases of behavior are closely related with the evolution of neuroendocrine mechanisms. Therefore, given the variety of equatorial environments and the large avian diversity inhabiting them, many possible mechanisms likely underlie the interactions between hormones and behavior in equatorial songbirds. Studies on wild tropical and equatorial songbirds are crucial to expand our knowledge about the environmental and neuroendocrine regulation of behavior.

Ethics

This research was conducted in accordance with permits of the Sistema de Autorização e Informação em Biodiversidade — SISBIO in

Table 1
Means (±standard error) of the physiological and morphological parameters measured during four sampling periods.

	April	July	October	January
Testosterone (pg/ml)	161.38 ± 73.5	500.58 ± 182.9	2279.12 ± 1243.5	1897.56 ± 760.7
Testis size (mm^3)	9 ± 0.29	5.5 ± 0.16	12.1 ± 0.25	14.6 ± 0.37
HVC volume Nissl (mm^3)	0.56 ± 0.03	0.6 ± 0.03	0.53 ± 0.03	0.61 ± 0.05
HVC volume AR (mm^3)	0.54 ± 0.03	0.79 ± 0.03	0.73 ± 0.02	0.59 ± 0.04
cell density (10^4/mm^3)	29.92 ± 1.87	27.82 ± 1.78	30.86 ± 1.49	33.30 ± 0.55
AR optical density	0.59 ± 0.14	0.53 ± 0.06	0.69 ± 0.27	0.45 ± 0.08

Brazil, number 26,444–1. All laboratory sample procedures were conducted according to German and European laws and authorized by the responsible governmental authority, the Regierung von Oberbayern.

Authors' contributions

RQ and MG wrote the manuscript. FS and MLS provided substantial input and revisions. RQ and MG conceived of and designed the study. RQ conducted the laboratory work and analyzed the data. RQ and FS carried out sample collection and field work. MLS processed the permits, and provided academic and technical guidance during field work.

Competing interests

The author declares no competing financial interests.

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Chapter 3

Investigating the effect of rainfall on male gonadal activity in an equatorial songbird

Abstract

In higher latitudes, predictable changes in the photoperiodic cycles are used as the primary predictive cue for the timing of reproduction in birds. The increase in day length in spring stimulates the hypothalamus to release gonadotropin-releasing hormone (GnRH) into the pituitary-portal blood system. GnRH secretion stimulates the release of luteinizing hormone and follicle-stimulating hormone from the anterior pituitary. These gonadotropins induce gonadal growth and the release of testosterone into the circulatory system of males, which in turn mediate the expression of reproductive traits. However, in the absence of significant changes in photoperiod, several studies have documented seasonal reproduction of tropical birds, but it remains unclear how those species detect and translate environmental cues into the neuroendocrine cascades regulating reproduction. Here, we investigated whether the precipitation influences the gonadal responses to GnRH in male silver-beaked tanagers, an equatorial songbird species. Males were maintained under semi-natural conditions in two different experimental regimes of precipitation, where they were treated with exogenous GnRH hormone. To examine testis activity in response to these different precipitation rates, we measured gonadal testosterone production before and after GnRH injection. We found that males kept in dry conditions had a larger magnitude of change from baseline testosterone to GnRH-induced levels. Our results suggest that predictable patterns of annual rainfall might act as a proximate environmental cue to time the reproductive physiology of male silver-beaked tanagers.

Prepared as: *Quispe, R.; Gahr, M.: Investigating the effect of rainfall on male gonadal activity in an equatorial songbird.*

Introduction

Most habitats over the biosphere exhibit temporal fluctuations in biotic and abiotic conditions. Since the reproductive stage is particularly demanding, birds usually breed when conditions are suitable for rearing the offspring (Dawson, 2008; Wingfield, 2008; Wingfield and Farner, 1993). After the breeding period many birds completely shut down their reproductive functions and enter into the next life-history stage, for example molt or migration (Wingfield, 2005). This inactivation of the reproductive system entails a temporal constraint because its seasonal re-activation usually requires time (Bernard et al., 1997; Dawson, 2015; Wingfield, 1993; Wingfield et al., 1992) . Accordingly, species living in seasonal habitats time their reproductive cycles by using environmental cues as a reliable signal (Dawson, 2008; Leitner et al., 2003). Thereby, in temperate-zone birds the reproductive stage is usually highly synchronous, within and among populations, as a result of the ubiquitous nature of photoperiod, the primary predictive and regulatory environmental cue (MacDougall-Shackleton et al., 2009).

The mechanisms regulating reproduction are well documented for birds living at temperate latitudes, mainly from the northern hemisphere (e.g. Follett et al., 1974; Wingfield, 1993; Wingfield et al., 1979). The increase of day length in spring activates the hypothalamic–pituitary–gonadal (HPG) axis, which begins with the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus. The GnRH then stimulates the secretion of hormones such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary, which in turn activate the production of gametes and steroid hormone production in the gonads (Hau, 2007). Gonadal steroids regulate important changes in morphology, physiology, and behavior associated with reproduction (Balthazart et al., 2010; Goymann and Hofer, 2010; Ramenofsky, 2011).

In general, little information is available about the HPG axis regulation and reproductive cycles of birds living in habitats with small photoperiodic changes. In particular for equatorial birds, the environmental control of seasonal reproductive mechanisms is poorly understood. Here, we studied the HPG axis sensitivity to rainfall of an equatorial songbird, the Silver-beaked tanager, (*Ramphocelus carbo*). This species is recognized as a non-migratory seasonal breeder, endemic to the Amazon region. We studied wild individual males that inhabit the eastern Amazon, a region where it rains almost every day, but there is a distinct rainy season that occurs annually and is highly predictable (Liebmann and Marengo, 2001; Moraes et al., 2005). In this region, silver-beaked tanagers typically breed during the dry season, but the peak in testosterone levels occur at the last part of this drier period, when the precipitation rate starts to gradually increase (Quispe et al., 2016). Therefore, in order to investigate whether the seasonal rainfall is a significant proximate factor in the reproductive control of equatorial silver-beaked tanagers, we experimentally subjected different males to a high or low precipitation treatment. We estimated the gonadal activity of males under these two simulated precipitation regimes by measuring gonadal testosterone production before and after the injection of exogenous GnRH hormone. The GnRH treatment is a method that allows us to assess the testis maximum physiological capacity to produce testosterone in males (Goymann et al., 2015). Thereby, we can examine the HPG axis responses to changing environmental factors, such as rainfall.

Methods

Study region and experimental rainfall rate manipulation

Two semi-natural outdoor aviaries of 2m x 2m x 2m were built in the surrounding area of our field biological station. Both aviaries were built on a framework of firm wood strips covered by rigid plastic mesh with natural soil as ground (Fig 1 and 2). We captured 12 adult males from the wild, of which six males were randomly placed in each of the two aviaries. All males were captured between the last two weeks of October, which is the period when males present a seasonal peak in circulating testosterone levels (Quispe et al., 2016).

The study region is located in the Amazon River basin, approximately 60 km to the northeast of Belem city in Brazil, very close to the equator (1°12′07″S 48°18′07″W). The annual variation in day length is approximately 9 minutes (on-line day length calculator; United States Naval Observatory, Astronomical Applications Department). This zone has an equatorial rainforest climate with average temperatures that vary little throughout the course of the year (26 ± 4 C°; INMET; weatherbase.com). Although it rains almost every day (i.e. no real dry season) in this region, there is a distinct rainy season with very high daily rainfall (INMET; weatherbase.com) that spans in average from December through May, while the drier season lasts from June until November (Liebmann and Marengo, 2001; Moraes et al., 2005).

In order to test the effect of rainfall on the testis activity of males, one aviary, the rainy aviary, was used to artificially increment the amount of precipitation. The other aviary, the dry aviary, was used to reduce direct contact to natural rain precipitation. The rainy aviary was equipped with an interconnected system of hoses that supplied five small rotor sprinklers placed on top of the roof. One sprinkler was located in each corner and a fifth sprinkler was situated in the center of the roof. Males were maintained inside the aviaries during a period of one month, from the 1st of November until the 1st of December 2012. During this period, we activated the system by supplying water for one hour in the morning (from 8 to 9 h) and one hour in the afternoon (from 16 to 17 h). As the roof of the rainy aviary was made of mesh material, animals were allowed to receive natural rain besides the precipitation received from the sprinkles. The dry aviary, on the other hand, did not have a hose precipitation system, and we reduced direct contact of birds with natural rain by covering the roof densely with large palm leaves. Inside the aviaries male individuals were provided with drinking water, fresh fruits and a mush made from a mixture of different fruits and cereals.

The experiment was conducted in the month of December, during the end of the dry season and beginning of the wet season, when birds are in their breeding stage (Quispe et al., 2016; Valente, 2000). After one month living under different precipitation treatments, we collected a blood sample to analyze testosterone levels in the plasma of each male. After this first bleeding, males were injected with 50 ml chicken GnRH-I (Bachem H 3106; 1.25 mg dissolved in 50 ml isotonic saline) into the pectoralis major muscle and kept in a holding bag. After 30 min, a second blood sample was taken.

Hormone assays

Testosterone concentrations were determined by radioimmunoassay following the procedures described in (Goymann et al., 2006). Samples were assayed in duplicate in one assay. The extraction recovery was 84 % ± 6.2 % (mean ± sd), the lower detection limit of the assay was

0.33 pg/ml and all samples were above the detection limit. The intra-assay coefficient of variation was 3.7 %, and the intra-extraction coefficient of variation of a chicken plasma pool was 1.4 %.

Statistical analysis

R version 3.2.0 (R Development Core Team 2015) was used for all statistical analysis. In order to estimate the influence of rainfall on the testis response to exogenous GnRH, we used four general linear models to analyze: (1) baseline testosterone levels of males before GnRH injection with aviary treatment (Dry and Rainy) as explanatory variable, (2) testosterone levels after the GnRH injection with aviary treatment as explanatory variable, (3) magnitude of the rise in testosterone levels in males after the GnRH injection with aviary treatment as a factor.

In addition, we analyzed (4) the variation in testosterone levels reached after GnRH injection within each aviary with baseline testosterone levels as explanatory variable (Dry and Rainy).

Testosterone data were log transformed to meet assumptions of normality.

Results

There was a significant effect of aviary treatment on the magnitude of increase in testosterone after the GnRH-injection ($F_{1,10}$ = 5.2, p = 0.04). Compared to the males from the dry aviary, individuals maintained in the rainy aviary had a lower magnitude of change between the testosterone levels before and after GnRH injections (Table 1) (Fig.3.).

There were no significant differences between males from the two aviaries in the testosterone levels before the GnRH injection ($F_{1,10}$ = 2.6, p = 0.13), and no significant differences either for testosterone levels after the GnRH injection ($F_{1,10}$ = 2.3, p = 0.15).

There was no effect of baseline testosterone concentrations on the testosterone levels reached after GnRH injection of males within each of the two aviaries.

Discussion

Male silver-beaked tanagers under different rain experimental conditions differed in their magnitude of increase in circulating testosterone levels after the injection of GnRH. The administration of exogenous GnRH induces a short-term rise in gonadal testosterone production from an individual's baseline to the respective individual's maximum. The magnitude of the testosterone increase in plasma represents the individual's hormonal reactive scope of testosterone (within an individual physiological range; Goymann et al., 2015). In our experiment, males kept in the dry aviary showed a larger magnitude of change in circulating testosterone compared to individuals maintained in the rainy aviary. Therefore, the results indicate that the males under the dry experimental conditions have a wider range of testosterone production, which is illustrated in their steeper slopes of GnRH-induced testosterone increase (Fig.3). On the other hand, males that were exposed to the rainy conditions had a more restricted capacity to further modulate testosterone levels. Therefore, the results suggest that seasonal rainfall patterns may affect the HPG axis regulation and the capacity of gonadal testosterone production in equatorial silver-beaked tanagers. It is of note that male silver-beaked tanagers showed lower levels of baseline testosterone compared to what is normally seen in their free-living conspecifics (see Chapters 1 & 2). This is probably due to the lack of supplementary factors and/or social cues under captivity experimental conditions. In general, testosterone levels are typically much lower in captive birds than in free-living individuals (Breuner et al., 1999; Wingfield and Farner, 1978).

Previous field studies showed that silver-beaked tanagers in the eastern Amazon initiate an extended breeding period during the dry season, which begins with the establishment of male's territories (Quispe et al., 2016; Valente, 2000). Males maintain their territories by displaying a dawn-song behavior during most of the dry season, and they undergo a peak of testosterone production only at the end of this drier period, when the rainfall starts to gradually increase (3 month after the onset of territoriality). This peak of gonadal testosterone production is probably related to an increase in copulatory activity of the males. Later on, during the rainy season there is a cessation of the reproductive functions of males, including a decrease in testosterone levels and absence of dawn-song displays (Quispe et al., 2016). For our experiment, we caught males at the end of the dry season, when the peak of gonadal activity occurs. Thus, by putting males in the rainy aviary we simulated the environmental conditions that they experience further in the rainy season, when their reproductive stage is completed. Accordingly, our results show that males in the rainy aviary had a smaller magnitude of testosterone increase, and a narrower capacity to modulate testosterone levels. This result, therefore, suggests that the increase of rainfall acts as a proximate environmental cue that affects the regulation of the HPG axis, constraining the range of gonadal testosterone production. In addition, two individuals of the rainy aviary decreased their levels of testosterone after GnRH injection (Fig. 3.), which suggests that some males were not able to further increase gonadal testosterone production. Besides, males in the rainy aviary had a larger degree of variability in their baseline testosterone (although there is no statistical difference of baseline among males from both treatments). This observation agrees with field studies that have indicated that during the rainy season (at the end of the breeding period of silver-beaked tanagers) testosterone levels of males start to decrease with some degree of individual variability (more asynchronous) (see **Chapter 1**), compared to what is normally seen in temperate zone species. On the other hand, the dry aviary mimicked the condition that males normally experience during the height of the dry season, which is the period of territorial establishment of males, previous to the occurrence of the peak of testosterone. We found that males in the dry aviary had more homogeneous concentrations of baseline testosterone. Interestingly, males in the dry aviary had a larger physiological range for gonadal testosterone production. These results agree with the assumption that, although male silver-beaked present low levels of circulating testosterone over most of the dry season, they maintain the HPG axis in an active functional state during this phase of breeding territorial settlement. This may allow them to circumvent the detrimental effects of having elevated testosterone during an extended breeding period (Wingfield et al., 2001).

In summary, GnRH treatments can induce increases in testosterone levels that are similar to the potential increases that could occur in the real life of birds and mimic the dynamic nature of the HPG axis in different life history stages (Goymann et al., 2015). We showed that higher rainfall rates may exert a restrictive effect on the hormonal reactive scope to produce testosterone regulated by the HPG axis. Overall, the results suggest that the predictable pattern of annual rainfall experienced by equatorial silver-beaked tanagers might act as a proximate environmental cue to time the reproductive physiology of males. Further studies are necessary to determine how other environmental cues could complement or interact with rainfall in the breeding phenology of equatorial silver-beaked tanager. To our knowledge very few studies have experimentally examined the influence of rain on the reproductive regulatory mechanisms of equatorial birds. Thus, the present work contributes to the scarce and necessary knowledge concerning the environmental control of reproductive cycles in animals from equatorial habitat.

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Figures and tables

Figure 1 . Rainy aviary with the interconnected hose system

Figure 2. Male silver-beaked tanagers inside an aviary

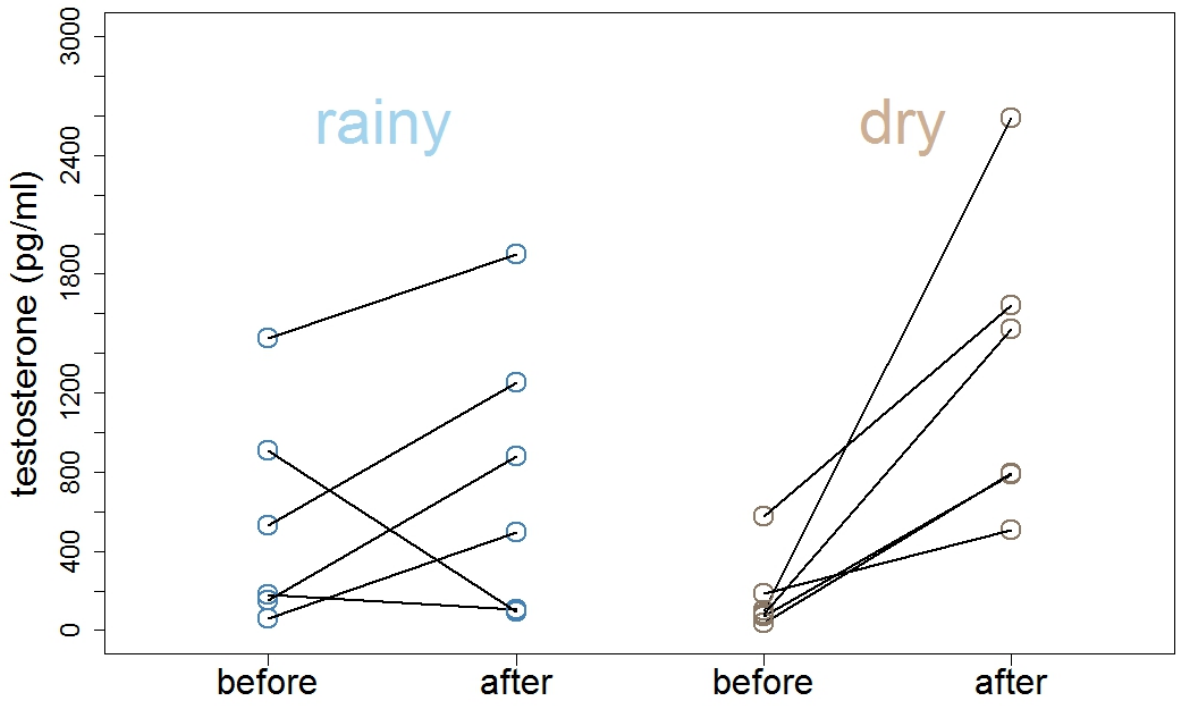

Figure 3. Testosterone levels in plasma before and after GnRH injection in males kept in two different experimental conditions, the rainy and dry aviaries.

Table 1.

Means (± standard error) of gonadal testosterone levels before and after GnRH injection in males kept under the two experimental treatments, rainy and dry aviaries.

	Rainy aviary	**Dry aviary**
Testosterone levels (pg/ml) ***before GnRH injection***	**550.9** ± 225	**175.3** ± 82.4
Testosterone levels (pg/ml) ***after GnRH injection***	**786.4** ± 288.1	**1305.5** ± 314.3

Chapter 4

Towards more physiological manipulations of hormones in field studies: Comparing the release dynamics of three kinds of testosterone implants, silastic tubing, time-release pellets and beeswax

Abstract

Hormone manipulations are of increasing interest in the areas of physiological ecology and evolution, because hormones are mediators of complex phenotypic changes. Often, however, hormone manipulations in field settings follow the approaches that have been used in classical endocrinology, potentially using supra-physiological doses. To answer ecological and evolutionary questions, it may be important to manipulate hormones within their physiological range. We compare the release dynamics of three kinds of implants, silastic tubing, time-release pellets, and beeswax pellets, each containing 3 mg of testosterone. These implants were placed into female Japanese quail, and plasma levels of testosterone measured over a period of 30 days. Testosterone in silastic tubing led to supraphysiological levels. Also, testosterone concentrations were highly variable between individuals. Time-release pellets led to levels of testosterone that were slightly supraphysiological during the first days. Over the period of 30 days, however, testosterone concentrations were more consistent. Beeswax implants led to a physiological increase in testosterone and a relatively constant release. The study demonstrated that hormone implants in 10 mm silastic tubing led to a supraphysiological peak in female quail. Thus, the use of similar-sized or even larger silastic implants in males or in other smaller vertebrates needs careful assessment. Time-release pellets and beeswax implants provide a more controlled release and degrade within the body. Thus, it is not necessary to recapture the animal to remove the implant. We propose beeswax implants as an appropriate procedure to manipulate testosterone levels within the physiological range. Hence, such implants may be an effective alternative for field studies.

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Short Communication

Towards more physiological manipulations of hormones in field studies: Comparing the release dynamics of three kinds of testosterone implants, silastic tubing, time-release pellets and beeswax

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ABSTRACT

Hormone manipulations are of increasing interest in the areas of physiological ecology and evolution, because hormones are mediators of complex phenotypic changes. Often, however, hormone manipulations in field settings follow the approaches that have been used in classical endocrinology, potentially using supra-physiological doses. To answer ecological and evolutionary questions, it may be important to manipulate hormones within their physiological range. We compare the release dynamics of three kinds of implants, silastic tubing, time-release pellets, and beeswax pellets, each containing 3 mg of testosterone. These implants were placed into female Japanese quail, and plasma levels of testosterone measured over a period of 30 days. Testosterone in silastic tubing led to supraphysiological levels. Also, testosterone concentrations were highly variable between individuals. Time-release pellets led to levels of testosterone that were slightly supraphysiological during the first days. Over the period of 30 days, however, testosterone concentrations were more consistent. Beeswax implants led to a physiological increase in testosterone and a relatively constant release. The study demonstrated that hormone implants in 10 mm silastic tubing led to a supraphysiological peak in female quail. Thus, the use of similar-sized or even larger silastic implants in males or in other smaller vertebrates needs careful assessment. Time-release pellets and beeswax implants provide a more controlled release and degrade within the body. Thus, it is not necessary to recapture the animal to remove the implant. We propose beeswax implants as an appropriate procedure to manipulate testosterone levels within the physiological range. Hence, such implants may be an effective alternative for field studies.

1. Introduction

Hormones are internal messengers that are released into the blood circulation after production and thereby can reach each single cell in the body. Thus, hormones can function as effective mediators of behavioral, physiological and morphological changes. The pleiotropic potential of hormones – that is their capacity to regulate multiple traits simultaneously – renders them particularly suited to control complex phenotypic changes such as transitions between different life-history stages (Hau, 2007). Their pleiotropic potential also qualifies hormones as effective mediators of trade-offs in resource allocation, for example the potential trade-off between reproduction and self-maintenance (Hau, 2007; Ketterson and Nolan, 1999; Zera et al., 2007). This is why the last two decades have seen a growing interest of evolutionary and ecological physiologists in hormones as a mechanistic link between changes in the environment and individual phenotypes. As a consequence, hormone manipulations in free-living animals to study the effect of hormones on fitness-relevant traits have become quite common.

The methods to manipulate hormones (in particular increasing the natural hormone level via artificial hormone implants) have been adopted from studies of classical endocrinology, which has been mainly concerned with the basic mechanisms of hormone action. In classical endocrinology, to study for example the effects of testosterone in males one would remove the testes and investigate the changes in the behavior, physiology and morphology (e.g. Berthold, 1849). In a second step, one would administer testosterone to restore the trait (e.g. Adkins-Regan, 2005; Ball and Balthazart, 2008; Balthazart et al., 2009). Often, the hormone dosages used in such studies have been in a pharmacological range, far beyond what an animal would experience under naturally relevant circumstances. If such methods are adapted in an

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ecological and evolutionary framework, the application of pharmacological doses of hormones may become particularly problematic (Fusani, 2008; Goymann and Wingfield, 2014). Natural and sexual selection can only act on phenotypes that are realized in nature. This means that only hormone manipulations within the existing physiological range can mimic existing conditions on which selection could act on. Of course, one could argue that by selecting hormone concentrations outside the range of natural levels one could produce a more extreme phenotype that could eventually evolve if higher levels of the hormone would mediate an extreme phenotype that may possess a selective advantage. Thus, a more extreme hormone manipulation may just mimic a potential for evolution and from this point of view adding supra-physiological levels may be justified. However, supra-physiological hormone concentrations may be problematic for other reasons and thus may not represent a good way of mimicking a more extreme phenotype. Effects of hormones often are not dose-dependent but can follow a step-function or a reverse U-shaped curve (Adkins-Regan, 2005; Hews and Moore, 1997). Very high concentrations of a hormone may even have non-specific effects (McDonnel and Murdoch, 2001; Purohit et al., 2000; Seyrek et al., 2007) that would not occur under normal physiological conditions, and that may not be relevant in ecological or evolutionary contexts. Thus, it may be particularly important for studies in ecology and evolution to manipulate hormone levels within or at least only slightly above their possible physiological range.

The most common method to increase hormone concentrations are hormones embedded in silastic tubing or time-release pellets which gradually release the hormone from a self-disintegrating matrix (Edler et al., 2011; Fusani, 2008). For field studies, the biodegradable time-release pellets have the major advantage that it is not necessary to recapture the animal to remove the implants after the study. Also, it appears that the pellets provide a more constant hormone release than silastic tubing (Fusani, 2008). Unfortunately, there are only a few studies that investigated the release dynamics of silastic implants and/or pellets (Christensen and Kesler, 1984; Edler et al., 2011; Fusani, 2008; Van Steenbrugge et al., 1984). Typically, investigators implant the hormone and take a blood sample of recaptures 1–3 weeks after implantation. After these periods hormone concentrations may be in a physiological range, but there are some indications that within the first few days after implantation this may not be the case and hormone concentrations may be supra-physiological instead (Edler et al., 2011; Fusani, 2008).

Our aim was to compare the temporal dynamics of the release of testosterone from silastic tubing and from time-release pellets over a period of 30 days. In addition, we searched for alternative biodegradable matrices that would allow for a more constant release of testosterone throughout this period of time. To this end, we tested beeswax implants and provide information regarding biodegradable injectable polymers (for the latter see Supplementary Material). Because most hormone manipulation studies in evolutionary and ecological physiology are conducted with birds, we used Japanese quail (*Coturnix japonica*) to compare the three kinds of implants in more detail. Japanese quail are large enough to be repeatedly bled within short periods of time, which was essential to compare the temporal release dynamics of the implants during the first few days after implantation. We used female Japanese quail maintained under short-day (non-reproductive) conditions to obtain a defined baseline of low endogenous plasma testosterone. The results demonstrate that the temporal dynamics of testosterone release from silastic tubing, time-release pellets, and beeswax implants differ substantially and suggest that beeswax is a suitable and low-cost procedure to study the effect of hormones on phenotypes in ecologically and evolutionary relevant settings.

2. Material and methods

2.1. Animal housing, implant devices and experimental design

Individual adult female Japanese quail (*C. japonica*) were kept in temperature-controlled rooms (21 ± 1 °C) on a 6 h light and 18 h dark cycle to maintain them in a non-reproductive status. In total there were 3 groups of 18 color-ringed females. Each group of females was housed in a mesh aviary of 2 m × 2 m × 2 m, and provided with food (quail breeder feed), fresh vegetables and water ad libitum. To compare the release rate of 3 different kinds of implants, individuals of each aviary group were either treated with (1) a silastic tube, (2) a 21 day time-release pellet, or (3) a beeswax implant. Individuals of each treatment group were randomly assigned to either a control group ($N = 9$, empty implants/pellets) or a testosterone treatment group ($N = 9$, implants or pellets filled with testosterone (Sigma T1500). All implants or pellets were inserted under local anesthesia with lidocaine hydrochloride (Xylocain Gel 2%, AstraZeneca) under the skin at the upper back of the birds.

Individuals of the silastic implant group received one implant made of silastic tubing of 10 mm length (Dow Corning, USA; 1.47 mm inner diameter, and 1.96 mm outer diameter) filled with 3 mg of crystalline testosterone and sealed on both sides with silicone. The surface area of silastic tubing implant was approximately 147 mm^3. Control animals were implanted with empty silastic tubing. Before implantation, the implants were soaked in sterile saline solution overnight. Individuals of the pellet group received two 21 day time-release pellets (Innovative Research of America, Sarasota, FL, USA) each containing 1.5 mg of testosterone. The surface area of time-release pellets was approximately 95 mm^3. Control animals received empty placebo pellets. Individuals of the beeswax group received one pellet made of beeswax/peanut oil. To prepare this pellet we mixed 2.72 g (80%) of beeswax (Spinnrad, Germany) and 0.68 g (20%) of hardened peanut oil (Fluka 93967, Sigma–Aldrich, Germany) in a glass beaker in a water bath at 65 °C. Once melted the liquid was added to a solution of 40 mg of testosterone in 50 μl of ethanol, and vortexed vigorously for 10 s. To ensure a homogeneous distribution of testosterone within beeswax/peanut oil, the mixture was heated in the water bath at 65 °C for a few seconds, and then vortexed again. This step was repeated 5 times. Finally, we filled a 1 ml syringe with the beeswax/peanut oil mixture, and waited until it became solid. Then, we cut off the narrow tip of the syringe with a scalpel and expelled the content. As result we obtained a solid cylinder of beeswax and hardened peanut oil with testosterone (termed "beeswax implant" throughout the paper). The beeswax cylinders were prepared in such a way that 1 mm contained 1 mg of testosterone (calculated according to the mass of the cylinder and the amount of testosterone included in the mixture). The surface area of the beeswax implant was approximately 113 mm^3. As a last step, we accurately cut the wax cylinder in small equal pieces with a scalpel to obtain several cylinders of 3 mm length (each of which contained 3 mg of testosterone used as implants). The control implants were prepared in a similar way, but not including testosterone. Individuals were implanted with one beeswax cylinder after soaking the cylinders overnight in saline solution.

During a period of 30 days we took a total of 11 blood samples per individual, on the day (0) before implantation, and on days 1, 4, 6, 8, 10, 12, 14, 18, 23, and 30 after the implantation. Blood samples were taken from the brachial wing vein and collected into heparinized capillary tubes. The blood was stored on ice until centrifugation at 6000 rpm for 10 min. Then, the plasma was drawn off and stored at −80 °C until hormone analysis. Immediately after blood sampling all individuals were weighed. All experimental procedures were conducted according to German and European laws and authorized by the responsible governmental authority, the *Regierung von Oberbayern*.

2.2. Hormone analysis

Testosterone concentrations were determined by radioimmunoassay following the methods of (Goymann et al., 2006). Samples were assayed in duplicates, in 8 assays (3 for silastic implants, 3 for pellets, and 2 for beeswax/peanut oil implants). The extraction recovery of testosterone was 90.0 ± 4.6% (mean ± sd, $N = 594$. The lower detection limits of the testosterone assay ranged from 0.32 to 0.42 pg/ml, and all samples were above the detection limit. The intra-assay coefficients of variation of a chicken plasma pool ranged from 0.6% to 10.2% in the 8 assays (mean ± SD: 4.8 ± 3.4%). The inter-assay coefficient of variation as determined by the variation of the chicken plasma pool between all assays was 10.9%. Hormone concentrations were calculated with Immunofit 3.0 (Beckmann Inc., Fullerton, CA, USA).

2.3. Statistical analysis

R version 3.0.3 (R Development Core Team 2014) was used for all statistical analyses, and the 'lme4' package (Bates et al., 2014) for the mixed-effect modeling. For the inferences of the models we used a Bayesian approach and obtained parameter estimates and their 95% credible intervals (CrI) using an uninformed prior distribution, which is the Bayesian equivalent of null hypothesis testing. Currently, the Bayesian approach is the only method that allows drawing exact inferences and avoiding the difficulties of determining the degrees of freedom in mixed model analyses (Bolker et al., 2009). Unlike null-hypothesis testing Bayesian statistics does not provide *p*-values. Instead, meaningful differences between groups can be assessed by comparing the range of the 95% credible intervals of one group with those of other groups. The 95% credible interval provides an estimate for the true range of a slope or group mean with a probability of 0.95. If the respective credible interval does not overlap with the posterior mean estimate of the group to compare with, the groups can be assumed to differ from each other. Using the sim function from the 'arm' package and non-informative prior-distribution (Gelman and Hill, 2006), we created a sample of 10,000 simulated values for each model parameter. Model fit was investigated and optimized through posterior model checking (Gelman and Hill, 2006; Gelman and Shalizi, 2013). Meaningful and comparable estimates were obtained following (Schielzeth, 2010).

We performed two different mixed models. First, we asked whether basal testosterone levels before implantation (DAY −1) differed significantly from levels on the day after implantation (DAY +1). Control and treatment groups, day, and the kind of implant (silastic tubing, time-release pellet and beeswax) were considered as fixed factors. Individual identity was included as a random factor. In the second model, we asked whether testosterone levels change throughout the experiment, starting from DAY 1 until DAY 30. This second model included the treatment (control and testosterone), and the type of implant (silastic, pellet, beeswax) as fixed factors and the sampling days (DAY 1 to DAY 30) as a numerical factor. Individual identity was included as a random effect. Residuals were analyzed using graphical methods (Cleveland 1993) for homogeneity of variance, violation of normality assumptions and departures from the model assumptions or other anomalies in the data and in the model fit. To meet these criteria testosterone values were log10-transformed prior to analysis. Conditional and marginal R^2 values were obtained using the method described by (Nakagawa and Schielzeth, 2013).

3. Results

3.1. Testosterone levels before and one day after implantation

First, we looked at the change in testosterone between the day before implantation (DAY −1) and the day after implantation (DAY +1). In individuals treated with hormone implants, testosterone concentrations were higher on DAY +1 than on DAY −1 (Fig. 1). In controls, there was no such difference. In individuals treated with testosterone implants, the 95% credible intervals indicated that on day 1 beeswax treated individuals expressed lower concentrations of testosterone than individuals treated with pellets or silastic implants (Fig. 1). Also, individuals treated with pellets expressed lower levels of testosterone than individuals treated with silastic implants (Fig. 1). Body mass had no effect (Table 1). The marginal and conditional R^2_{glmm} of the model explained a large proportion of the variance in the data and were identical ($R^2 = 0.925$), because all of the variance in the data was explained by the fixed effects alone (Table 1).

3.2. Temporal profile of testosterone concentrations between treatments

The animals treated with empty silastic implants, empty time-release pellets or empty beeswax pellets showed low levels of testosterone throughout the 30 days period (means ± 95% CI of silastic tubing: 0.11 ± 0.04 ng/ml ($N = 98$); time-release pellet: 0.05 ± 0.004 ng/ml ($N = 97$); and beeswax pellet: 0.09 ± 0.02 ng/ml ($N = 98$)) indicating that the matrices themselves did not elevate testosterone in female quail. We restricted the statistical comparison to the different testosterone implants, because our focus was on the release dynamics of different kind of implants rather than their general effectiveness in comparison to their respective controls.

The results show that the temporal dynamics of testosterone release from silastic tubing, time-release pellets, and beeswax implants differ substantially (Fig. 3). The linear mixed effect model indicated that testosterone concentrations were higher in quails implanted with silastic tubing ($F = 56.553$; back-transformed intercept [95% credible interval]: 19.8 ng/ml [12.8–30.3 ng/ml]) than in

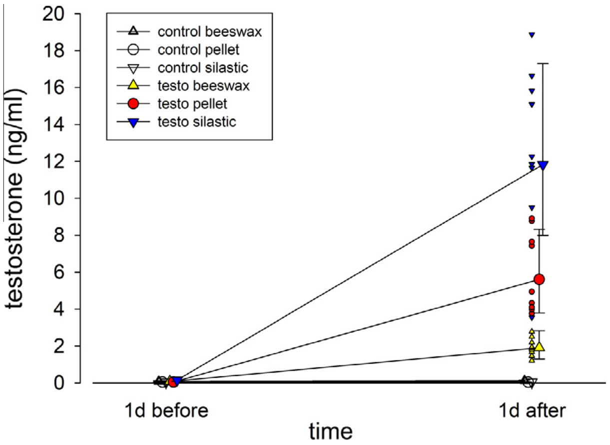

Fig. 1. Testosterone levels one day before and one day after implantation. Back-transformed estimates ± credible intervals obtained with the three different implants tested. Open symbols represent individuals treated with empty implants, colored symbols represent individuals treated with testosterone implants. Each different color represents different implants. For day 1 after implantation, individual data points for the three groups of testosterone-treated birds are shown (small symbols). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the pellet group (back-transformed intercept: 6.5 ng/ml [4.1–10.1 ng/ml]) and testosterone concentrations of both of these groups were higher than the beeswax group (back-transformed intercept: 0.63 ng/ml [0.42–0.96 ng/ml]; Table 2; Fig. 2). Testosterone concentrations decreased over the 30 days treatment period (Table 2, Fig. 2), but the slope of the silastic implant differ from the others. The model explained a large proportion of the variance with a marginal $R^2_{\text{glmm}} = 0.815$ and a conditional $R^2_{\text{glmm}} = 0.907$.

4. Discussion

In this study, we used female Japanese quail to compare the effectiveness of three kinds of matrices – silastic tubing, time-release pellets, and beeswax implants – in elevating circulating testosterone concentrations over a period of 30 days. All three kinds of implants contained the same amount of testosterone (3 mg) and effectively increased testosterone over a period of 30 days. However, the temporal level dynamics differed to a large degree. The physiological levels of plasma testosterone described for male quails under reproductive conditions are in the range of 3–5 ng/ml (Biswas et al., 2007; Cornil et al., 2009; Henare et al., 2011). Testosterone in silastic tubing led to the steepest increase in testosterone, with a supra-physiological peak on day 1 and high levels maintained for a period of about 2 weeks. Thereafter, plasma levels of testosterone started to decline. Testosterone in time-release pellets also led to a slightly supra-physiological peak in testosterone after 1 day. However, pellet implants peaked at lower levels compared to testosterone in silastic tubing, and resulted in a more or less constant decline during the rest of the implantation period. Testosterone in beeswax implants peaked within a physiological range on day 1 and then, elevated levels of testosterone were maintained during the 30 days period of implantation also with a more or less constant decline. In contrast, all individuals with control implants maintained circulating testosterone concentrations with some variability at non-breeding baseline throughout the 30 days period. Preliminary experiments with biodegradable polymers that can be injected under the skin were unfortunately not very promising and hence we discontinued using them (see Supporting Information).

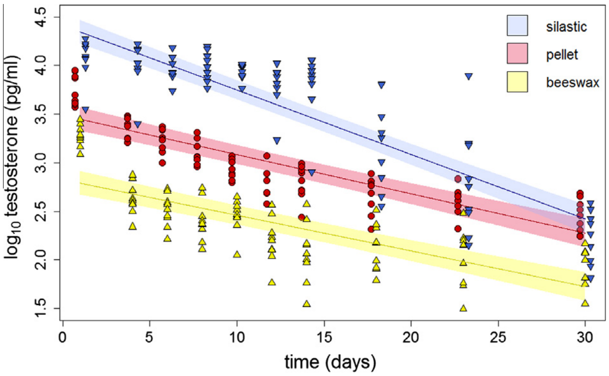

Fig. 2. Temporal profiles of testosterone concentrations after implantation. Decrease in testosterones levels over time (day 1 to day 30). Only testosterone-treated individuals are shown. Testosterone data were log10-transformed, solid lines represent the linear model fit, with the shading representing 95% credible intervals. Each different color represents different implants. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Testosterone concentrations one day before and one day after implantation. Linear mixed model of posterior means of log10-transformed testosterone concentrations before and after implantation depending on the kind of implant (beeswax, time-release pellet, silastic tubing), hormone treatment (empty control, 3 mg testosterone) and body mass. Each posterior estimate is provided with its corresponding Bayesian credible interval. If credible intervals of one group do not overlap with the posterior estimate of another respective group the groups can be assumed to differ from each other (see Fig. 1 for back-transformed mean testosterone concentrations and the respective credible intervals). 'Significant' differences are shown in bold.

Fixed effects	Time	Posterior mean	2.5%	97.5%
Beeswax control	Day before	1.914	1.745	2.079
	Day after	2.064	1.893	2.232
Pellet control	Day before	1.678	1.501	1.850
	Day after	1.622	1.437	1.802
Silastic control	Day before	1.746	1.539	1.945
	Day after	2.018	1.896	2.134
Beeswax testosterone	Day before	1.881	1.710	2.047
	Day after	**3.280**	**3.112**	**3.451**
Pellet testosterone	Day before	1.678	1.501	1.850
	Day after	**3.749**	**3.579**	**3.920**
Silastic testosterone	Day before	2.190	1.908	2.480
	Day after	**4.073**	**3.903**	**4.238**
Body mass		−0.0003	−0.003	0.002
Random effects		Variance		
Individual (intercept)		*0.000*		
Residual		*0.055*		

Table 2

Temporal profiles of testosterone concentrations between treatments. Linear mixed model posterior means of log10-transformed testosterone concentrations in relation to kind of implant (beeswax, time-release pellet, and silastic tubing), the slope of each curve, and body mass as a covariate. Each posterior estimate is provided with its corresponding Bayesian credible interval. The fact that the credible intervals of time-release pellets and silastic tubing did not include the posterior mean estimate of beeswax implants indicates that testosterone concentrations in plasma of quail implanted with time-release pellets and silastic tubing were higher than those of beeswax implanted quail. Also, the slopes differed with the steepest decline in testosterone concentrations in the silastic tubing group. Body mass had a slight positive effect on testosterone concentrations. The variance estimates of the random effects are included. 'Significant' differences between groups are shown in bold.

Fixed effects	Posterior mean	2.5% CrI	97.5% CrI
Beeswax (intercept on day 1)	**2.800**	**2.620**	**2.981**
Time release pellet (intercept on day 1)	**3.811**	**3.615**	**4.012**
Silastic (intercept on day 1)	**4.297**	**4.110**	**4.482**
Slope (beeswax)	−0.038	−0.044	−0.032
Slope (pellet)	−0.045	−0.051	−0.039
Slope (silastic)	**−0.063**	**−0.069**	**−0.057**
Body mass	0.011	0.007	0.032
Random effects	Variance		
Individual (intercept)	*0.0530*		
Residual	*0.0537*		

Testosterone concentrations in individuals treated with silastic tubing were 2–3 times higher than mean maximum levels of testosterone described for male quail during reproduction (Biswas et al., 2007; Cornil et al., 2009; Henare et al., 2011). If implanted in a reproductively active male quail, testosterone concentrations most likely would have been even higher than the actual levels reached in non-reproductive females. Further, we used only 10 mm implants in quail, but many studies use even longer implants in much smaller-sized birds. Because the release rate of silastic implants may be largely determined by their surface area, we expect that such large implants could release even more testosterone. Thus, depending on the regular testosterone concentration and body size of the respective species studied, larger or smaller sized implants may be appropriate. Also, because we measured plasma testosterone only about 24 h after implantation, the initial increase in testosterone during the first couple of hours after implantation may have been even higher than the concentrations measured after 24 h. Finally, testosterone concentrations of quail implanted with silastic tubing were much more variable between individuals than those of quail implanted with pellets or beeswax

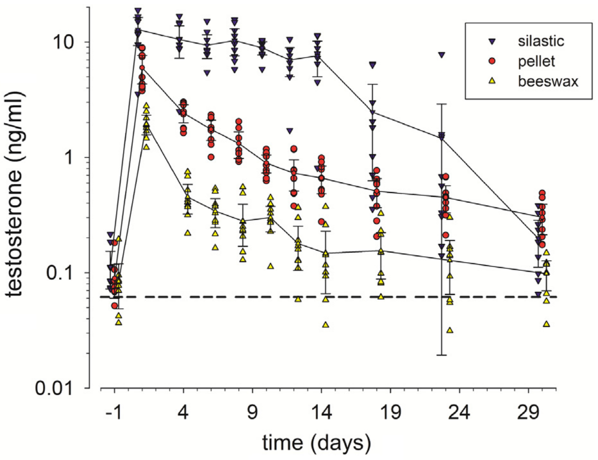

Fig. 3. Temporal profile of testosterone. Means (smaller symbols) ± 95% confidence intervals of testosterone-treated female quail. Each larger symbol represents the levels of one individual per day. Please note the logarithmic scale of the *y*-axis. Data are shown from day-1 (before implantation) until day 30 (last day of sampling). Each different color represents different implants. The dashed line shows the mean pre-implant level of plasma testosterone of all animals as a reference for comparison. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

after day 1 (Fig. 1) and throughout the 30 days measurement period (Figs. 2 and 3). Overall, the testosterone release pattern over 30 days was least variable between individuals in quail implanted with time-release pellets (Fig. 3).

All three kinds of implants contained the same amount of testosterone. The testosterone concentrations measured in the plasma of individual birds suggest that silastic tubing had the fastest, time-release pellets an intermediate, and beeswax the slowest release of testosterone. The results of our study confirm a previous comparison that reported the release rate for silastic tubing and time-release pellets on a weekly basis. Also in this study on female canaries (*Serinus canaria*), silastic tubing implants produced very high testosterone levels with low consistency over time, whereas time-release pellets produced lower levels with a higher consistency (Fusani, 2008). Edler et al. (2011) found that 90-day time-release pellets produced supra-physiological levels of testosterone in southern red bishops (*Euplectes orix*) at least within the first 2–4 weeks after implantation.

The time-release pellets and beeswax implants showed a very similar constancy of testosterone release over time. Given that beeswax and hardened peanut oil are very malleable materials, the release rate of beeswax cylinders can probably easily be modified by changing the surface of the implant, e.g. increasing the surface area should lead to a higher release. Before applying any implant method to a new species it is certainly advisable to test-implant a couple of individuals to check the concentration of circulating hormones and maybe modify the testosterone content of the implant according to the need of the respective study. The beeswax implant offers the advantage to be very plastic and easily adjustable in shape. Also, the beeswax method seems to reliably elevate testosterone only for a period of less than two weeks. After this period of time, testosterone concentrations of some individuals had returned to baseline levels (see Fig. 3).

5. Conclusions

Time-release pellets and in particular beeswax implants performed better than silastic tubing in avoiding supra-physiological concentrations of testosterone. Both pellets and beeswax also offer a more constant release of testosterone into the circulation. Finally, both time-release pellets and beeswax implants degrade within the body of birds, so that it is not necessary to recapture the animals to remove the implants. We consider implants made out of beeswax a low-cost and easy-to-fabricate alternative to elevate testosterone concentrations in captive or free-living birds. Beeswax pellets seem to be especially suited for moderate elevations of testosterone or potential other hormones. Because the release rates of the various implants (silastic, pellets, and beeswax) are hardly predictable it is advisable to do validate the appropriate release before applying either method in the context of a specific study question.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2015.01.007.

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General discussion

In the present thesis I investigated the regulatory mechanisms underlying the annual cycle and song behavior of the equatorial silver-beaked tanager, an endemic Amazon songbird with an entirely tropical phylogenetic background. I show that males have marked seasonal schedules of molt and dawn-song behavior. In addition, males exhibited important seasonal changes in testis size and gonadal testosterone production, which denote a marked seasonal breeding pattern (**Chapters 1 & 2**). Particularly interesting is the neuroendocrine regulation of seasonal dawn-song, a behavior directly involved in the breeding territoriality of males, whose seasonal activation appears as decoupled from the seasonal peak of testosterone levels in plasma (**Chapter 2**). Further, although at equatorial latitudes environmental photic cues have a very small magnitude of variation over time, they do seem to influence the occurrence of dawn-song in male silver-beaked tanagers on a daily basis, as well as at a seasonal level. Besides this, the results of this thesis confirm the significance of rainfall for the timing of song phenology and seasonal phenotypes of equatorial songbirds. On the other hand, the habitat of the equatorial silver-beaked tanager imposes singular environmental conditions, including unconstrained food availability over the year. Accordingly, males exhibited uniform annual levels of baseline corticosterone (**Chapter 1**). However, individual baseline corticosterone and testosterone levels are clearly associated with the molting status of males. Finally, in **Chapter 4** I complemented my research by proposing a new method of steroid hormone implantation to be used for the study of wild birds. Overall, the present thesis demonstrates a concerted orchestration of seasonal events in equatorial males. The phenology of silver-beaked tanagers is mediated by conserved neuroendocrine regulatory networks that function with a different temporal and relational pattern when compared with widely studied temperate-zone bird species.

Environmental cues and phenology at the equatorial Amazon

The phenology of animals represents the temporal dimension of their life histories, in close relationship with the seasonal environment. It is hypothesized that the phenology of birds, particularly the timing of breeding, is ultimately dependent on the temporal availability of food sources on which young offspring are dependent (Illera and Díaz, 2006). Thereby, the increase in day length is used as a proximate predictive cue to anticipate the increment of food availability in the habitat, which typically coincides with the arrival of the spring in temperate regions (Dawson, 2008; Dawson et al., 2001; Gwinner, 1989; Wingfield, 2012; Wingfield et al., 1992). In order to identify seasonal changes in nutrients flux at the equatorial habitat of silver-beaked tanagers, in **Chapter 1** I tracked isotope signatures in blood cells and feathers of males over the course of a year. There were no changes of $\delta^{13}C$ and $\delta^{15}N$ isotope ratios in blood and feathers, which indicate that silver-beaked tanagers do not experience major dietary fluctuations across seasons. Additionally, these results do not support seasonal shifts in the trophic level of males. Overall, these results suggest that silver-beaked tanagers inhabiting the eastern Amazon do not experience seasonal restrictions on food availability, and maintain a uniform diet over the year. Nevertheless, I found significant changes in the $\delta^{34}S$ ratios of both tissues analyzed, which very likely indicates a seasonal variation in the mixing water composition of the Amazon estuary (see discussion of **Chapter 1**). This finding suggests an interesting possibility about the potential use of the seasonal composition of estuary's water to time seasonal processes of birds.

Since annual photoperiod cycles are very small at equatorial latitudes, it has been proposed that annual fluctuations in alternative photic cues, such as light intensity or solar time (sunrise and twilight timing) can be used as environmental cues to time life history processes of birds (Goymann et al., 2012; Gwinner and Scheuerlein, 1998). In fact, at equatorial latitudes those alternative photic signals present a larger magnitude of variation than day length, and fluctuate in a predictable way over the year. However, there was no effect of civil twilight time on the seasonal occurrence of dawn-song of males (**Chapter 1**). Surprisingly, the subtle annual change of day length (of 9 minutes) correlated with the seasonal increase in gonadal production of testosterone and the activation of dawn-song in males (**Chapter 2**). This finding opens a question concerning the degree of sensitivity that equatorial species have to small variations in annual photoperiod. So far, the smallest change of day length that has been experimentally demonstrated to be detected by a tropical bird is of 17 minutes (Hau et al., 1998). If an annual photoperiodic cycle of 9 minutes amplitude can be perceived by silver-beaked tanagers, a species from a purely tropical lineage, it would provide substantial evidence about the prevalence of photoperiodicity among songbirds. To distinguish such small annual changes in day length, equatorial silver-beaked tanagers would need a precise biological time-measuring system. One possible explanation is based on the Bünning hypothesis (Bünning 1936), which assumed that circadian oscillations consist of light (photophil) and dark (scotophil)-requiring phases. This hypothesis postulates that the photoperiodic effect on a given light-dark cycle is not dependent on the absolute length of the photoperiod, but rather on which portion of an underlying circadian rhythm is illuminated. Hence, a major feature of this hypothesis is the assumption of the existence of a circadian rhythm of sensitivity to the inductive effects of light. For instance, as days become a little longer, the ambient light may impinge the scotophil phase of equatorial silver-beaked tanagers, which may encode the critical photoperiod that induces their seasonal responses Indeed, this hypothesis has been successfully tested in other songbird species (Follett et al., 1974; Menaker and Eskin, 1967).

In addition to photoperiod and food sources, precipitation patterns can also provide temporal cues to bird life histories (Dawson, 2008; Leitner et al., 2003; Moore et al., 2005; Oppel et al., 2013). This seems to be particularly important for equatorial birds, since the variation in the intertropical convergence zone (where the northeast and southeast trade winds come together at equatorial regions) affects rainfall, resulting in predictable rainy and dry seasons. Equatorial silver-beaked tanagers exhibited a clear tendency to molt during the rainy season, and on the other hand, dawn-song behavior and the peak of testosterone levels in males occurred during the drier season (**Chapters 1 & 2**). This suggests that the annual patterns of rainfall exert an important influence on the seasonal traits of silver-beaked tanagers. Similarly, drastic changes in rainfall rate can elicit changes in the reproductive physiology of some opportunistic songbird species (Hau et al., 2004; Small et al., 2007). Particularly famous is the case of the zebra finch (*Taeniopygia guttata*) and their ability to breed in response to rain at any time of the year (Immelmann, 1971; Perfito et al., 2007).

The results obtained in **Chapter 3**, in which individual males were exposed to different experimental conditions of precipitation, support the assumption that increased rainfall rates constrain the production of gonadal testosterone. Males that were placed in a rainy aviary, after injection of exogenous GnRH presented smaller magnitudes of change in their testosterone levels compared to males that were in the dry aviary. This outcome provides important experimental evidence concerning the effect of rain on the reproductive cycles of equatorial birds. In general, the absolute timing of wet and dry seasons is not as predictable as is photoperiod because other factors such as wind direction or ocean currents affect them. Therefore it is likely that the influence of seasonal rainfall on the phenology of equatorial birds is complemented with additional environmental cues.

In summary, the phenology of silver-beaked tanagers in the eastern lowland Amazon seems to be governed by the annual pattern of rainfall in combination with the small variations in day length (**Chapter 1**). There is an inhibitory effect of the rainy season on the reproductive functions of males, including seasonal dawn-song and gonadal activity (**Chapters 1, 2 & 3**). It has been theorized that during rainy periods there is higher risk of nest destruction, lower survival of eggs, and increased mortality of chicks, which might ultimately influence the breeding phenology of tropical birds (Monadjem and Bamford, 2009; Oppel et al., 2013; Radford and Du Plessis, 2003; Siikamäki, 1996). In addition, it is reported that seasonal changes in light intensity caused by the cloud cover during the rainy season could serve as an proximate cue for equatorial birds (Gwinner and Scheuerlein, 1998). Besides that, the seasonal increase of δ^{34}S isotopes ratios found in the tissues of silver-beaked tanagers coincides with the period of higher fresh water discharge into the Amazon estuary. This result shows an indirect effect of rainfall on the isotope signatures of males, through changing the mixed-water composition of the Amazon estuary. On the other hand, there is a very interesting association between the seasonal timing of dawn-song, gonadal activity, and the small annual increase in day length (**Chapter 2**). Taken all together, it is very likely that equatorial silver-beaked tanagers, in interaction with their endogenous circannual rhythm, integrate several environmental cues such as rainfall, estuarine variations, light intensity, and the small annual changes in day length as complementary components in their annual scheduling process.

Annual cycle and steroid hormones production

Male silver-beaked tanagers showed marked seasonality of gonadal testosterone production. Further, the seasonal elevation of circulating testosterone was positively correlated with a seasonal increase in testis size (**Chapter 2**), a pattern normally observed in temperate zone birds as well (Garamszegi et al., 2005). Thus, these results indicate that equatorial males undergo pronounced cycles in their reproductive physiology. Previous comparative studies have described male tropical birds as having lower concentrations of plasma testosterone involving low amplitude cycles (Garamszegi et al., 2005; Goymann et al., 2004; Hau et al., 2008a). However, this generalization could represent an overestimation, because data on testosterone levels of tropical birds are scarcer and sporadic. Further, it is theorized that among tropical birds testosterone levels are related to the degree of habitat seasonality and the length of the breeding stage; larger habitat seasonality and shorter breeding periods involves higher testosterone levels of a given species (Goymann et al., 2004; Hau et al., 2008a). However, that seems not to be the case with silver-beaked tanagers, because males showed peak concentrations of testosterone well within the range of higher latitudes birds (**Chapter1**) despite their relatively stable habitat and extended breeding period (**Chapters 1 & 2**). Therefore, this result points out that, the assumption of tropical birds having lower levels of testosterone is not a universal rule, but depends on particular characteristics of each species. In relation, it is interesting that male silver-beaked tanagers maintained low levels of testosterone during the first part of their breeding stage and throughout most of the period of territorial settlement (**Chapter 1 & 2**). These low concentrations may represent a way of avoiding potential detrimental effects of maintaining elevated concentrations of testosterone throughout the extended breeding season of silver-beaked tanagers (Wingfield et al., 2001) (**Chapter 2**). Male silver-beaked tanagers presented peak concentrations of testosterone only in the last phase of the breeding stage, when high levels might be critical for gamete production and the execution of consummatory sexual behaviors, such as copulation (**Chapter 2**). Further, gonadal testosterone production was down-regulated in molting males, which occurred mainly during the rainy season. This result suggests the existence of a physiological constraint between reproduction and molting, as both stages require large mobilization of energy and nutrients (Romero et al., 2005).

Baseline corticosterone levels in male silver-beaked tanagers (the primary glucocorticoid of birds) did not vary significantly over the year. This is an interesting result since most avian species studied in the wild modulate baseline corticosterone levels on a seasonal basis (Landys et al., 2006; Romero, 2002). In general, changes of baseline glucocorticoid concentrations in vertebrates are associated with the maintenance of the internal homeostatic level. Accordingly, it is hypothesized that seasonal adverse effects of the environment (i.e due to climatic or food constraints), and/or changes in the individual energetic demands over the year determine seasonal changes in baseline levels of corticosterone in birds (Landys et al., 2006; Romero, 2002). Thus, the uniform baseline levels showed by silver-beaked tanagers over seasons suggest that the environmental conditions of the eastern Amazon do not impose major seasonal challenges for birds, probably due to a benign climate and the high productivity of the Amazon estuary. Nevertheless, corticoid levels were significantly affected by the molting state of males, which indicate that males may adjust their levels to particular life history stages. In addition, males showed a diel rhythm of adrenocortical activity with daily elevations of corticosterone productions during the mornings. This pattern is typically observed in temperate zone species, and suggests that as the day progresses corticosterone influences the physiological and behavioral responses of equatorial silver-beaked tanagers. For instance, increased circulating levels of corticosterone in the morning may affect activity cycles and daily onset of foraging in male silver-beaked tanagers (Breuner et al., 1999; Landys et al., 2006).

Neuroendocrine regulation and timing of dawn-song

There is a large diversity of passerine birds that perform a relatively short burst of intense singing activity in the early morning, called the dawn-song (Staicer et al., 1996). Despite its ubiquity, the mechanisms and timing of dawn-song remains poorly understood for tropical and equatorial bird species. Equatorial male silver-beaked tanagers exhibited a precise timing of dawn-song behavior, and the seasonal pattern of dawn-song was directly related to the establishment of the breeding territories of males (**Chapter 1**). This association between breeding territoriality and dawn-song behavior is a common characteristic observed among passerines (Amrhein and Lerch, 2010; Foote et al., 2008; Kunc et al., 2005; Voigt et al., 2006). Hence, the dawn-song behavior of male silver-beaked tanagers showed temporal and functional features that are similar to the ones widely described for higher latitudes species. However, there are aspects of their regulatory mechanisms that involve a differential neuroendocrine and environmental control of dawn-song.

Neuroendocrine mechanisms

In general, seasonal differences in male song of temperate-zone songbirds are positively correlated with seasonal variations in gonadal size and sex steroid production (Ball et al., 2004; Gahr, 2014; Wingfield and Farner, 1993). However, in silver-beaked tanagers the seasonal onset of dawn-song occurred with low levels of circulating testosterone. In fact, males showed a seasonal peak of circulating testosterone that correlated with an increase in testis size, but it occurred two month later than the seasonal activation of dawn-song (**Chapter 2**). Therefore, these results suggest that dawn-song behavior, and the associated territoriality, was initiated long before the reproductive system was fully developed. Thereby, seasonal activation of dawn-song in males appeared to be decoupled from high gonadal testosterone levels. This disassociation may be a mechanism for circumventing possible negative effects of maintaining elevated levels of testosterone (Wingfield et al., 2001), given that silver-beaked tanagers perform their territorial dawn-song for an extended period of 6-7 months. It is of note, however, that testosterone may exert a booster effect on the behavior, since the increase in testosterone levels correlated with the increase in dawn-song rate of males, which occurs later in the breeding period (**Chapter 2**).

At a neural level in the song control system, the HVC of male silver-beaked tanagers presented seasonal differences in the expression of androgen receptors, which was defined through the analysis of their mRNA expression. The Nissl stained sections indicated no seasonal changes in morphology and cell density in the HVC of males. During the seasonal dawn-song onset, males presented an extended area of androgen receptor expression in HVC (**Chapter 2**). The extended HVC area defined by its androgen receptor expression was maintained during most of the dawn-song period, but decreased towards the last phase of the breeding season (**Chapter 2**). Visual comparison of adjacent Nissl stained and androgen receptor labelled HVC sections denoted that the androgen receptor HVC area extended beyond the medial and caudo-ventral border of the Nissl-defined HVC. Therefore, based on previous studies it is hypothesized that the seasonal increase in the androgen receptor area of HVC in male silver-beaked tanagers involves changes in a number of androgen target cells among RA-projecting neurons (Bottjer and Johnson, 1997; Johnson and Bottjer, 1993; Sohrabji et al., 1989). These results indicate that seasonal activation of dawn-song in male silver-beaked tanagers coincided with an increased expression of androgen in HVC, which occurs with low levels of gonadal testosterone. This larger absolute number of receptors means increased chances for the hormone (testosterone and its androgenic metabolites) to bind receptors on HVC. This condition can potentially function as a mechanism to allow low levels of testosterone to activate seasonal dawn-song.

Environmental control

The study site is characterized by the constant occurrence of rain over the year. However, there is a dramatic change in precipitation rate over seasons, which occurs in a rather predictable manner. Although there is a minor degree of variability in the timing, every year the region undergoes two main seasons: the rainy and the dry seasons (Liebmann and Marengo, 2001; Moraes et al., 2005). The seasonal onset of dawn-song occurred with the arrival of the dry season, and then males continued performing dawn-song throughout the whole dry season until the beginning of the next rainy period (**Chapters 1 & 2**). Other studies in equatorial rufous-collared sparrows have previously shown that the annual rainfall can exert important influences on the annual timing of song behavior (Moore et al., 2005, 2004). Thus, the results of this thesis confirm the significance of rainfall for the song phenology of equatorial songbirds, and suggest an incompatibility between heavy rainfall and the seasonal dawn-song of silver-beaked tanagers. On the other hand, the seasonal onset of dawn-song behavior coincided with the small increase in photoperiod that males experience in the region. Once dawn-song was seasonally activated, males continued singing until annual day length started to decrease. Therefore, seasonal dynamics of dawn-song behavior in equatorial silver-beaked tanagers seems to be influenced by the very small annual changes in day length. There is important evidence indicating that the photoperiod can directly influence seasonal song in temperate-zone songbirds (Bentley and Ball, 2000; Robertson et al., 2014; Smith et al., 1997). In relation, it is demonstrated that the song control system of oscine songbirds expresses melatonin receptors in some nuclei, including HVC (Bentley, 2003; Bentley and Ball, 2000; Gahr and Kosar, 1996). These observations point to a role in song behavior for melatonin associated with day length variations. However, a photoperiodic control of seasonal behaviors at an equatorial scale has never been proved in any vertebrate. Taken together, the results suggest that seasonal dawn-song of male silver-beaked tanagers is controlled by an integrated perception of the rainfall and photoperiodic cycles of the equatorial eastern Amazon (**Chapter 1 & 2**).

Within the period when males display their dawn-song behavior, there was a precise timing in the daily onset of dawn-song. Male silver-beaked tanagers accurately mirrored the civil twilight time to initiate their daily dawn-song activity (**Chapter 1**). A close relationship between daily dawn-song activity and ambient light has been reported for many passerine species at higher latitudes (Bruni et al., 2014; Silva et al., 2014; Thomas et al., 2002; York et al., 2014). However, daily timing of dawn-song behavior has never been described for songbirds in equatorial habitats, where the amplitude cycles of the annual variation in solar time are much smaller. In **Chapter 2**, I show that equatorial silver-beaked tanagers are able to perceive small daily changes in civil twilight time to initiate their daily dawn-song. I am not aware of a previous study that integrates seasonal and daily timing of dawn-song in equatorial songbirds, under subtle fluctuations of photoperiod and solar time. In **Chapter 1 & 2** it is shown that equatorial silver-beaked tanagers align seasonal dawn-song with rainfall cycles, subtle photoperiodic changes, and, on a daily basis, according to small fluctuations in civil twilight time.

Steroid hormone implants: looking for physiological ranges

Hormone manipulations to study how hormones modulate behavior, developmental stages and life history stages in free-living animals have become quite a common research practice (Fusani et al., 2005; Ketterson et al., 1996, 1991). Given that in the field, it is often difficult to capture the same individual more than once, the use of implanting devices that can deliver the hormone continuously is an effective approach. Despite this situation, in the literature there are very few methodical descriptions about releasing properties of different steroid hormone implants (for discussion see Fusani, 2008). For the purposes of this thesis, I experimentally manipulated testosterone levels in male silver-beaked tanagers by using silastic tubing implants, and the consequences were evidently noxious. This situation encouraged the execution of the study presented in **Chapter 4**.

The aim of the last chapter of my thesis (**Chapter 4**) was to examine the temporal dynamics of the release of testosterone from silastic tubing and from time-release pellets over a period of 30 days. In addition, I searched for alternative biodegradable matrices that would allow for a more constant release of testosterone throughout the same period, and within a physiological feasible range. To this end, implants built out of beeswax material were developed, and a biodegradable injectable polymer tested. I used female Japanese quail (*Coturnix japonica*) as a model. Time-release pellets and in particular beeswax implants performed better in avoiding supra-physiological concentrations of circulating testosterone. In addition, both pellets and beeswax also offered a more constant release of testosterone into the circulation, and degraded within the body of birds. The beeswax implant has the advantage of being very flexible and easily adjustable in shape. Also, the beeswax method seems to reliably elevate testosterone for shorter periods of less than two weeks. Thus, beeswax implants are a low-cost and easy-to-fabricate alternative to elevate testosterone (or other steroid hormones) concentrations in captive or free-living birds. Due to the time limits of my PhD I was not able to use this method with silver-beaked tanagers. However, the results presented in **Chapter 4** have paved the way for the realization of future studies.

Conclusion and future directions

In the present thesis I examined the mechanisms underlying seasonality of an equatorial songbird, with a main focus on steroid hormones and song behavior. This work required an extensive (and exciting) period of field research exploration, in a non-traditional study model, from which were obtained quite singular outcomes that integrate environmental and internal regulatory aspects. For instance, it was shown that dawn-song behavior in males in an equatorial habitat takes place seasonally, as a result of the interaction between recurrent environmental signals (rain and photic cues), social context (territory establishment) and neuroendocrine processes. On the other hand, the thesis generated several new questions, which could not be answered within the timeframe of my PhD work. Hence, I hope to have contributed inspiring findings that can be further investigated. Some logical further steps to be addressed are: (1) the different kinds of cues that are perceived and integrated in order to time seasonal phenotypes in this equatorial habitat, (2) the mechanistic relationship between territoriality and dawn-song behavior, (3) the aspects/components determining seasonal changes in steroid hormone sensitivity in the HVC of males, (4) the genetic networks underlying mechanisms of song when gonadal testosterone levels are low, and (5) the potential similarities in neuroendocrine mechanisms among closely related equatorial songbirds, and the differences with temperate-zone species. All these topics are fascinating and might provide valuable insights in the integration of different regulatory aspects of seasonal phenotypes.

Overall, free-ranging birds experience a rich suite of complex interactions with their habitat, which involves equally complex neuroendocrine responses. Despite the difficulties presented by field studies, I believe that they are indispensable for a better understanding of the interactions between brain, hormones, behavior and the environment. Besides that, in the face of an accelerated global climate change there is a critical need to understand regulatory mechanisms of biological cycles. Indeed, outcomes obtained from “neuroendocrine field-research” can provide many answers ranging from a reductionist to a systemic level, which are useful for revealing the importance of these mechanisms in evolution.

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Rene Quispe

Seewiesen, 17th April 2016

Curriculum vitae

Rene Quispe Valdes

Education

- 2010 to date: PhD student, International Max Planck Research School for Organismal Biology (IMPRS), Max Planck Institute for Ornithology, Seewiesen, Germany

  Thesis: Environmental and neuroendocrine control of seasonal phenotypes and song behavior of an equatorial songbird

- 2006-2007: Degree of Veterinary Physician, Universidad de Chile; graduation with honors

  Thesis: Geographic variation of foraging behaviors associated with food hoarding in *Octodon degus*

- 1997 – 2006: BSc in Veterinarian Medicine, Universidad de Chile

- 1993 – 1996: High School, The Angel's School, Santiago, Chile

Publications

Peer-reviewed

- **Quispe, R**.; Sebe, F.; da Silva, M.L.; Gahr, M.: Dawn-song onset coincides with increased HVC androgen receptor expression but is decoupled from high circulating testosterone in an equatorial songbird. Physiology and Behavior 156, 1-7 (2016)

- **Quispe, R.;** Trappschuh, M.; Gahr, M.; Goymann, W.: Towards more physiological manipulations of hormones in field studies: Comparing the release dynamics of three kinds of testosterone implants, silastic tubing, time-release pellets and beeswax. General and Comparative Endocrinology 212, 100-105 (2015).

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- Ebensperger, L. A.; Sobrero, R.; Quirici, V.; Castro, R. A.; Ortiz Tolhuysen, L.; Vargas, F.; Burger, J. R.; **Quispe, R**.; Villavicencio, C. P.; Vasquez, R. A. et al.: Ecological drivers of group living in two populations of the communally rearing rodent, *Octodon degus*. Behavioral Ecology and Sociobiology 66 (2), pp. 261-274 (2012)
- Villavicencio, C. P.; Márquez , I. N.; **Quispe, R.;** Vásquez , R. A.: Familiarity and kinship influence kin discrimination in *Octodon degus* . Animal Behaviour 78, 377-384 (2009)
- **Quispe, R.;** Villavicencio, C. P.; Cortes , A.; Vásquez , R. A.: Inter-population variation in hoarding behaviour in degus, *Octodon degus*. Ethology 115, 465-474 (2009)

Talks

- UFA-DFH (French-German University) meeting Paris, France (2015)
- 9th topical meeting of the Ethologische Gesellschaft, Tutzing, Germany (2014)
- 17th International Congress of Comparative Endocrinology, Barcelona, Spain (2013)
- UFA-DFH (French-German University) meeting with the IFE (Francilien Institute for Ethology) workshop, Paris, France (2013)
- IMPRS symposium: Grand challenges in behavioral ecology and neurobiology (2011)
- LXVII Annual meeting of Biology Society of Chile, Pucon, Chile (2005)

Posters

- 9th topical meeting of the Ethologische Gesellschaft, Hamburg, Germany (2015)
- 11th International Congress of Neuroethology, Sapporo, Japan (2014)
- SICB Annual Meeting - Society for Integrative and Comparative Biology , Austin, Texas, USA 2014
- 10th International Mammalogical Congress, Mendoza-Argentina (2009)

- 12th International Behavioral Ecology Congress, Cornell University, Ithaca, New York, USA (2008)

Research activity:

- 2004-2005: Research assistant, project FONDECYT 1020550: The ecologic-evolutive context of learning: foraging and information use. Faculty of Science, University of Chile.

- 2006-2007: Research assistant, project FONDECYT 1060186: Geographic variation in behavioral profiles. Faculty of Science, University of Chile.

Awards and funding

- 2014: 11th International Congress of Neuroethology, Sapporo, Japan “Best poster presentation”

- 2009: Scholarship Becas-Chile, Conicyt, Chile