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Minireview

Endocrinology in field studies: Problems and solutions for the experimental design

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ABSTRACT

The increasing interest in hormones among field biologists can be frustrating because of the difficulties of applying classical endocrinological methods to natural settings. A few thoroughly tested methods have become popular because of their simplicity of use. This does not mean that such methods are the best or the appropriate ones for all studies. In this brief review I will examine some common problems encountered by field biologists who want to study the relationships between a morphological, behavioral, or physiological trait and a hormone. First, I will discuss why questions asked in the field often differ substantially from those asked in the laboratory, and how to adapt the design of the experiment accordingly. Second, I will review alternative methods to study hormone–trait relationships and how to combine them to strengthen the conclusions that can be drawn from the study. Then, I will discuss how to find the right control for a hormonal manipulation. Finally, I will examine the pitfalls associated with long-term hormonal treatment and the available methods for such types of studies.

1. Introduction

There is an increasing interest in hormones among ecologists, ethologists, and other researchers who work with free-living species. The birth date of field endocrinology can be traced back to 1802, when George Montagu observed that in male songbirds singing activity was higher in the periods during which birds had larger testes (Montagu, 1802, cited in Armstrong, 1963). By the 1950, there were several studies on hormones in free-living animals, and particularly in birds (Collias, 1950). However, the major impulse to the development of the field came from the studies of John C. Wingfield in the 1970s. Wingfield developed methods to measure hormone concentration in small blood samples taken from wild birds without the need for killing the animals (Wingfield and Farner, 1975) and used the technique to analyze the circulating concentration of androgen, estrogen, and corticosteroids in relation to season, territorial behavior, and life cycle stages. Beside his fundamental contributions to the theoretical aspects of wildlife endocrinology (Wingfield et al., 1990, 1997, 2001; Wingfield and Farner, 1993; Wingfield and Moore, 1987), Wingfield has been particularly successful in developing methods to address questions that had been previously investigated only in laboratory animals. The great success of field endocrinology in the last years has resulted in an increase in the number of researchers from other areas who study how hormones modulate behavior, developmental stages, life history stages, and immunological parameters. Very often these researchers look for simple methods to study the action of hormones, and realize that most of classical laboratory studies are based on methods that cannot be easily applied in the field. This led to the strategy of sticking to few published methods. However, this approach might result in adopting methodologies that are not the most appropriate ones for the specific aim of the study. In addition, studies involving hormones are often published in journals without a focus on endocrinology, which might favor the publication of studies based on uncomplicated tests of hormone action. In this brief article, I would like to address some common problems encountered by field biologists when designing experiments involving hormones. Because my work has focused mainly on androgen and estrogen, I will base my review on these hormones. However, most of the concepts developed here refer to hormones in general. A more general review on methodological issues in field endocrinology has been published recently (Fusani et al., 2005).

2. With or without gonads?

Classic studies of behavioral endocrinology typically involved the removal of the natural source of the hormone and the replacement with exogenous hormone. This approach served mainly two types of studies, those testing hormone-dependent traits, and those testing the effects of hormone agonists and antagonists. The oldest example of the first type of studies is the pioneering work of Berthold on caponization (Berthold, 1849). Farmers have known for centuries that if the testicles are removed from young male chicks, masculine traits will fail to develop, which in the case of fowls is called caponization. Berthold demonstrated that if the

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testicles were reimplanted in castrated chicks they will develop normal masculine traits (Berthold, 1849). Later it was discovered that the testicles are the main source of androgen in males, thus the traits that were lost as a result of castration but restored by androgen treatment were called androgen-dependent. This experimental protocol has been used successfully in a large number of key studies, including the classical paper of Phoenix and coworkers describing the principles of sexual differentiation of brain and behavior in mammals (Phoenix et al., 1959). However, there are several reasons for which such an approach might be difficult or inappropriate in field endocrinology. First, gonadectomy might be challenging to perform in small animals, particularly under field conditions. Secondly, it is an invasive operation that requires some recovery period, which might expose the individuals to increased predation risks and is undesired when behavioral tests have to be performed shortly afterwards. Finally, because of its surgical nature gonadectomy is not always allowed by licensing authorities. The problem of studying if a trait is androgen-dependent when gonadectomy is not possible or desirable has been addressed in several ways. Correlational evidence can be collected for example by showing that the trait is modified seasonally in concurrence with changes in androgen levels. However, this kind of observations can be only indicative as other seasonal factors like the photoperiod could be directly responsible for the variation of the trait (Dawson et al., 2001; Kirn and Schwabl, 1997). The latter hypothesis can be tested by treating the animals with the hormone when its natural levels are low and see whether the trait changes in the expected direction. Alternatively, an androgen antagonist can be used to inhibit receptor-mediated responses. Also this approach is not immune to the effects of confounding variables, and can be used only for short-term experiments because receptor inhibition alters the regulatory feedback on the hormone. The real problem, however, is not how to find an alternative to gonadectomy and hormone replacement but rather whether this protocol is the appropriate one for the study in question. The removal of the gonads has several side effects and a gonadectomized animal is not simply an animal without gonadal hormones. Moreover, we now know that 'gonadal hormones' can be produced in large amounts also at extragonadal sites (Callard et al., 1978; Naftolin et al., 1975; Schlinger and Arnold, 1991). Thus, unless the study is specifically aimed to know what happens when the gonads are removed, other experimental approaches might be preferable. In fact, a behavioral ecologist or an ecological immunologist are probably more interested in asking how the studied trait is modulated by the hormone, i.e. how variations in the trait are related to hormone variations. In this perspective, the alternative approaches listed above and discussed in the next section are likely to be more effective than the castration-replacement protocol.

3. Alternative approaches to studying hormone function

Let's say we have decided to adopt one or a combination of the alternative approaches presented above: seasonal or life history stage correlations between the trait(s) and the hormone, hormone treatment in periods and/or physiological conditions of low hormone levels, and hormone or hormone receptor manipulation in intact animals. Each of these approaches has some pitfalls that deserve to be examined in details. Correlational evidence should always imply a good knowledge of the seasonal or ontogenetic stages for a correct interpretation of the data. For example, male canaries show large changes in androgen and androgen receptor levels between autumn and spring which are correlated with differences in song activity and song structure (Fusani et al., 2000; Gahr and Metzdorf, 1997; Leitner et al., 2001a,b; Nottebohm et al., 1986). Therefore it would be tempting to conclude that low androgen levels together with low androgen receptor expression are related to song instability. However, this correlation holds for the early autumn but not for the later autumn: in November canaries have very low levels of androgen yet the song is already stable and the expression of androgen receptor in the neural song system does not differ from spring (Fusani et al., 2000). In fact, the decrease in circulating androgen, brain androgen receptors, and song stability in the early autumn are all expression of a general 'shutdown' of reproductive traits which is typical of the molt period (Dawson, 2006; Dawson et al., 2001; Nicholls et al., 1988). All these data do not challenge the notion that song is regulated by androgen in male canaries, on the contrary, they illustrate how dynamic the system is. At the same time though, they tell us that it would be inexact to conclude that song stability is proportionally related to concentrations of androgen in the blood.

The second type of approach is to treat the animal with a hormone when its endogenous levels are naturally low. A good example is the stimulation of song development in female songbirds by testosterone. Already in the 1939 Leonard showed that the development of masculine song could be 'induced' in female canaries by injecting the recently discovered male hormone, testosterone (Leonard, 1939). A straightforward generalization of these results is that song development in males depends on the action of gonadal androgen. This interpretation was challenged by the discovery that the action of testosterone within the brain is often mediated by its conversion into estrogen by the enzyme aromatase (Hutchison, 1971; Naftolin et al., 1975), which is particularly abundant in the brain of male songbirds (Metzdorf et al., 1999; Schlinger and Arnold, 1991). Nevertheless, female songbirds have lower concentrations of aromatase in their brain, thus it could be concluded that song development in females is not mediated by aromatization but depends on the androgenic action of testosterone. Intact females, however, do also have very high aromatase activity in their ovaries so when they are given testosterone there is a significant increase in the concentration of circulating estrogen which is produced by the ovarian aromatization (Fusani, 2000; Fusani et al., 2003). This phenomenon would last a few days but if testosterone treatment is prolonged estrogen levels decrease to basal, probably because of the negative feedback of estrogenic metabolites of testosterone on LH production (Fusani et al., 2003). Thus also in female canaries song development depends on both androgenic and estrogenic actions of testosterone. Indeed when the estrogenic action is blocked by giving an aromatase inhibitor together with testosterone, song development is altered (Fusani et al., 2003).

Hormone manipulation in intact animals can sometime provide unexpected results. It is generally assumed that 'hormone-dependent traits' means 'traits whose expression varies proportionally with hormone concentration', although we have seen above that the definition is traditionally based on the removal-replacement protocol. In reality, proportional relationships between hormones and morphological or behavioral traits are less common than thought, and in many cases a threshold mechanism seems to be involved (reviewed by Adkins-Regan, 2005; Fusani and Hutchison, 2003; reviewed by Hews and Moore, 1997). Thus it is not unusual that the same hormonal treatment that leads to an increase in trait expression in animals which have low endogenous concentrations of the hormone has no effect on the trait when the animals have high endogenous levels. For example, testosterone can induce courtship behavior in juvenile and female Golden-collared manakins and in non-breeding males (Day et al., 2006) but does not affect courtship activity in breeding males (Day et al., 2007). Similarly, in male ring doves courtship is testosterone-dependent following the classical definition (Hutchison, 1970) but testosterone treatment of courting males does not result in further increases in courtship activity (Fusani and Hutchison, 2003). Sometime administration of exogenous hormones can actually