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SCIENCE DIRECT

Hormones and Behavior 48 (2005) 484 – 491

Hormones and Behavior

www.elsevier.com/locate/yhbeh

Difficulties and special issues associated with field research in behavioral neuroendocrinology

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Received 2 March 2005; revised 26 April 2005; accepted 3 May 2005
Available online 16 June 2005

Abstract

Classical behavioral neuroendocrinology has focused on a limited number of domestic mammals and birds. The model systems used in these studies represent a very small proportion of the diversity of hormone–behavior interactions found in nature. In the last three decades, an increasing number of researchers have concentrated their efforts on studying behavioral neuroendocrinology of wild animals. Field behavioral neuroendocrinology presents a series of challenges ranging from the design of the experiments to sample preservation and transportation. The constraints of field conditions limit the number of factors that can be controlled for and the questions that can be addressed. On the other side, many behaviors can be studied only in the field, and only a few species can be kept in captivity. Thus, field studies are necessary to understand the complexity and variety of interactions between hormones, brain, and behavior. In this article, we will review some of the peculiarities and challenges of field behavioral neuroendocrinology, including solutions for some of the most commonly encountered technical issues.

Keywords: Behavioral neuroendocrinology; Wild animals; Field experiment

Introduction

Most studies in behavioral neuroendocrinology are conducted in a laboratory setting. In the laboratory, variables such as climate, nutritional condition, social interactions, dominance, and reproductive status can be controlled. However, in contrast to captive animals, free-ranging populations are exposed to a wide array of ecologically relevant environmental and social stimuli. Thus, free-living animals experience a rich suite of complex interactions with their environment, which results in equally complex neuroendocrine responses. Consequently, laboratory studies face the quandary of producing reliable data under controlled conditions while severely limiting the exposure to natural stimuli and thus the expression of the complete set of behavioral and neuroendocrine traits. For example, wild dusky-footed woodrats (*Neotoma fuscipes*) build 'houses' from twigs that are essential for survival and may be used by generations of woodrats (Monaghan and Glickman, 1992). When the territorial behavior of intact and castrated woodrats was compared in an open field test, both groups of woodrats fought with the same intensity, and the likelihood of becoming dominant was equal, suggesting that territorial behavior was independent of testosterone (Monaghan and Glickman, 1992). However, when the researchers changed the setting and offered the woodrats a 'house', intact woodrats fought more intensively and were more likely to be dominant compared to castrated conspecifics. Hence, in the context of defending a "house", testosterone played a role in the control of territorial behavior, but it appeared to be less important in regulating aggressive behavior displayed in an open field test.

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0018-506X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.yhbeh.2005.05.005

Not only can the context of behavior in captivity differ from that in the wild, captivity itself can also have tremendous effects on behavior and physiology. For example, deficits in social experience during ontogeny can cause abnormal behavior in the black-headed gull (*Larus ridibundus* Groothuis and Vanmulekom, 1991). Furthermore, in both mammals and birds, behavioral deprivation may alter brain structures (see Barnea and Nottebohm, 1994; Healy et al., 1996; Rosenzweig and Bennett, 1996). For instance, hippocampal volume is reduced in captive as compared to free-living dark-eyed juncos (*Junco hyemalis* Smulders et al., 2000). Furthermore, comparative studies indicate that testosterone levels are generally higher in free-living than in captive birds (Wingfield et al., 1990), and the identical pharmacological treatment results in different behavioral responses in free-ranging as compared to captive male European stonechats (*Saxicola torquata rubicola*) (Canoine and Gwinner, 2002a,b). Hence, deprivation of important environmental and social cues, restraint in space, a limited nutritional spectrum, absence of predation and its perceived risks (Bednekoff and Lima, 1998), and many other factors can dramatically influence the neuroendocrinological and behavioral output of an organism in the laboratory (Künzl and Sachser, 1999). These findings emphasize the need for field experiments in behavioral neuroendocrinology. Or, as Fernando Nottebohm put it: "Unless you understand the needs, the habits, the problems of an animal in nature, you will not understand it at all. Take nature away and all your insight is in a biological vacuum." (in Specter, 2001). However, field experiments come with their own set of drawbacks and challenges. The purpose of this review is to highlight some of the issues that this group of researchers has come across while conducting field studies in behavioral neuroendocrinology on vertebrates (mostly birds) in various locations around the world. We also offer specific recommendations to overcome logistical problems frequently encountered in field neuroendocrine research.

Experimental design

Field studies often generate unique data on the neuroendocrinology of a wild animal in its natural habitat but face the problems discussed above. Almost everything changes all the time in the environment of a wild animal and can introduce considerable variability in the data set, e.g., climate, nutrition, or dominance status, to name but a few. Thus, field experiment needs to be designed carefully to obtain meaningful results in light of the anticipated variability in the data.

Designing field studies

Small samples sizes (6–10 or lower) are common in field behavioral neuroendocrinology due to the need to limit the impact on wild populations. Final sample sizes can be further reduced due to the difficulty of observing and catching wild animals. Small sample sizes and high variability in the data make careful experimental design a crucial part of successful neuroendocrine field studies. A simple design with only few treatment groups is therefore advantageous because it will maximize the power of statistical tests. Examples include comparisons between reproductive states (Canoine and Gwinner, 2002a; Foidart et al., 1998), sexes (Schultz and Schlinger, 1999), phenotypes (Miranda et al., 2003; Schlinger et al., 1999; Wikelski et al., 2005), or endocrinologically manipulated versus control animals (Romero et al., 1998; Semsar et al., 2001; Soma et al., 2002). In some experiments, a repeated-measures design is feasible where the same individual is measured repeatedly under different conditions or before and after a treatment. This can also be a means to improve the power of detecting differences in a data set with a low sample size. However, one problem often encountered in field studies using a repeated-measures design is that not all individuals can be recaptured each time. At the same time, additional data points may be available from individuals that originally were not included in the study. Attempts at solving this issue statistically include estimating missing data from repeated measurements, combining data from different time points, taking the mean of repeated measures for each individual, or randomly choosing just one measurement for each individual for statistics on independent data (Hau et al., 2002, 2004b; Sands and Creel, 2004).

Conducting field studies and coping with stochastic events

For most studies, animals will have to be caught at some time to obtain a biological sample (e.g., blood or tissue). In most laboratory studies, animals are habituated to human disturbance and handling and can swiftly be removed from their cages. However, to catch an individual in the field can take a considerable amount of time and effort and can become the most challenging aspect of the study (e.g., Hau et al., 2004a). The various methods of catching animals all have both benefits and drawbacks. In bird research, it is common to use Japanese mistnets to capture birds (but there are many other methods, see Bub, 1995). In many instances, passive mistnetting—without attracting the bird to the net—is an excellent method. In some situations, however, the bird needs to be attracted using social stimulation either because the sample needs to be obtained at a certain time (e.g., immediately after a behavioral observation Hau et al., 2004a) or because passive capture rate is exceedingly low (Wikelski et al., 2000). A typical social attractant is the playback of conspecific song with or without simultaneous presentation of a decoy (Wingfield, 1985). Social stimulation in itself, however, can stimulate hormone secretion within minutes (Oliveira et al., 2001; Wingfield and Wada, 1989), which in turn might affect a range of physiological parameters. If the time after which hormones increase due to social stimulation is known, care can be taken to capture the subject before the critical time (Wikelski et al., 2003a). Else, one needs to resort to another method of capture or at the very least keep detailed records to later account for these issues with statistical tools (for example, see Dittami and Gwinner, 1990). An efficient passive capture method for many vertebrates consists of walk-in traps, which may contain a food bait as an attractant. However, even such apparently benign traps have their drawbacks as they can impose stress on the animals once they realize they are trapped (Place and Kenagy, 2000; Romero and Romero, 2002). Additionally, the bait may alter the endocrine responses of the trapped animal since it can provide additional nutritional resources (e.g., Romero and Romero, 2002). Even swift immobilization procedures such as darting, often used in studies on wild mammals, can influence the stress axis (Devilliers et al., 1995; Sapolsky, 1985).

After a subject is caught in the field, the time it takes until the biological sample is obtained can be longer than in laboratory studies. This could be because standard methods take longer in difficult field conditions or because the animal needs to be transported to the laboratory for further processing. However, the stress resulting from capture and transport and the resulting increase in glucocorticoids, which typically occurs within minutes after capture, can alter the physiology of the individual (Martin et al., 2004; Sapolsky et al., 2000; Wingfield et al., 1997). For example, corticosterone can suppress the secretion of gonadal steroids in a wide variety of vertebrates (Moore et al., 1991; Wingfield, 1988). In some cases, testosterone may increase after acute stress (Heiblum et al., 2000; Place and Kenagy, 2000; Sapolsky, 1986). Again, careful advance planning and meticulous record taking are important. If an unstressed condition is important for the experiment, taking the sample within 2 min is of the essence (Romero and Reed, 2005; Romero and Romero, 2002). Alternatively, if the time elapsed from capture to obtaining the sample is recorded, one might be able to control for effects of stress on the biological sample using regression statistics.

Despite careful experiment planning, there are many ways in which natural events can impinge on a project. The most constructive way of dealing with stochasticity is to take advantage of unpredictable events as 'natural experiments'. Examples in field endocrinology where researchers have made excellent use of such natural experiments include studies on the endocrine effects of inclement weather (Smith et al., 1994; Wingfield et al., 1983), social stimulation (Goymann et al., 2001; Wikelski et al., 1999), environmental contamination (Wikelski et al., 2002), or drought (Romero and Wikelski, 2001).

Special techniques required in field neuroendocrinology

Fieldwork has requirements that are very different from those for laboratory studies and vary depending on the geographic location of the study area, accessibility, available facilities, species studied, etc. In this review, therefore, we will focus on special technical requirements of field behavioral neuroendocrinology studies. Typically, experiments of this kind involve hormonal manipulations that may need to be continued for a certain period. In the laboratory, this can be achieved by repeated (i.e. daily) administration of the substance. In the field, it is often difficult or impossible to capture the same individual more than once or twice. Thus, it is necessary to implant devices that deliver drugs continuously. One of the most commonly used devices consists of a piece of silicon tubing (Silastic Medical Grade silicon tubing, Dow Corning Inc.) that is filled with the substance to be administered after which the two ends are closed with silicon glue. The tube is then inserted subcutaneously and releases the substance for a period that depends on the length of the tubing, the thickness of the walls, and the amount of drug. The rate of passage from the inside of the tubing to the outside, however, depends mainly on intrinsic properties of the substance itself, in particular on its lipophilic nature. Thus, while silastic tubing is useful for lipophilic hormones like steroid hormones, it cannot be used for hydrophilic substances. If one or both ends of the tubing are left open, silastic can be used also for hydrophilic substances, however, in this case, the release is relatively fast. Alternative drug delivery devices are osmotic pumps and time-release pellets. The release rate of an osmotic pump is more or less independent of the solubility properties of the drug. However, the drug must form a stable solution for the whole duration of the experiment. Osmotic mini-pumps (Alzet, Charles River) which can release drugs for up to 4 weeks have been now used successfully in several studies (Fusani et al., 2001a; Soma et al., 2000). Time-release pellets are also a good way to deliver drugs for longer periods (Fusani et al., 2003). Innovative Research of America (Sarasota, Florida, USA) produces pellets for the most common hormones and anti-hormones at several dosages and release period length. The big advantage of these pellets is that the drug is included in an organic matrix that is slowly reabsorbed and thus it is not necessary to recapture the animals to remove the implant like for silastic tubing or osmotic mini-pumps.

Another limitation of field studies is the difficulty of using invasive techniques in animals that need to be released in their home area immediately after the treatment. Procedures that involve long recovery times must be excluded to avoid the risk of hypothermia, increased predation, and social/territorial challenges to the experimental subject. Sometimes it is not possible to observe the animal in the hours following the treatment, therefore any procedure that may potentially put at risk the health of the animals should be first tested on few individuals when used in a new species.

Experimental animals could be fitted with radio transmitters to ascertain their location (Cochran et al., 2004;

Goymann and Wingfield, 2004) or even physiological parameters such as heart rate and respiration, and these techniques have just become available for the use on small animals (Bowlin et al., in press).

Certain methods such as blood sampling of birds from the wing vein have now become standard field procedures and are relatively harmless provided that the experimenter has been properly trained. In a few cases, researchers have developed methods that transfer complex laboratory techniques to the field. One such example is the method developed by L. Michael Romero for intracranial drug administration in wild songbirds (Romero et al., 1998). Many neuroactive hormones and drugs do not pass the blood–brain barrier and therefore cannot be administered peripherally. In the laboratory, this can be bypassed by intracranial administration using a stereotaxic apparatus. Romero developed a small, portable stereotaxic device that he used successfully to inject neuropeptides into the third ventricle of small passerine birds in harsh field conditions (Romero et al., 1998).

Technical issues in field neuroendocrinology

In the following sections, we will briefly review some technical challenges commonly encountered in field neuroendocrinology. This is not meant to be an exhaustive review but rather an overview of available techniques and solutions.

Field stations

A good field station or field base can greatly simplify fieldwork. The best field stations can provide safe storage of samples and equipment, bench space for sample processing, electricity, water, telephone and Internet connection, and above all the knowledge and experience of station managers and other researchers. In addition, the station manager can provide researchers with updated information about research permits, safety of study areas, and contacts with local authorities. Thus, the availability of a field station should be highly valued when planning a field study.

Blood sampling and processing

Blood sampling is probably the most common procedure in field neuroendocrinology studies. Sampling methods differ according to species, however, processing almost invariably involves centrifugation and separation of plasma for hormone measurements. There are portable centrifuges on the market which can be operated with batteries (e.g., ZipSpin, LWScientific, Inc., Lawrenceville, GA) or even manually (Armin Baack, Schwerin, Germany).

Tissue preparation

To maintain the structure of the tissue as intact as possible, it is necessary to fix the samples. The fixative rapidly penetrates the cell membranes and immobilizes the macromolecular material. This can be done in several ways:

Perfusion

The most commonly used method is intracardiac perfusion with paraformaldehyde or neutrally buffered formalin (e.g., Goodson et al., 2004; Leitner et al., 2001). In the laboratory, a peristaltic pump is generally used, but at many field locations, electricity may not be available. Alternatives to peristaltic pumps are large syringes or gravity. Generally, a good perfusion can be achieved by hanging the bottles containing the formalin 2 m above the ground. For some histological techniques, such as in situ hybridization, it is essential to use sterile solutions and materials (Leitner et al., 2001). In such cases, it is more difficult to transfer laboratory methods to a field setting. Hence, depending on the technique that will be later used to process the tissue, the following alternative fixation methods can be considered.

Acrolein

Recently, acrolein has been used to fix tissue in field studies. Acrolein fixation is obtained by simple immersion of the tissue in the fixative. We still know little about potential interferences of acrolein with staining techniques, i.e. immunohistochemistry. Hence, artifacts caused by acrolein cannot be excluded (Butler et al., 1999). However, the few studies that have used this fixative have provided satisfactory results (Butler et al., 1999; Iturriza and Thibault, 1994; Maney and Ball, 2003). Acrolein is highly toxic but could be a valid alternative to formalin in field work situations when perfusion is unpractical.

Freezing

Rapid freezing is not only an alternative to fixation in histological studies but also the method of choice for procedures such as enzyme assays or receptor binding. The best results will be obtained using liquid nitrogen (Fusani et al., 2001b; Soma et al., 1999). The block of tissue to be frozen is placed on a small aluminum weighing dish that is floating on the liquid nitrogen. Liquid nitrogen can be kept in special containers for long periods of time, but transportation can be a logistic challenge. Alternatively, tissues can be frozen on dry ice (Riters et al., 2001; Schlinger et al., 1989). It is important that the tissue freezes as quickly as possible to avoid formation of water crystals that might alter the histological structure. Thus, the dry ice should be kept in a deep styrofoam box so that cold air will surround the tissue. This is critical when working in hot climates. The best results can be obtained by filling a metal container with ethanol and putting it in a styrofoam box surrounded by dry ice. Evaporation of the alcohol will decrease the temperature even further. The tissue is frozen on a small aluminum weighing dish that is floating on the ethanol.

Storage and transportation

Tissue or plasma are best stored at very low temperatures, i.e., −80°C or lower. Only few field biologists will be lucky enough to conduct their fieldwork at a place where they can store their samples in a −80°C freezer. Even if there is a freezer available at the field station, eventually the samples will have to be transported from there to the laboratory. After the hard work of properly collecting the samples, the transportation is a very critical step for the samples (and the investigator!). Transportation on an airplane can be particularly challenging because security regulations may require that the container is opened to inspect the content.

Liquid nitrogen

Liquid nitrogen is the best and easiest solution to store samples. Liquid nitrogen can be kept in special containers for long periods of time—from a couple of weeks up to 6 months or longer, depending on the size and model of container—and should be seriously considered for storing tissue and plasma when conducting field studies in remote places. The initial expenses are high, and both the pressure-sealed container and the liquid nitrogen itself are not easy to handle. In many countries, liquid nitrogen can only be transported in special safety-containers. These are indispensable for transportation on airplanes. The disadvantage of these containers is that they do not allow storing liquid nitrogen for long periods of time. It is not allowed to transport liquid nitrogen in the passenger cabin of airplanes. Some airlines do not allow liquid nitrogen on board at all.

Dry ice

Dry ice is a good alternative for short-term storage and transportation, and handling is less problematic than with liquid nitrogen. The disadvantage is that dry ice evaporates rapidly and cannot be used to store samples for longer than a few days. Dry ice should be kept in a styrofoam container that is well closed but not sealed to avoid the building-up of pressure as the dry ice evaporates. Dry ice will last longest if stored at −80°C or at the lowest possible temperature. Transportation of samples on dry ice is simple, but not all airlines allow passengers to carry dry ice and if they do, they usually restrict it the amount to 2.5 kg. It is recommendable to enquire about dry ice restrictions before booking the flight.

Transportation of animals

A general problem of field studies is the difficulty to control the experimental conditions. If possible, it is desirable to set up an experimental room at the field site or nearby (e.g., Hau et al., 2000a,b). For studies involving specific recording equipment that cannot be taken to the field, it is necessary to transport the wild caught animals to the laboratory. Such complex studies were conducted, for example, by E. Gwinner and his co-workers at the Max Planck Institute for Ornithology in Andechs (Germany) (Fusani and Gwinner, 2004; Gwinner and Scheuerlein, 1999; Partecke et al., 2004; Wikelski et al., 2003b). Different bird species were caught in several parts of the world and transported back to the home laboratory. This is a very difficult task and requires long-term advance preparations. Permits for catching, export, transportation, and import are essential. Quarantine regulations have to be fulfilled, and certificates from veterinarians are required. Furthermore, in some situations, special animal care is indispensable, for example, when working with bird hatchlings that need to be fed at frequent intervals. In such cases, the animals need to be with the passengers during the flight. In the past, the airlines usually gave permissions to do so, but recent changes in security policies render such undertakings increasingly difficult. Apart from the formalities that have to be dealt with in advance, the actual transportation of the animals has to be planned in detail. Special cages are required to reduce the space needs and to minimize the possibility of injury for the animals. The International Air Transport Association (IATA) provides guidelines for transportation of animals on airplanes which vary according to the species, and most airlines will not allow the animals to be boarded unless these guidelines are respected. Researchers should consider that transportation represents a stressor, especially for wild-caught animals, and this could have tremendous effect on their behavior and physiology during and after the trip. Thus, an appropriate period of acclimatization should elapse between the animals have reached the laboratory and the beginning of the experiments.

Conservation considerations

Neuroendocrinology studies on wild animals are sometimes designed to improve our knowledge of the species reproductive physiology. In many cases, conservation itself can be the goal of the research (cf. Cochrem, this issue). Manipulating wild animals in their natural habitat can have widespread consequences not only for the experimental individual itself, but also for its neighbors and potentially for its offspring. For example, testosterone-manipulated individuals increase their interactions with neighbors, who in turn increase their endogenous testosterone, possible to live up to the challenge (Wingfield et al., 1990). Similar effects can occur in females that live in dense and thus interaction-rich colonies (Eising et al., 2001). As maternal hormones deposited in the egg can significantly affect offspring phenotype in birds (Schwabl, 1996), it is clear that endocrine manipulations in the field can have long-term and even cross-generational consequences at least on local populations. Experimental manipulations will also have direct implications for individuals. Repeated capture can induce avoidance strategies in animals which again could have negative effects on populations but also make recaptures more difficult reducing sample sizes. Manipulated individuals may also be preferentially selected by predators. Capture itself may affect the characteristics of the stress response because it can be perceived as a predation attempt (cf. Canoine et al., 2002; cf. Scheuerlein et al., 2001). Some neuroendocrinological experiments require terminal sampling of individuals. Obviously, such invasive studies cannot be conducted on populations that are endangered or small. Overall, ethical considerations should rank highly in the decision making process of whether and how a neuroendocrinological study should be conducted in the wild. Ethical concerns will also affect the decision of how many individuals will be used for the experiments. Sample sizes should take into account that field experiments have intrinsically more variation compared to laboratory experiments due to imponderabilities of environmental and social conditions. Furthermore, some animals will vanish for unknown causes (emigration, predation). Thus, to conduct solid experiments, field neuroendocrinologists need to plan for considerably larger sample sizes than would normally be necessary to achieve sufficient statistical power. Pilot experiments are therefore a strong requisite of any field study involving invasive techniques (in the broadest meaning). Fortunately, the ecological community has already put forward guidelines for the use of wild animals in research that should be adhered to (Oring et al., 1988). To date, the authors are not aware of neuroendocrinolgical studies that endangered or harmed local populations of wild animals, a tribute to the ethics of researchers in this field.

Permits and health considerations

An often overlooked complication for field experiments is the fact the multiple agencies need to be contacted for acquiring the necessary permits for catching animals, for experimental manipulations, and for terminal sampling. In the case the field study is conducted in a country different from that in which the samples will be analyzed, export and import permits for animal samples need to be applied for well in advance. Regulations not only differ between countries but also between regions, like among US states. Obviously, the primary and main concern of the regulatory agencies is the health and well-being of humans, domestic animals, and livestock that could be affected by animal-borne diseases introduced in the country via research samples. For example, mammalian and avian samples could potentially contain viruses that are detrimental to humans or livestock, such as West Nile or New Castle disease germs. Agencies are less concerned about fish and reptilian samples because fewer possibilities for cross-infections of livestock exist. Even if the possibility of cross-infections and spread of diseases is generally extremely rare, serious precautions should be taken, in particular in the packaging of samples for transportation.

Conclusions

In this brief review, we have addressed the most common issues associated with studying behavioral neuroendocrinology in the field. We have also provided solutions to some typical technical and logistic problems that we have encountered while conducting our studies. Despite the challenges presented by field studies, we believe that they are necessary for understanding the complex interactions between brain, hormones, and behavior in an evolutionary perspective.

Acknowledgment

We dedicate this paper to the late Ebo Gwinner, who impressed upon us the importance of studying behavioral endocrinology in the field. Ebo was a most inspiring and enthusiastic mentor for all of us.

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