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Gonadotropin Inhibitory Hormone: New Insights into the Neuroendocrinology of Stress and Social Conditions

Author
Calisi, Rebecca Marie

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Gonadotropin Inhibitory Hormone: New Insights into the Neuroendocrinology of Stress and Social Conditions

By

Rebecca Marie Calisi

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Integrative Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor George E. Bentley, Chair
Professor Eileen A. Lacey
Professor Lance J. Kriegsfeld

Spring 2010

Abstract

Gonadotropin Inhibitory Hormone: New Insights into the Neuroendocrinology of Stress and Social Conditions

by

Rebecca Marie Calisi

Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Professor George E. Bentley, Chair

Stress is a known inhibitor of reproductive function and its associated behaviors. How and why stress acts to influence reproduction have been intensely studied and appear to be extremely varied, though we do not fully understand how these processes operate at the level of the brain. My dissertation work examined the role of the novel neurohormone gonadotropin inhibitory hormone (GnIH) in response to stress and social environment in reproductively active birds. To review the actions of GnIH in brief, GnIH inhibits the activity of gonadotropin releasing hormone (GnRH) neurons in the brain in addition to reducing the release of the gonadotropins luteinizing hormone and follicle-stimulating hormone from the pituitary gland. GnIH also reduces testosterone release from the gonads. Central administration of GnIH can decrease both copulation solicitations in birds and sexual behaviors in rodents. However, how GnIH fluctuates naturally in response to stress and social environment is not well understood. My questions were as follows:

1) Does capture-handling stress affect GnIH, and is there a seasonal effect?
2) Does social environment affect GnIH, and does this vary with breeding stage?
3) Is there a mechanism in place for the stress response to affect reproduction via GnIH? Specifically, are receptors for glucocorticoids expressed in avian GnIH cells? And what are the conservation implications of such neuroendocrine research?

My work is the first to demonstrate the effects of stress (in birds) and social environment (in any organism) on GnIH. My work is also the first to report the co-localization of glucocorticoid receptors in avian GnIH cells, providing a mechanism by which the stress response can influence reproduction at the level of the brain. Because GnIH presence and function are conserved throughout all vertebrates studied, these findings can create new avenues into studies of vertebrate stress and reproductive physiology.

Dedication

To my grandmother, Rebecca Raquel Rodríguez Garza Vela (1912-1995)

As a Mexican-American woman born and raised on the Texas-Mexico border, things could not have been easy for my grandmother. However, her constant passion for knowledge and adventure could not be bridled. After being awarded her degree in nursing in 1934 from McAllen, Texas, Municipal Hospital, she became the first female airline hostess for Braniff Airways (in was mandatory in that time for hostesses to be registered nurses) and traveled "from the Great Lakes to the Gulf". She later became a surgical nurse until retiring in 1968. During her life, Rebecca was an active participant in and staunch supporter of the Democratic Party. She was a member of the Rio Grande City Consolidated School District School Board and served as School Board President for three terms, all the while raising six children. Rebecca was a Distinguished Member of the Hidalgo-Starr Medical Society, Women's Auxiliary and Pan American Round Table. She was an amazing, unstoppable, inspirational woman who broke through many boundaries.

Although our time together was too short, I cannot help but feel my abuela has passed on to me her *ganas* for science, education and adventure. I hope I can one day be half the woman she was, and I am proud to dedicate my doctoral work to her.

Table of contents

Introduction

The stress (hypothalamic-pituitary-adrenal [HPA]) axis and the reproductive (hypothalamic-pituitary-gonadal [HPG]) axis are two hormone systems vital for individual and species survival in all vertebrates studied. When stress is detected by the brain, the hypothalamus releases corticotropin releasing hormone which ultimately stimulates the release of glucocorticoids, or "stress" hormones (cortisol, corticosterone), from the adrenal glands. Glucocorticoids send signals to the rest of the body to prepare it to respond to the stressor. For example, glucocorticoids promote the metabolism of fat stores for energetic demands and inhibit temporarily 'unnecessary' activities, such as digestion and reproduction, until the stressor has passed. At the onset of reproduction, the same area of the brain that produces corticotropin releasing hormone during a stress response (the hypothalamus) produces gonadotropin releasing hormone (GnRH), a neurohormone responsible for regulating reproduction and its associated behaviors,. GnRH is released from the hypothalamus into the anterior pituitary gland, where it causes the release of gonadotropin hormones into the bloodstream. Gonadotropins cause the gonads to develop and produce gonadal steroids (androgens and estrogens). These steroids feedback onto the reproductive axis to regulate it.

The recent discovery of the novel neurohormone gonadotropin inhibitory hormone (GnIH; Tsutsui et al. 2000) is changing the way we view how reproduction and sexual behavior are regulated by the brain. GnIH, first discovered in birds (Tsutsui et al. 2000), inhibits pituitary gonadotropin release *in vitro* and *in vivo* (Tsutsui et al. 2000, Osugi et al. 2004, Ciccone et al. 2004) in both birds and mammals (Kriegsfeld et al. 2006, Johnson et al. 2007, Murakami et al 2008, Clarke et al 2008), indicating the conserved nature of its actions (reviewed in Bentley et al. 2006). GnIH also inhibits gonadotropin synthesis in birds (Ubuka et al. 2008, Ciccone et al. 2004). Located in the hypothalamus, GnIH-producing neurons appear to make direct contact with GnRH- producing neurons in both birds and mammals (birds: Ubuka et al. 2008, sheep: Smith et al. 2008, rodents: Johnson et al. 2007, Kriegsfeld et al. 2006, primate: Ubuka et al. 2009, humans: Ubuka et al 2010). GnIH receptor mRNA is expressed by GnRH neurons (Ubuka et al. 2008), suggesting that GnIH can directly affect or regulate GnRH, and thus the reproductive axis. In mice, direct application of mammalian RF-amide related peptide (RFRP; the mammalian form of GnIH) to GnRH cells in cultured brain slices decreases firing rate in a subpopulation of cells, further suggesting a direct action of GnIH on GnRH neurons (Ducret et al. 2009; Wu et al. 2009). Thus, the discovery of GnIH and its actions provides us with an exciting and novel tool with which to study the mechanisms controlling reproduction.

Stress is a known inhibitor of reproductive function and its associated behaviors, but how stress acts to influence reproduction at the level of the brain is not well understood. Because of the inhibitory nature of GnIH on the reproductive axis, I hypothesized that stress and social effects upon reproduction are associated with the hypothalamic GnIH system. I utilized two avian neuroendocrine model systems, the house sparrow (*Passer domesticus*) and the European starling (*Sturnus vulgaris*). To examine effects of stress on the GnIH system, I conducted experiments using a captive population of house sparrows. Experiments concerning social effects on the GnIH system were conducted using a semi-natural captive population of European starlings.

In my first chapter, "Lab and field experiments: are they the same animal?" I review instances in which 1) results from captive experiments differ as a result of different laboratory housing conditions, and 2) results gained from captive animals differ from those gained from

wild animals sampled in their natural environment. My goal was to promote discussion of the influence of housing environment on physiology to highlight the need for experiments in both lab and field when looking at complex biological systems. This semi-natural experimental set-up is a powerful way to examine the physiological relevance of the question at hand.

Chapter 2, "Seasonal differences in hypothalamic EGR-1 and GnIH expression following capture-handling stress in House sparrows (*Passer domesticus*)", examines the effects of capture-handling stress in the hypothalamus of male and female adult house sparrows at the start (spring) and end of the breeding season (fall). Additionally, I quantified the expression of the protein product of the immediate-early gene, *EGR-1*, using this as an indicator of neuronal activation surrounding the GnIH neuron population (the paraventricular nucleus). My results imply an influence of stress upon the paraventricular nucleus and the GnIH system that changes over the annual cycle of reproduction.

Chapter 3, "Social environment influences gonadotropin inhibitory hormone (GnIH) in the brain," examines how the outcome of competition for limited resources needed for reproduction affects the GnIH system in European starlings. By limiting the number of nestboxes per semi-natural enclosure and thus the number of social pairing and nesting opportunities, I observed that birds which had nestboxes ("winners") had significantly different numbers of GnIH cells than those without nestboxes ("losers"), and this relationship varied with breeding stage. At the beginning of the breeding season, winners had fewer GnIH peptide cells than losers. At the middle of the breeding season, when eggs were being incubated by both males and females, winners had more GnIH cells than losers. Thus, my data indicate that GnIH has the ability to respond and/or serve as a modulator of reproductive behaviors in response to social environment. Additionally, I provide evidence of the adaptive value of this mechanism.

Chapter 4, "Stress, the brain and reproduction: implications for conservation neuroendocrinology," reviews work on the effects of stress on the brain and how knowledge of the GnIH and other neuroendocrine systems may have applications for conservation. In order to minimize or alleviate the negative effects of stress on the reproductive system, we need to understand better the neural control of reproduction and sexual behaviors. Examination of the brain is largely neglected because of the challenges of studying it in endangered species. However, the use of non-endangered model species can greatly increase our knowledge of important reproductive neuroendocrine processes. This type of information has profound conservation implications as many species, endangered or otherwise, exhibit a decrease in or termination of reproductive activity and behaviors under captive conditions, stressful or otherwise. Evidence suggests that the neurohormones kisspeptin (KISS) and GnIH play important complementary roles in precisely driving reproductive function, with KISS serving as an accelerator and GnIH serving as a restraint on GnRH production and secretion and the reproductive system. Both KISS and GnIH belong to the RFamide peptide family and are affected by stress, though evidence exists only for the direct effect of stress on reproduction at the level of the brain via GnIH. GnIH cells co-express receptors for glucocorticoids in mammals, and I am the first to report they are co-expressed in avian hypothalamic GnIH cells. I discuss the role of KISS and GnIH in the stress response and their conservation implications. By understanding the role of these RFamide peptides in reproduction and sexual behaviors in response to stress, we can develop pharmacological tools with which to improve the success of captive breeding programs.

In sum, studies of the effects of stress and social behavior on the reproductive axis are seminal to understanding behavioral neuroendocrinology. The recent discovery of the novel

neurohormone GnIH is changing the way we view how reproduction and sexual behavior are regulated by the brain. Until now, little was known about how stress affects the GnIH system, and nothing was known about whether GnIH could be influenced by social environment. Here, I report that stress, social environment, reproductive stage and seasonal environment all combine to influence GnIH content, and thus reproductive output and I provide evidence as to the functional significance of this phenomenon. These data provide a paradigm shift for work in stress and reproductive physiology, with implications for conservation physiology.

Acknowledgements

I thank my co-authors and funding sources, each of which is listed at the end of the appropriate chapter. In general, I thank my loving husband, Samuel Díaz-Muñoz, for not only his help as a colleague, but for his kind patience, support and understanding. I thank my immediate and extended family (the Calisi, Rodríguez and Muñoz branches) for making me laugh and keep things in perspective. I thank Jeanette Boylan, Kenneth Kaemmerer and Todd Bowsher (Dallas Zoo and Aquarium) for encouraging me to go to graduate school in the first place, and I thank John Malone for teaching me early on what it means to be a scientist. Many thanks to Audrey Knowlton, Colette Patt and the Berkeley Edge Program, and to the wonderful staff of the Department of Integrative Biology for all their help over the years, especially Michael Schneider, Emily Howard, and who I consider to be my (and many others) academic fairy godmother, Mei Griebenow. Also, I am grateful to have the following amazing people in my life who have supported me in more ways than one: Jennifer Bialkowski, Natalie Thompson, Kelly Ayres, Mark McGee, Matt Watson, Ben Carter, Yonatan Munk, Julie Woodruff, Dustin Rubenstein, Lindsey Kaplan, Omulu Capoeira Guanabara, the Gillettes, the Bustamantes, the Alarcons and the McGuires.

I am thankful for all the help I received in the lab and field from everyone in the Bentley Lab. I am especially thankful for all the incredibly smart and talented undergraduate students that aided in my research: Josemine Miranda, Norma Rizzo, Noel Cruz, Emanuel Zamora, Susanna Fenstermacher and Fei Liang. I am indebted to my lab sisters Patricia Soares Castro Lopes, Nicolette McGuire and Nicole Perfito for more than I have space to write here. My academic committee members, Lance Kriegsfeld and Eileen Lacey, were invaluable mentors – I am forever changed in the way I ask questions, interpret data and view the field in general. Also, I thank Daniela Kaufer, Tyrone Hayes, Kevin Padian and John Wingfield for our discussions, and I thank John for accepting me as a post-doc in his lab at UC Davis. Finally, and most importantly, I thank my academic research advisor, George "Jorge" Bentley, the most stubborn person I have ever met, for his mentorship and support – but most of all, for his tough love, kind ear and fervent attitude to defend us all "to the hilt"!

REBECCA M. CALISI

The University of California at Berkeley • Department of Integrative Biology
3060 Valley Life Sciences, Building #3040 • Berkeley, CA 94720-3140
Lab Tel: 510-642-1530
Fax: 510-643-2180 • Email: calisi@berkeley.edu
Lab website: http://ib.berkeley.edu/labs/bentley

EDUCATION

Post-doc (Neurology, Physiology and Behavior)
The University of California at Davis, begin Aug 2010 • Advisor: Dr. John C. Wingfield

Ph.D. (Integrative Biology)
The University of California at Berkeley, May 2010 • Advisor: Dr. George E. Bentley

M.S. (Biology)
The University of Texas at Arlington, May 2006 • Advisor: Dr. Daniel R. Formanowicz

B.A. (Psychology)
Boston College, (*cum laude*), May 2001 • Advisor: Dr. Hiram Brownell

PUBLICATIONS

JOURNAL ARTICLES

Calisi, R.M., Perfito, N. and G.E. Bentley. 2010. Gonadotropin inhibitory hormone (GnIH): conservation implications. *In prep*.

Calisi, R.M., Díaz-Muñoz, S.L., Wingfield, J.C., Bentley, G.E. Social environment influences gonadotropin inhibitory hormone (GnIH) in the brain. *Submitted*.

Calisi, R.M. and G.E. Bentley. 2009. Lab and field experiments: Are they the same animal? *Hormones and Behavior,* 56:1-10. ****FEATURED ON FACULTY OF 1000; PICKED AS TOP 25 HOTTEST ARTICLES, NEUROSCIENCE, HORMONES AND BEHAVIOR****

Bentley, G.E., Ubuka, T., McGuire, N.L., **Calisi, R.M**., Perfito, N., Kriegsfeld, L.J., Wingfield, J.C. and Tsutsui, K. 2009. GnIH: A Multifunctional Neuropeptide. *Journal of Neuroendocrinology*, 21:276-281.

Calisi, R.M., J. H. Malone, D.K. Hews. 2008. Female secondary coloration in the Mexican boulder spiny lizard (*Sceloporus pyrocephalus*) is associated with nematode load. *Journal of Zoology.* 276:358-367. ****PICKED EDITORIAL BOARD MEMBER'S CHOICE FEATURE****

Calisi, R.M., Rizzo, N.O., Bentley, G.E. 2008. Seasonal differences in hypothalamic EGR-1 and GnIH expression following capture-handling stress in house sparrows (*Passer domesticus*). *General and Comparative Endocrinology*. 157:283-287.

Ubuka,T., McGuire, N.L., **Calisi, R.M.**, Perfito, N. and Bentley, G.E. 2008. The control of reproductive physiology and behavior by gonadotropin-inhibitory hormone. *Integrative and Comparative Biology*. 1-10.

Calisi, R.M. and D.K. Hews. 2007. Steroid correlates of multiple color traits in the spiny lizard, Sceloporus pyrocephalus. *Journal of Comparative Physiology* 177:641-654.

Calisi, R.M. 2005. Variation in Bidder's organ volume is attributable to reproductive status in *Bufo woodhousii*. *Journal of Herpetology* 39(4): 656-659.

Calisi, R.M. 2004. Geographic distribution. *Hyla squirrela*. *Herpetological Review* 35:282.

BOOK CHAPTERS:

McGuire, N.L., **Calisi, R.M.** and G.E. Bentley. *Invited chapter, in press.* Hormones and behavior in association with seasonality. J.C.Wingfield (ed.) *Encyclopedia of Animal Behavior.*

SELECTED GRANTS & AWARDS

2010-2013 National Science Foundation: Postdoc Research Fellowship in Biology	$189,000
2010-2011 University of California's President's Post-doctoral Fellowship finalist	-
2010 National Science Foundation travel award to speak at IOC, Brazil	$500
2009-2010 Ford Diversity Dissertation Fellowship	$21,000
2009-2010 University California Dissertation-Year Award	$10,000
2009-2010 University California Dissertation-Year Fellowship: *declined*	$20,970
2010 Best Student Paper, Div. Neurobiology, SICB. Seattle, WA.	$100
2009 National Science Foundation travel award to speak at ABS, Brazil	$1,000
2008 Mentor Research Award, UC Berkeley	$14,000
2008 Outstanding Graduate Student Instructor Award, UC Berkeley	-
2008 Ford Predoctoral Fellowship Honorable Mention	-
2008 Society of Integrative and Comparative Biology, Div. Neurobiology - Best Poster	$100
2008 Society of Integrative and Comparative Biology Student Research Grant	$1,000
2006 Univ. California, Berkeley, Berkeley Edge Summer Fellowship	$6,000
2006-2008 Univ. California, Berkeley, Chancellor's Fellowship	$92,000
2006-2007 Arizona State Univ., Ph.D. Fellowship: *declined*	$20,000
2006 Phi Sigma Student Research Grant, Univ. Texas, Arlington	$1000
2005 Nominated by D.K. Hews for Student Associate Member, Society of Sigma Xi	-
2005 Howard McCarley Student Research Award, SWAN	$1000
2005 Texas Academy of Science Student Research Grant	$500
2004 Phi Sigma Student Research Grant, Univ. Texas, Arlington	$1000
2004 East Texas Herpetological Society Student Research Grant	$500

SELECTED PRESENTATIONS

PAPERS:

Calisi, R.M., Bentley, G.E. **2010**. The effects of social environment on avian gonadotropin inhibitory hormone (GnIH) during the breeding season. International Ornithological Conference. Campos de Jordão, SP, Brazil, Aug. 22-28.

Calisi, R.M., Perfito, N. and Bentley, G.E. **2010**. How can stress affect the neural control of reproduction? An examination of gonadotropin inhibitory hormone (GnIH) and glucocorticoid receptors (GR) in songbirds. Society for Integrative and Comparative Biology. Seattle, WA, Jan. 3-6. ****BEST STUDENT PAPER, DIVISION OF NEUROBIOLOGY****

Calisi, R.M. 2009. How can stress affect reproduction? A look at endocrine control by the brain. Invited speaker at the Conference of Ford Fellows. Meeting theme: Feeding the fire in challenging times: cultivating community and leadership for positive change. Newport Beach, CA, Oct 16-17.

Calisi, R.M and Bentley, G.E. 2009. What's GnIH got to do with it? Social stress, neuroendocrinology and reproductive behavior in songbirds. Animal Behavior Society, Pirenópolis, Brazil.

Calisi, R.M. **2009**. Social stress, neuroendocrinology and reproductive behavior in songbirds. Nueroendocrine Joint Lab Summit, University of California at Davis.

Bentley, G.E., Ubuka, T., McGuire, N.L**., Calisi, R.M.,** Perfito, M.N., Tsutsui, K., Wingfield, J.C. **2009**. Regulation of vertebrate reproduction by GnRH and GnIH . Society for Integrative and Comparative Biology, Boston, MA.

Bentley, G.E., McGuire, N., **Calisi, R.M.**, Perfito, N. and Ubuka,T. **2008.** Gonadotropin-inhibitory hormone: A synopsis. Society for Integrative and Comparative Biology, San Antonio, TX.

Calisi, R.M., J.H. Malone, and D.K. Hews. **2006**. Female secondary sexual coloration in a spiny lizard is associated with nematode load, testosterone, and reproductive state. Animal Behaviour Society, Snow Bird, Utah.

Calisi, R.M. and J.H. Malone. **2006**. Female secondary coloration elicits particular behavioral responses from conspecific males and females in a spiny lizard. Texas Academy of Science, Lamar University, Beaumont, Texas.

Calisi, R.M., J.H. Malone, J. W. Malcom, and D.R. Formanowicz. **2005**. Size and secondary color correlates to area coverage in the Mexican Boulder Spiny Lizard, *Sceloporus pyrocephalus*. Texas Herpetology Society Meeting, Austin, Texas.

Calisi, R.M. and D.K. Hews. **2005**. Plasma hormone concentrations and sexual color traits in male and female *Sceloporus pyrocephalus* lizards. Animal Behavior Society, Snow Bird, Utah.

Calisi, R.M. and J.H. Malone. **2005**. Female coloration and parasitism in the Mexican boulder spiny lizard, *Sceloporus pyrocephalus*. Texas Academy of Science, Pan American University, Edinburgh,Texas.

INVITED LECTURES:

Calisi, R.M., Díaz-Muñoz, S.L., Wingfield, J.C., Bentley, G.E. **2010**. Social environment influences gonadotropin inhibitory hormone (GnIH) in the brain. Departmental Speaker. Department of Biology, California State University, East Bay.

Calisi, R.M and G.E. Bentley**. 2009.** The effects of seasonal and social stress on the reproductive neuroendocrinology of European starlings. Invited speaker in symposium entitled "From phylogeny to physiology: integrative studies of starlings and mockingbirds," American Ornithologists' Union. Philadelphia, Aug 12-14.

Calisi, R.M. 2007. Neural pathways of reproductive strategies: A review. Neurocircuitry Seminar Series, Univ. California at Berkeley.

Calisi, R.M. 2006. Proximate and ultimate mechanisms associated with female secondary coloration in the Mexican Boulder Spiny Lizard, *Sceloporus pyrocephalus*. Animal Behavior Seminar, Univ. California at Berkeley.

Calisi, R.M. 2005. The importance of studying secondary sexual color: a look at female coloration in the Mexican Boulder Spiny Lizard. Comparative Vertebrate Anatomy course guest lecturer, Univ. Texas at Arlington.

Calisi, R.M. 2004. Determining enrichment effects on captive Tree lizards, *Urosaurus ornatus*, using corticosterone as an index of stress reactivity. Phi Sigma Society, Univ. Texas at Arlington.

Calisi, R.M. 2004. Glucocorticoids and the Adrenal gland. Stress Hormones and their role in reproduction. Endocrinology course guest lecturer, Univ. Texas at Arlington.

POSTERS:

Calisi, R.M and Bentley, G.E. **2009**. What's GnIH got to do with it? Social stress, neuroendocrinology and reproductive behavior in songbirds. Univ California at Berkeley, Neuroscience Conference, Tahoe, CA.

Perfito, N., Jeong, S., **Calisi, R.M**., Silverin, B., Bentley, G.E. and M. Hua. **2009**. Mechinisms underlying flexibility in seasonal time measurement. Univ California at Berkeley, Neuroscience Conference, Tahoe, CA.

Calisi, R.M., N.O. Rizzo and G.E.Bentley. **2008**. Capture-handling stress and its effects upon hypothalamic EGR-1 and GnIH expression in House sparrows (*Passer domesticus*). Society for Integrative and Comparative Biology, San Antonio, TX. ****AWARDED BEST POSTER, DIVISION OF NEUROSCIENCE****

Calisi, R.M. and G.E. Bentley. **2007**. Lab and field experiments: are they the same animal? Society for Integrative and Comparative Biology, Phoenix, Arizona.

Calisi, R.M. and J.H. Malone. **2005**. Relationships between body size and territory acquisition in Mexican boulder spiny lizards, *Sceloporus pyrocephalus*. Texas Academy of Science, Pan American University, Edinburgh, Texas.

Calisi, R.M. and J.H. Malone. **2004**. Relaciones entre tamaño corporal y adquisición de territorio en lagartijas espinosas de las rocas, *Sceloporus pyrocephalus*, hembras y machos. Mexican National Herpetology Conference held by La Sociedad Herpetológica Mexicana, A. C. (SHM) at Universidad Autónoma de Juárez, Villahermosa, Tabasco, México.

APPOINTMENTS

2007, 2008 Graduate Student Instructor, Comparative Endocrinology, Univ. California, Berkeley
2007 Graduate Student Instructor, Animal Behavior, Univ. California, Berkeley
2006 Graduate Research Assistant, Bentley Lab, Univ. California, Berkeley
2006 Graduate Teaching Assistant, Structure and Function of Organisms, UT, Arlington
2005 Graduate Research Assistant, Tropical Herpetofauna Catalogue, Univ. Texas, Arlington
2004 Head Graduate Teaching Assistant, Cell and Molecular Biology, Univ. Texas, Arlington
2003 Graduate Teaching Assistant, Cell and Molecular Biology, Univ. Texas, Arlington
2002-2003 Instructor, Education and Research Department, Dallas Zoo and Aquarium

SERVICE

2010-2011
Graduate Student & Post-doc Representative, Div. Comparative Endocrinology, Society for Integrative & Comparative Biology

2008-2010
Grad Student Co-Chair, Graduate Diversity Committee, Dept. Integrative Biology, UC Berkeley

2009
Co-organizer for Univ. California, Berkeley & Univ. California, Davis Joint Neuroendocrine Lab Summit

2007, 2008
Volunteer, Co-coordinator for prospective graduate student visits, Dept. Integrative Biology, UC Berkeley

2007
Mentor to college student Emanuel Zamora, NSF Research Experience for Undergrads (REU), UC Berkeley

2006
Mentor to high school student Noel Cruz, Eastside Project (increasing diversity in academics), UC Berkeley

2006
Volunteer, Texas Science Olympiad (Grades 6-12): Genetics Section, Univ. Texas, Arlington

2005-2006
Graduate Student Senator for M.S. Biology program, Univ. Texas, Arlington

2005-2006
Co-Chair, Guest Speaker Committee, Phi Sigma Biology Honors Society, Univ. Texas, Arlington

2000-2001
President of Psi Chi (Psychology Honor Student Society), Boston College

Chapter 1

Lab and field experiments: Are they the same animal?

Lab and field experiments: Are they the same animal?

Abstract

To advance our understanding of biological processes we often plan our experiments based on published data. This can be confusing though, as data from experiments performed in a laboratory environment are sometimes different from, or completely opposite to, findings from similar experiments performed in the "real world". In this mini-review, we discuss instances where results from laboratory experiments differ as a result of laboratory housing conditions, and where they differ from results gathered in the field environment. Experiments involving endocrinology and behavior appear to be particularly susceptible to influence from the environment in which they are performed. As such, we have attempted to promote discussion of the influence of housing environment on the reproductive axis, circadian biology and behavior, immune function, stress biology, neuroplasticity and photoperiodism. For example, why should a rodent species be diurnal in one housing environment yet nocturnal in another? Are data that are gathered from experiments in the laboratory applicable to the field environment, and vice-versa? We hope not only to highlight the need for experiments in both lab and field when looking at complex biological systems, but also to promote frank discussion of discordant data. Perhaps, just as study of individual variation has been gaining momentum in recent years, data from variation between experimental arenas can provide us with novel lines of research.

The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact.
Thomas H. Huxley (1825 - 1895)

Introduction

The vast majority of experiments involving vertebrates are performed in a laboratory environment. The main reason behind this is straightforward: to control as many environmental variables as possible, facilitating interpretation of the resulting data. In general, this type of experiment tends to yield relatively "clean" data and allows us to investigate the effects of a specific manipulation. If, after careful statistical analysis, we can reject our initial null hypothesis, then we have a foundation upon which we can devise further experiments. Hopefully in time we are able to gain some insight into the biological workings of our model organism, whether we are studying behavioral, physiological or molecular mechanisms, or a combination of all three. What happens if we take our initial experiment out to the model organism's natural environment, where there are numerous uncontrollable physical and socio-environmental factors? We would hope that our initial findings are the same as those gathered in the laboratory, or at least leaning in the same direction. Often they are not. This review attempts to highlight a few of the many instances where results from laboratory experiments differ to some degree from their field counterparts (see Table 1 for examples of this).

Ricklefs and Wilkelski (2002) state that understanding life-history traits requires an understanding of how individuals respond to particular environments, and studies of such should, "integrate behavior and physiology within the environmental and demographic contexts of selection." In the same vein, "an animal that is not in its natural habitat is out of context…(animals) only make sense when they are living in the natural environments in which they evolved and to which they have become adapted," (Eric Pianka, *pers. comm*.).

Sometimes the differences in data from lab and field experiments are extreme; at other times they are more subtle and more easily overlooked/explained away. Whatever the degree of difference, each raises questions about how we should interpret lab data, and to what extent we should use them as generalizations about what happens in the real world. The reverse is also true: we might miss important factors in field experiments because of data "noise" from uncontrolled factors. This review is neither intended to be critical of either laboratory or field experiments, nor to give the impression that one type of experiment is more pertinent than another. Rather, we hope to highlight the need for experiments in both lab and field when looking at complex biological systems. We should perhaps view the laboratory and field experimentation arenas as being complementary to one another, rather than as independent entities.

Gonadotropins, gonadal and adrenal steroids

When an animal is taken out of its natural environment it is common to observe changes in its basic endocrine milieu. The endocrine system is a chemical communication network that has evolved to be sensitive to environmental perturbations. It is intended for the relay of external and internal messages to specific tissues and elicits appropriate chemical and behavioral responses. A change in an individual's immediate environment can thus induce a quantitatively or qualitatively different endocrine response. For example, free-living male kestrels (*Falco tinnunculus*) have 3-fold higher androgen concentrations during the breeding season than in captivity, while the gonadotropin luteinizing hormone (LH) during the winter is elevated in

captive males and females as compared to their free-living conspecifics (Meijer and Schwabl 1989).

Even changes from a natural to a captive diet can alter behavior and physiology. For instance, most lab chow for rodents contains soy. Soybean isoflavone extracts can affect growth and reproductive system development in growing rats (Guan et al. 2008). Phytoestrogens found in soy can also reduce female sexual behavior in rodents (Kudwa et al. 2007), as well as decrease cloacal protuberance growth rate in songbirds (cloacal protuberance is an androgen-dependent sperm storage and non-intromittent copulatory organ) (Corbitt et al., 2007).

What happens when a natural environment is simulated within a captive setting? Territorial interactions can be differentially androgen-dependent in wild and captive male dusky-footed woodrats (*Neotoma fuscipes*; Caldwell et al. 1984, Monaghan and Glickman 1992, Glickman and Caldwell 1994). In their natural habitat, castrated and intact male dusky-footed woodrats fought with equal intensity over self-built twig shelters, suggesting that territorial behavior in this species is independent of gonadal steroids. However, when offered a shelter in captivity, intact males fought more intensely than castrated males, demonstrating that territorial behavior may be dependent on gonadal steroids only in this type of artificial situation (Caldwell et al. 1984, Monaghan and Glickman 1992, Glickman and Caldwell 1994). The reasons for this differential response to gonadal steroids remain unclear.

Another important factor to consider is the influence of social interactions on the physiology and resulting behavior (or vice-versa) of an organism. Wingfield et al. (1990) proposed the Challenge Hypothesis which emphasized the impact of social stimuli on hormonal and behavioral responses. Simplified, the Challenge Hypothesis predicts that circulating testosterone should be higher in birds during periods of social instability, whether that instability is a result of a territorial intrusion or progression from one life-history stage to the next (i.e., transition from non-breeding to establishment of breeding territories). Initially over 20 species of birds were examined, encompassing six orders and 14 families, representing various mating systems and breeding strategies. Results generally demonstrated that testosterone concentrations were many times higher 1) in response to social instability, and 2) in free-living, breeding birds compared to those in captivity. Behaviors that are believed to lead to greater fitness - such as territory and mate acquisition and defense - are responsive to and themselves influence circulating androgen concentrations. These behaviors are either altered or cease completely when lab animals are maintained in solitary or artificial group sizes or sex ratios. For example, wild, paired male cowbirds (*Molothrus ater*) had higher concentrations of testosterone than unpaired males early in the season, with testosterone peaking during social hierarchy formation and mate acquisition (Dufty and Wingfield 1986a). However, paired males kept in captivity and exposed to long days had a more rapid increase and longer maintenance of testosterone, enhanced gonadal growth and longer maintenance of enhanced gonadal size than unpaired males, though maximal gonadal weights were lower than those of free-living males (Dufty and Wingfield 1986b).

Stress

Environmental factors and social interactions (or lack thereof) in captive versus wild settings can yield differences in the stress response. We define stress here as that which occurs in association with unpredictable and life-threatening perturbations in the environment and invokes an "emergency life-history stage" (Wingfield et al. 1998, Wingfield and Sapolsky 2003).

Glucocorticoids such as corticosterone (CORT) and cortisol, are steroid hormones commonly measured to quantify the stress response. Baseline and stress-induced plasma glucocorticoid concentrations can change when an organism is brought into a captive setting. In a study examining baseline CORT in wild and captive white-throated sparrows (*Zonotrichia albicollis*) and white-crowned sparrows (*Zonotrichia leucophrys*) during the non-breeding season, both species had baseline plasma CORT levels that were 2-3 times higher than wild birds, even after being held in captivity for 35 days to acclimate (Marra et al. 1995). Wild and captive brown-headed cowbirds do not differ in baseline CORT concentrations; however, birds housed in solitude as opposed to being housed with other conspecifics have elevated corticosterone levels, regardless of photoperiod (Dufty and Wingfield 1986b). In contrast, there are reports of corticosterone concentrations being higher in free-living birds (Romero and Wingfield 1998; Wingfield and Kitaysky 2002; Cyr and Romero 2008) and mammals (Kunzl and Sachser 1999). For example, Cyr and Romero (2008) found fecal glucocorticoid concentration differences between captive and wild female European starlings (*Sturnus vulgaris*) in response to stress. In this study, experimentally-induced chronic stress did not alter captive female fecal glucocorticoid concentrations during the day or night, though induced stress was associated with higher concentrations in the field. In sum, the social and physical environment can influence an organism's endocrine status. We consider that it is important to bear these findings in mind when interpreting results from organisms observed in either a lab or field environment.

Brain morphology and function

The environment in which an animal is raised and/or housed can affect brain morphology and function. Captive experimental manipulations of rodent housing environments have resulted in differences in brain morphology. Diamond et al. (1987) examined the effects of lab conditions on rodent brain by varying their captive environment, with treatments consisting of a standard lab environment, an enriched lab environment and a crowded, enriched lab environment. Crowding in rodents was already known to have deleterious effects, such as destructive behavior, retardation of sexual maturity and increased infant mortality (Calhoun 1973) which enrichment could abate, if not totally alleviate (Calhoun 1962, Christian and Davis 1964). Enrichment in the study by Diamond et al. (1987) included running wheels, ladders, tunnels, mazes and various other objects. Cortical thickness, which has been associated with certain enriched versus impoverished environmental conditions (Diamond et al. 1972, Diamond 1976, Diamond et al. 1976), was greater in the two enriched groups as compared to the standard control group. The authors posited that enrichment in a laboratory setting may mitigate the stress of crowding. Kempermann et al. (1997) later demonstrated that neurogenesis in the dentate gyrus of the rodent hippocampus can occur differentially according to laboratory environment. Mice housed in an enriched, or environmentally complex, laboratory environment had a greater abundance of dentate gyrus cells and a larger hippocampal granule cell layer than littermates housed in standard cages. Thus, enriched environments could promote survival of neural hippocampal precursor cell progeny, higher granule cell numbers and a larger hippocampal volume. The authors tested performance using a Morris water maze and found a significant enhanced performance in the enriched environment group, implying that the neural changes influence behavioral performance.

Gonadal steroid differences in wild versus captive animals may be the cause or result of changes in the brain. Seasonally-breeding vertebrates, for example, experience various

neurochemical and neuroanatomical changes according to the stage of their reproductive cycle. Nottebohm (1981) first noticed that certain brain regions involved in vocal control of male canaries, such as the HVC, robust nucleus of the arcopallium (RA), and Area X, are larger in the spring than in the fall. During the spring, the testes secrete higher amounts of androgens and males sing more frequently (Nottebohm and Nottebohm 1978). The elevation of testosterone induces rapid and sequential growth of the song control system (Tramontin et al. 2003). Seasonal volumetric growth of HVC largely involves increased recruitment of new neurons and their survival (Tramontin et al. 2000, Thompson et al. 2005). Increases in the volumes of RA and Area X involve increases in neuronal size and spacing, with no apparent change in neuron number. Both RA and Area X are efferent targets of HVC and depend upon HVC for trophic support (Nottebohm 1981). However, this volumetric growth of song nuclei may be affected by lab and field settings. For example, captive studies of the seasonally breading black-capped chickadee (*Poecile atricapillus*) revealed photoperiodic effects in HVC and Area X (MacDougall-Shackleton et al. 2003), but wild caught conspecifics did not exhibit such change (Smulders et al. 2006). Phillmore et al. (2006) found minimal to no seasonal differences in captive black-capped chickadee song control nuclei, although captive breeding birds did have slightly larger HVC and RA as a proportion of their telencephalic volume than non-breeding captive birds. Phillmore et al. (2006) and Smulders et al. (2006) suggest one possibility for this lack of nucleus volume change is that captive birds lack exposure to the year-round vocalizations and social interactions of their wild counterparts, although these hypotheses have yet to be tested.

Synaptogenesis within the song control system may result in the ability to learn new syllables each year and increase song repertoire. However, this effect of learning can be muted in captivity, possibly due to low levels of circulating androgens (Baker et al. 1984, Brenowitz et al., 1998). Captive animals are generally documented as having lower testosterone concentrations as compared to their wild-caught conspecifics (Wildt 1993, Wingfield 1980, Wingfield 1983, Wingfield and Farner 1980, Wingfield and Moore 1987). Baker et al. (1984) asked whether Nuttall's white-crowned sparrow (*Zonotrichia leucophrys nuttali*) exhibits volume increases in specific song control nuclei. Males were exposed to song in the wild when they were juveniles and then captured when they were 30 days old. Birds were then kept in captivity and housed under long days, in which they were exposed to recorded song, and short days, in which recorded song was not played. These captive birds gave evidence of seasonally fluctuating androgen levels in captivity, as seen from cloacal protuberance and testes weight, though hormone concentrations were not assayed. Histological examination revealed no seasonal change in the volumes of the song control nuclei HVC and RA (Baker et al. 1984). The authors claim their results to be consistent with the hypothesis that seasonal changes in volume of HVC and RA may be specifically related to the capacity to learn new syllables each breeding season (which Nuttall's white-crowned sparrows do not). However, when Brenowitz et al. (1998) examined the same subspecies of white-crowned sparrow in the wild, they found that birds caught in the spring had significantly greater HVC and RA volumes than birds caught in the fall. The results of the study by Baker et al. (1984) may have been a result of low circulating steroid levels in captive birds or differences in social cues (Tramontin et al. 1999). The fact that gonadal steroid-dependent secondary sexual characteristics such as cloacal protuberance were measurable implies to us that circulating gonadal steroids were at sufficiently high concentrations to cause neural changes. Thus, some aspect of captive housing is likely to have influenced volumetric changes of song control nuclei via some unknown mechanism.

Neuroanatomical changes in wild dark-eyed juncos (*Junco hyemalis*) were thought to also be androgen-dependent, but observations from birds in captive conditions suggest otherwise (Smulders et al. 2000). In the field, dark-eyed juncos treated with testosterone feed their young less often than sham-treated controls, sing more (Ketterson et al. 1992, Chandler et al. 1994) and have larger home ranges (Chandler et al. 1997). The hippocampus, which is involved in the processing spatial and relational information in birds and mammals (O'Keefe and Nadel 1978, Bingman 1990, Wood et al. 1999), was thus thought to be influenced by testosterone. In the lab, hippocampal volume was reduced compared to free-living birds but was, however, independent of testosterone treatment (Smulders et al. 2000). Smulders et al. (2000) propose that environmental stressors in captivity may have been attributed to increased corticosterone, which could decrease hippocampal volume. However, work by Klukowski et al. (1997) on captive juncos and by Schoech et al. (1999) on free-living juncos implies that captive juncos might have lower corticosterone concentrations than free-living juncos (but note that this is a result of inter-assay interpretation). Perhaps spatial and behavioral deprivation, which can alter brain function and morphology (Rosenzweig and Bennett 1996), may have influenced hippocampal volume changes. Smulders et al. (2000) conclude, "laboratory studies alone may not accurately reflect the actual neural processes that underlie behavior in the wild, and thus field studies may be indispensable in characterizing brain-behavior relations."

Circadian Rhythms

Circadian rhythms are endogenous physiological oscillations which occur within a period of approximately 24 hours and persist under constant conditions. In mammals, these rhythms are coordinated by the body's master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus and can be maintained by a combination of genetic, cellular, and neural regulatory mechanisms (Kriegsfeld and Silver 2006). Circadian rhythms, such as sleep/wake cycles and hormone production and secretion, are adaptive in that they aid in the synchronization of an organism's internal environment with its fluctuating external environment (Rusak and Zucker 1979). For example, DeCoursey et al. (2000) found that a significantly higher proportion of SCN-lesioned free-living eastern chipmunks (*Tamias striatus*) were killed by weasel predation as compared to surgical and intact controls. This is presumably an effect of temporally inappropriate activity, as chipmunks may have been more active when predators were present as a result of the loss of their SCN. Authors questioned if nighttime restlessness in SCN-lesioned chipmunks may have alerted predators to their whereabouts more easily than the behavior of controls, although this has yet to be tested fully.

Many times, mechanistic studies of circadian rhythms take place in controlled lab settings. Multiple reports in mammals suggest circadian rhythms can differ in captivity from those in a natural setting (Nevo et al. 1982, Labyak et al. 1997, Blachong et al. 1999, Rezende et al. 2003, Urrejola et al. 2005, Rattenborg et al. 2008, Lacey, *pers. comm.*). For example, naked mole rats (*Heterocephalus glaber*) exhibit short bursts of activity over a 24 h cycle in captivity compared to more temporally structured behavior in the field (Urrejola et al. 2005). One change in circadian rhythmic activity commonly reported is the shift from diurnal activity in the wild to nocturnal activity in captivity. Cururus (*Spalacopus cyanus*) are naturally diurnal (Reig 1970, Geiser et al. 2000, Rezende et al. 2003, Urrejola et al., 2005) but exhibit nocturnal or arrhythmic activities in captivity (Bengall et al. 2002, Rezende et al. 2003). Tuco-tucos (*Ctenomys sociabilis*) are also naturally diurnal (Lacey et al. 1997) but often become more nocturnal when

brought into a lab setting (Lacey et al. *pers. comm*.). Blanchong et al. (1999) report that unstriped nile rats - also named African grass rats - (*Arvicanthis niloticus*) exhibit diurnal activity in the field but are both diurnal and nocturnal in the lab. Nocturnal activity could be brought about by supplying a running wheel in the animal's enclosure as well as being dependent on parentage and sex. Degus (*Octodon degus*) also will switch from diurnal activity to nocturnal activity when supplied a running wheel in the lab (Kas and Edgar 1998, 1999). This species exhibits crepuscular behavior in the field (Fulk 1976, Labyak et al. 1997, Kenagy 2002) but diurnal, nocturnal or crepuscular activity in captivity (Labyak et al. 1997), a pattern also found in blind mole rats (*Spalax ehrenbergi*; Nevo et al. 1982, Ben-Shlomo et al. 1995). More recently, Rattenborg et al. (2008) reported that brown-throated three-toed sloths (*Bradypus variegatus*) living in tropical rainforest canopies sleep about six hours less than their captive conspecifics. These examples raise the question of how circadian rhythms can be influenced by the housing environment. What influence do "abnormal" social cues have on patterns of rhythmicity? How can something as alien to a rodent's natural environment as a running-wheel shift something as apparently hard-wired as circadian rhythmicity? Perhaps circadian rhythms are more plastic than has been considered. Are some of the phenomena that can be observed in the lab under specific conditions (e.g., "splitting" of circadian rhythms into 2 activity bouts per 24h cycle under constant light in some species) simply a representation of the plastic and adaptable nature of the underlying rhythm? These examples also raise the question of whether commonly-used lab strains of rodents would even exhibit the same rhythmic behaviors in the wild as in the lab. All these are intriguing questions that are waiting to be tested.

Photoperiodic responsiveness

Many mammalian species are photoperiodic. That is, their reproductive system responds to changing photoperiod so as to time parturition to the time of year most conducive to raising young (Baker 1938). Over 30 rodent species are characterized as "long-day" breeders (Prendergast et al. 2001). During exposure to short, winter-like day lengths, an increased duration of the nocturnally-secreted pineal hormone melatonin causes a suite of physiological changes associated with reproductive quiescence. There is a decrease in synthesis and release of the neuropeptide gonadotropin-releasing hormone (GnRH), with consequent reductions in circulating gonadotropic hormones (luteinizing hormone, LH; follicle-stimulating hormone, FSH), gonadal regression and reduction in sex steroids and reproductive behaviors (see Goldman and Nelson, 1993 for review, Prendergast et al. 2001). For some time in mammalian photoperiodic research, a dogma stood that the costs of breeding at other times of year were too great for this strategy to be maintained over evolutionary time. Despite the dogma, in many laboratory experiments involving photoperiodic rodents such as microtine voles, mice (*Peromyscus* spp.) and Siberian hamsters (*Phodopus sungorus*), subsets of the experimental populations did not regress their reproductive systems under short, winter-like photoperiods (see Prendergast et al., 2001 for review). This phenomenon was generally considered to be a lab artifact, and thought most likely to be a result of constant food availability, or generally "less harsh" conditions in the laboratory compared to the animals' natural setting (see Nelson 1987). As a result, the so-called "non-responders" to changing photoperiod were identified as animals with gonad sizes that fell outside 2 standard deviations above the mean (during exposure to short photoperiods), and excluded from data analysis. Sample-sizes in experiments were increased to account for this phenomenon, although approximately 30% of individuals in some of these

species can now be classified as photoperiodic non –responders (Hoffman 1978, Puchalski and Lynch 1986, Prendergast et al. 2000). Eventually, Lynch and colleagues clearly demonstrated that photoperiodic non-responsiveness was heritable in Siberian hamsters (Lynch et al. 1989; Klyman and Lynch, 1992). Thus it is likely that 30% of individuals in wild populations of these species retain the ability to breed year-round, regardless of photoperiod. However, as Prendergast et al. (2000) state in their review, the highly inbred laboratory animals of these species might be, "a very peculiar animal model that may be different from the same species living in the wild". The fitness payoffs of photoperiodic non-responsiveness in the wild have yet to be determined, and of course these benefits will vary according to the prevailing environmental conditions other than photoperiod (Prendergast et al. 2001).

Even within a laboratory environment, the individual housing conditions can affect the reproductive response to photoperiod or even melatonin treatment (melatonin is the pineal hormone which is secreted at night and which, in photoperiodic mammals, is responsible for gonadal regression during short photoperiods via an unknown link to the GnRH system). Traditionally, rodents have been supplied with exercise wheels within their cages as a form of environmental enrichment, or as a way of measuring nocturnal activity levels via electrical contacts between the rotating wheel and the stationary cage. In golden hamsters, wheel-running prevents melatonin-induced gonadal regression (Pieper et al., 1988) and reverses short-day-induced anestrus (Borer et al. 1983), most likely via increased gonadotropin secretion (Pieper et al. 1995). In contrast, the absence of exercise wheel-running can also cause a loss of photoperiodic responsiveness in a PNRj line of Siberian hamsters (Freeman & Goldman 1997). Thus it is possible that somewhat unexpected factors can influence the expression of physiological and behavioral traits in a given environment. How the brain integrates the seemingly disparate stimuli of exercise and ambient photoperiod to influence reproductive status in a non-intuitive direction (exercise stimulating the reproductive axis, for example) is a matter for debate.

Many avian species are also photoperiodic. It is noteworthy that males of strictly photoperiodic wild bird species tend to exhibit much more uniform gonadal responses to changing day length than the mammalian species already discussed, both in the laboratory and the wild. A possible reason for this uniform response in males is that the costs of maintaining an active reproductive system year-round are simply too high for this strategy to remain viable within a population. Females of these species often show a muted gonadal response to changing photoperiod in lab conditions. Presumably this is because they do not receive sufficient supplementary information and/or fulfillment of their dietary requirements (Wingfield et al. 1990). Ovarian and follicular development in captive European starlings (*Sturnus vulgaris*) (Dawson 1997) and several sparrow species (*Zonotrichia* spp) (King et al. 1966., Wingfield 1984b, Wingfield and Farner 1993, Dawson 2001) is often only 20-30% of its maximum in the wild in response to identical day lengths. Thus, at the gonad level, one might say that 50% of the population (females) shows a dramatically muted response to changing photoperiod in the laboratory. Having said that, many of the other physiological changes associated with the photoperiodic reproductive response to changing photoperiod do occur in lab-housed females, and these are often reduced to a lesser degree than ovarian development. These include changes in the gonadotropin-releasing hormone system, changes in circulating hormones such as luteinizing hormone, testosterone and prolactin, and feather molt at the termination of the reproductive period (Dawson 1997, Bentley et al. 2000). Thus in wild birds, the underlying neurobiology and physiology of reproductive responsiveness to photoperiod is similar to that

seen in the lab (see Leitner et al. 2003), but some aspect of the laboratory environment prevents full expression of the response at the level of the gonads. As a result, females housed in a laboratory environment often will not exhibit behaviors that depend upon high concentrations of circulating gonadal steroids. An example of this lack of steroid-dependent behavior in the laboratory is the copulation solicitation display which females usually only will exhibit in captivity if their circulating estradiol is supplemented with a subcutaneous implant (Moore 1983, Maney et al, 1997). It may be that female expression of the full suite of endocrine responses to photoperiod depends on the birds' prior experience. Baptista and Petrinovich (1986) describe an experiment on female white-crowned sparrows which were collected as nestlings in the wild and hand-reared in the laboratory. They readily ovulated in captivity when housed in a situation under which ovulation had never been reported in wild-caught adult females. They suggest that young female white-crowned sparrows undergo an imprinting process during their first year in terms of what stimuli supplementary to photoperiod are required for ovulation. When hand-reared in captivity they "imprinted" on substitute supplementary stimuli and therefore would ovulate under these conditions. Kern (1971) studied morphological differences between ovaries from free-living and captive white-crowned sparrows. This author's main findings were that the ovaries of captive birds lack pre- and postovulatory follicles; they contain more atresias; the stroma is dense and poorly vascularized year-round; the membrana granulosa of developing follicles becomes proliferative earlier in the spring and is always less active mitotically than in wild birds; thecae do not develop beyond formative stages; thecal gland cells become abundant earlier in spring and stromal gland cells are less numerous. Kern concluded that many of these differences suggest that white-crowned sparrows do not breed in captivity because of an abnormal synthesis and/or secretion of hypophysial gonadotropin. How many environmental cues are involved in the final maturation of the ovaries and how the brain integrates all of these cues into an endocrine response is not known, nor is how early imprinting might influence the interpretation of a laboratory versus field environment.

Despite the tenet that even if the gonadal response is muted in the laboratory, but the underlying neurobiology and physiology is relatively unchanged as compared to the wild, domestication of some bird species appears to have reduced the response to changing day length, even at the level of the brain. Canaries (*Serinus canaria*) in the wild exhibit robust photoperiodic responses (Leitner et al. 2003), as do many of the domesticated strains. However some domesticated strains appear to have lost the ability (or need) to alter their reproductive status according to the prevailing photoperiod, even at the level of the gonadotropin-releasing hormone (GnRH) system (Bentley et al., 2000).

Perfito et al. (2008) considered cues other than solely photoperiod as sources for reproductive timing. The opportunistically breeding Lesser Sundas zebra finch (*Taeniopygia guttata guttata*) lives in an Australian environment that experiences unpredictable pulses in food. This species can breed year round, both in the lab and the field. Using a semi-domesticated strain of males to examine whether long days or food availability was the prevailing factor or stimulate gonadal growth, Perfito et al. (2008) found that extended feeding times or increased food abundance during long days, not just the long days themselves, is the driving permissive cue for gonadal growth. These results beg the question of what cues are needed in a captive environment, be it photoperiod, food availability, etc., to truly mimic an organism's natural environment? If studying reproductive timing in Lesser Sundas zebra finches, we now know it is incorrect to assume photoperiodic treatments in a captive setting completely mimic reproductive cues in the wild (Perfito et al. 2008), thus permitting a more revealing analysis of reproductive

mechanisms in this opportunistically breeding species. Another example showing how possible factors that can influence results and their interpretation can be found in Table 2.

Immune Function

Variation in environmental conditions can accompany variation in immunocompetence. Factors such as photoperiod, temperature, food availability and social environment may create energetic tradeoffs in order to maintain critical physiological processes. For example, immune function may become enhanced during winter months to aid in survival during climatic and nutritionally harsh times (Sinclair and Lochmiller 2000). It may also become enhanced because a long-day breeder does not have reproductive energetic requirements in the short days of winter and thus can allocate more energy towards others physiological processes such as immune function (Greenman et al. 2005). Social environment can also affect immune function: Weil et al. (2007) reported that housing adult male Siberian hamsters (*Phodopus sungorus*) with ovariectomized females prevented short-day enhancement of delayed-type hypersensitivity immune responses, independently of photoperiod-mediated reproductive changes (Weil et al. 2007). How the social environment is translated into an immune signal is not known.

In a controlled lab setting, enriched environments are positively associated with immunocompetence (Diamond 1964, 1967; Rosenzweig 1966). Mlynarik et al. (2004) gave captive male juvenile Wistar rats an immune challenge (*Escherichia coli* lipopolysaccharide in increasing amounts) which resulted in high levels of corticosterone and decreases in body weight. However, rats housed in enriched conditions had low levels of corticosterone and maintained body weight, despite the immune challenge. In a related study by Kozlowski et al. (2004), Sprague-Dawley rats raised in enriched conditions and then given a controlled cortical impact suffered greater neurodegeneration and deficit in their forelimb use than controls raised in standard environments. However, the enriched group demonstrated an increased capacity for recovery. Studies such as these emphasize the need to be cautious when interpreting results for clinical use, as results vary depending on the captive lab conditions.

Given the topics already covered in this mini-review, it is of no surprise that the immunocompetence of captive organisms can also differ from their free-living counterparts. The evolution of host resistance is speculated to, in part, be the result of a continuous arms race between pathogens and their hosts (Hamilton and Zuk 1982). Secondary sexual characteristics can be costly to maintain and thus can serve as an honest signal of quality. Testosterone, which can mediate these secondary sexual characteristics, often has a negative correlation with aspects of immune function (Folstad and Karter 1992). Hence, a trade-off may exist between immunocompetence and the expression of elaborate traits. To test, Peters (2000) examined the primary antibody response of captive control and testosterone implanted male superb fairy-wrens (*Malurus cyaneus*) to sheep red blood cell (SRBC) immunizations. Peters (2000) also examined a cross-section of free-living males with elevated or basal testosterone, as determined by their plumage phase. Captive males implanted with testosterone had a decreased SRBC antibody response, which in itself lends support to the immunocompetence handicap hypothesis. However, free-living males with naturally elevated testosterone had an increased likelihood to respond to SRBCs than males with basal testosterone concentrations, which is contrary to Folstad and Karter's (1992) hypothesis. The free living males of this study had undergone early molting and nuptial plumage development, which is associated with an increase in testosterone concentrations. Early molting and high testosterone may be a sign of high quality and good

endurance, which females show preferences for (Dunn and Cockburn 1999). Peters (2000) posits that these particular wild males exhibited an increased SRBC response because they were of high quality beforehand. While this hypothesis may be the reason behind this discrepancy, we must also consider the possible social and environmental factors denied to captive animals which may play a role in their immunocompetence.

Summary

It seems clear that experimental arena and design can influence the outcome of certain experiments. We expect that there are equally as many examples of experiments that are repeatable in the lab and the wild, although it is not the goal of this review to highlight such studies. Context-dependent rejection of null hypotheses might well influence the direction of research programs, and just as lines of research that are not applicable to an organism's natural environment might be developed and sustained as a result of housing context, opportunities for investigating behavioral or physiological phenomena in the wild might be missed. We highlighted only a small subset of examples to emphasize the need for experiments in both lab and field when looking at complex biological systems, or at least caution when interpreting a study's results. Pros and cons exist for both lab and field experiments (Figure 3), therefore understanding the subject or behavior in question may be best attempted by a combination of both settings. By comprehending how and why these various results occur may lead to an even greater understanding of the question at hand. Of course, specific phenomena might have no relation to what occurs in an animal's natural setting but may help us elucidate mechanisms of physiology and behavior. As such, lab models remain very powerful tools for investigation and in no way do we wish to imply otherwise.

Semi-natural environmental settings might be the one of the best ways to study an organism's physiology and behavior. This type of experimental arena allows an animal to remain in and be exposed to its natural setting, yet still allows for some control by the researcher. To deprive an animal of its ecology may deprive the researcher of a true result, or introduce an artefactual one. It is difficult, if not mostly impossible, to control various confounding social and environmental factors, therefore we must remain ever cognizant of these issues and open-minded when designing experiments and interpreting their outcomes. As mentioned in the Introduction, we assume that there are many more examples of discrepancies between lab and field data that have not been published, simply because investigators find them hard to explain. Perhaps, just as study of individual variation has been gaining momentum in recent years, data from variation between experimental arenas can provide us with unprecedented lines of research.

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Table 1. Examples of result discrepancies between data collected from wild animals in the field and captive animals in the lab.

Gonadotropins, gonadal and adrenal steroids		
Species	Field data	Captive data
kestrels (*Falco tinnunculus*)	Male androgens higher during breeding season Male and female LH lower in winter (Meijer and Schwabl 1989)	Male androgens lower during breeding season Male and female LH higher in winter (Meijer and Schwabl 1989)
dusky-footed woodrats (*Neotoma fuscipes*)	Castrated and intact males fight over shelters with equal intensity Territorial behavior may be androgen independent (Caldwell et al., 1984, Monaghan and Glickman 1992, Glickman and Caldwell 1994)	Intact males fight more intensely over shelters than castrated males Territorial behavior may be androgen dependent (Caldwell et al., 1984, Monaghan and Glickman 1992, Glickman and Caldwell 1994)
20 species of birds (6 orders, 14 families)	T higher in response to social instability in breeding birds (Wingfield et al. 1990)	T lower in response to social instability in breeding birds (Wingfield et al. 1990)
cowbirds (*Molothrus ater*)	Paired male T higher early in breeding season Male T peaks during social hierarchy formation and mate aquisition (Dufty and Wingfield 1986a)	Paired males had more rapid T increase and longer maintenance, enhanced gonad growth and size maintenance than unpaired Maximal gonadal weights less than wild males Males housed in solitude had higher CORT than those housed with conspecifics (Dufty and Wingfield 1986b)
white-throated sparrows (*Zonotrichia albicollis*)	Baseline CORT lower in non-breeding season (Marra et al. 1995)	Baseline CORT higher in non-breeding season (Marra et al. 1995)
white-throated sparrows (*Zonotrichia leucophrys*)	Baseline CORT lower in non-breeding season (Marra et al. 1995)	Baseline CORT higher in non-breeding season (Marra et al. 1995)
European starling (*Sturnus vulgaris*)	Female fecal glucocorticoids altered by induced stress (Cyr and Romero 2008)	Female fecal glucocorticoids not altered by induced stress (Cyr and Romero 2008)
Brain morphology and function		
Species	Field data	Captive data
black-capped chickadee (*Poecile atricapillus*)	No photoperiodic effects in HVc and Area X (Smulders et al. 2006)	Photoperiodic effects in HVc and Area X (MacDougall-Shackleton et al. 2003) Minimal to no seasonal differences in song nuclei, though breeding birds had slightly larger HVc and RA as a proportion of their telencephalonic volume than non-breeding birds (Phillmore et al. 2006)
Nuttall's white-crowned sparrow (*Zonotrichia leucophrys nuttali*)	Greater HVc and RA volumes in spring than fall (Brenowitz et al. 1998)	No seasonal change in HVc or RA in wild males captured at 30 days and put in captivity (Baker et al. 1984)

dark-eyed juncos (*Junco hyemalis*)	Hippocampal volume larger than captive birds, independent of T treatment (Smulders et al. 2000)	Hippocampal volume smaller than wild birds, independent of T treatment (Smulders et al. 2000)
Long-Evans rats		Cortical thickness was greater in those housed in enriched environments versus standard (Diamond et al. 1987)
Harlan Sprague Dawley mice		Dentate gyrus cells more abundant and hippocampal granule cell layer larger if housed in enriched environment versus standard (Kempermann et al. 1997)
Photoperiodic responsiveness		
Species	Field data	Captive data
golden hamsters (*Mesocricetus auratus*)		Melatonin dependent gonadal regression under short photoperiod (Reiter 1980, Goldman and Darrow 1983) Wheel-running prevents melatonin-induced gonadal regression and reverses short-day anestrus (Borer et al. 1983, Pieper et al. 1988)
canary (*Serinus canaria*)	Robust photoperiodic responses (Leitner et al. 2003)	Photoperiod non-responsiveness in some domesticated strains (Bentley et al. 2000)
Circadian rhythms		
Species	Field data	Captive data
naked mole rat (*Heterocephalus glaber*)	Temproally structured activity (Urrejola et al. 2005)	Short bursts of activity over a 24 h cycle (Urrejola et al. 2005)
cururus (*Spalacopus cyanus*)	Diurnal (Reig 1970, Rezende et al. 2003, Urrejola et al. 2005)	Nocturnal or arrhythmic activities (Bengall et al. 2002, Rezende et al. 2003)
tucos (*Ctenomys sociabilis*)	Diurnal (Lacey et al. 1997)	Nocturnal (Lacey, pers comm.)
unstriped nile rats (*Arvicanthis niloticus*)	Diurnal (Blanchong et al. 1999)	Diurnal, but nocturnal if supplied running wheel and dependent on parentage and sex (Blanchong et al. 1999)
brown-throated three-toed sloths (Bradypus variegatus)	Sleep 9.63 hours per day (Rattenborg et al. 2008)	Sleep 15.85 hours per day (Galvão deMoura Filho et al. 1983)
degus (*Octodon degus*)	Crepuscular (Fulk 1976, Labyak et al. 1997, Kenagy 2002)	Diurnal, nocturnal, crepuscular. Will alter diurnal activity if supplied running wheel. (Labyak et al. 1997, Kas and Edgar 1998, 1999)
Immune function		
Species	Field data	Captive data
Wistar rats		High CORT and decrease in body weight in juveniles given immune challenge; CORT low and body weight maintained when housed in enriched environment (Mlynarik et al. 2004)
Sprague-Dawley rats		Greater neurodegeneration and forelimb use deficit after cortical impact, though increased recovery capacity, in rats raised in enriched environment compared to controls (Zoklowski et al. 2004)

superb fairy-wrens (*Malurus cyaneus*)	Greater immune response in males with naturally high T than males with basal T concen. (Peters 2000)	Decreased immune response in males with T implants as compared to controls (Peters 2000)

Figure 1: An example of possible factors that can influence results and their interpretation in lab and field experiments. While a lab setting may yield cleaner data, a field setting may yield more biologically relevant data. Both testing realms can reveal important separate though relatively equal aspects. Thus, both can bring about a more comprehensive understanding of the question at hand.

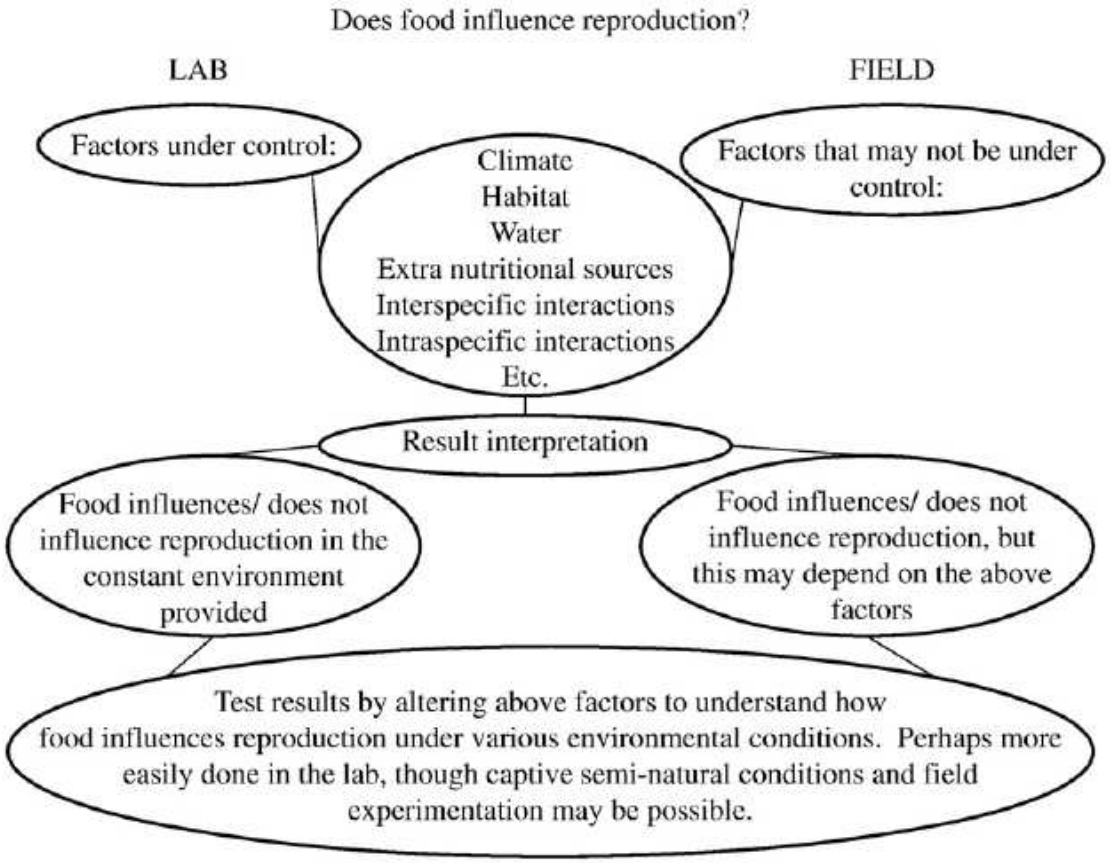

Figure 2. Pros and cons of experimenting in the lab, field or semi-natural environment.

Pros and cons of different experimental arenas

	Pros	Cons
Lab:	controlled setting allows for clearer understanding of direct cause and effect	biological and physiological response in an unnatural setting may not be biologically relevant to organism
Field:	natural biological and physiological responses occur	difficult to understand direct cause and effect in an uncontrolled setting due to complex environmental interactions
Semi-natural:	semi-controlled setting, such as an outdoor, size-appropriate enclosure in the organism's natural environment, may yield natural biological and physiological responses; social, nutritional, etc. factors can be mostly controlled	some factors, like climate, light, noise, etc. may still confound results and hinder clear interpretations

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Chapter 2

Seasonal differences in hypothalamic EGR-1 and GnIH expression following capture-handling stress in House sparrows (*Passer domesticus*)

Seasonal differences in hypothalamic EGR-1 and GnIH expression following capture-handling stress in House sparrows (*Passer domesticus*)

Abstract

Stress is a known inhibitor of reproductive function. The mechanisms by which stress acts to influence the reproductive axis have been intensely studied and appear to be extremely varied. Gonadotropin-releasing hormone (GnRH) is a critical component of the vertebrate reproductive axis and directly causes pituitary gonadotropin synthesis and release. A second neuropeptide, gonadotropin-inhibitory hormone (GnIH), directly inhibits pituitary gonadotropin synthesis and release in birds. We hypothesized that stress effects upon reproduction are mediated via the hypothalamic GnIH system. We examined the effects of capture-handling stress in the hypothalamus of male and female adult house sparrows (*Passer domesticus*) at the start (spring) and end of the breeding season (fall). We quantified numbers of GnIH neurons to provide an estimate of hypothalamic GnIH content. In addition, we quantified the expression of the protein product of the immediate-early gene, *EGR-1*, using this as an indicator of neuronal activation. We saw an increase in EGR-1 positive cells in the paraventricular nuclei of stressed birds as opposed to controls at both collecting times, but this stress response was more apparent in the spring as opposed to the fall. There were more GnIH-positive neurons in fall birds versus those sampled in the spring, and a significant increase in GnIH positive neurons was seen in stressed birds only in spring. GnIH cells show little to no activation of EGR-1, suggesting that EGR-1 is not involved in GnIH transcription in response to capture-handling stress. These data imply an influence of stress upon the paraventricular nucleus and the GnIH system that changes over the annual cycle of reproduction.

Introduction

Stress can have negative impact upon the reproductive system (Siegel, 1980; Moberg, 1991; Moore and Jessop, 2003; Boonstra, 2004). Our definition of stress here is in line with that used by John Wingfield and colleagues: that which occurs in association with unpredictable and life-threatening perturbations in the environment and invokes an "emergency life-history stage" (Wingfield et al., 1998; Wingfield and Sapolsky, 2003). Demands associated with specific stages of the predictable life-history cycle (as per Jacobs and Wingfield, 2000) are also considered stressful by some, but it is perhaps more useful to consider these demands as more predictable physiological demands, and thus not "stressful" per se. The concepts of allostasis and allostatic load permit differentiation between physiological states in the contexts of predictable and unpredictable energetic and behavioral demands (Sterling and Eyer, 1988; McEwen, 2002; McEwen and Wingfield, 2003). For passerine birds, perturbations such as capture-handling can induce a massive stress response that results from apparent perception of a life-threatening situation (Wingfield et al., 1992). In this paper we provide evidence in birds that stress can activate gonadotropin-inhibitory hormone (GnIH), a neuropeptide system that inhibits reproductive physiology and behavior in birds and mammals. Thus, we propose that stress-induced inhibition of reproduction might, to some degree, be mediated via the GnIH system.

For all vertebrates studied, reproduction is regulated by gonadotropin-releasing hormone (GnRH) of one form or another. GnRH is released from neurons in the preoptic area of the hypothalamus to the median eminence, where it causes the anterior pituitary gland to release the gonadotropic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream. LH and FSH act on the gonads and cause them to develop and increase in activity.

Gonadotropin-inhibitory hormone (GnIH) is a neuropeptide that inhibits gonadotropin release from cultured quail anterior pituitary (Tsutsui et al., 2000). GnIH inhibits pituitary gonadotropin release in vitro (Tsutsui et al., 2000; Ciccone et al 2004), and in vivo in birds (Osugi et al., 2004; Bentley et al., 2006a; Ubuka et al., 2006) and mammals (Kriegsfeld et al., 2006; Johnson et al., 2007). GnIH also inhibits gonadotropin synthesis in vivo in birds (Ubuka et al., 2006), and GnIH and its mammalian homolog inhibit reproductive behaviors in birds and mammals (Bentley et al., 2006b; Johnson et al., 2007).

GnIH producing neurons are located in the paraventricular nucleus (PVN) of birds and dorsomedial hypothalamus (DMH) of rodents and appear to make direct contact with GnRH-I producing neurons in birds and mammals (Bentley et al., 2003; Bentley et al., 2006a,b; Kriegsfeld et al., 2006; Ubuka et al., 2008). Furthermore, GnIH receptor mRNA is expressed in GnRH-I and –II neurons (Ubuka et al., 2008). Thus there is potential for GnIH to influence the activity of GnRH neurons directly, in addition to the action of GnIH upon pituitary gonadotropin release. Significantly for the study of stress and its role in regulating reproduction, GnIH-producing neurons in rats also express glucocorticoid receptors (Kirby et al., 2007). This suggests that the glucocorticoid stress response could be directly communicated to GnIH cells and from there to the reproductive axis, given the neural architecture of the GnIH/GnRH systems.

Physiologically, a stress response generally involves an increase in adrenal glucocorticoid secretion, which can reduce activity of the hypothalamo-pituitary-gonadal (HPG) axis (Moberg, 1985; Sapolsky, 1987; Sapolsky, 1992; Romero and Sapolsky, 1996; Wingfield and Romero, 2000; Wingfield and Sapolsky, 2003). The action of corticotropin releasing hormone (CRH) and

glucocorticoid feedback on the hypothalamus may thus inhibit GnRH-I release (Williams et al., 1990).

The glucocorticoid stress response varies seasonally in many species (birds: Wingfield, 1994; Breuner and Orchinik, 2001; Romero, 2002; Romero et al., 2006; mammals: Kenagy and Place, 2000; herpetofauna: Licht et al., 1983; Smith and John-Alder, 1999). We used a seasonally breeding songbird species, house sparrow (*Passer domesticus*), to examine GnIH at both the beginning (spring) and end (fall) of the breeding season in association with a capture-handling stress response. A capture-handling stress paradigm is known to elevate glucocorticoid levels in many species, including house sparrows (Wingfield et al., 1992; Wingfield, 1994; Breuner et al., 1999; Wingfield and Romero, 2001). Therefore we further assessed the physiological effects of this manipulation by evaluating the neuronal response within the PVN by measuring the expression of the protein product of the immediate early gene *EGR-1*. Activation of immediate-early genes, such as *EGR-1*, is thought to be one of the first measurable neurobiological responses to external stimuli. As such, immediate-early genes are excellent markers of neuronal activity in response to environmental input (Morgan and Curran, 1989, 1995). Therefore we measured EGR-1 positive cells in the PVN, the only area in the avian brain known to contain GnIH positive neurons, as a measure of that area's response of stress. Given our hypothesis that GnIH is involved in mediation of stress-induced reproductive dysfunction, we predicted that GnIH-producing neurons would be more abundant in stressed animals. Due to seasonal differences in the stress response as well as possible seasonal differences in the presence and role of GnIH (as per Bentley et al., 2003), we predicted a difference in the response of GnIH-producing neurons to stress at the beginning versus the end of the breeding season.

Materials and methods

Animals

Adult male and female house sparrows (*Passer domesticus*) were collected in 2007 in Lodi, California using Japanese mist nets. Birds were sampled during the second week of February (9 males: 6 females) and the last week of July though the first week of August, just prior to molting, (10 males: 7 females), which are the beginning and end of the northern California house sparrow breeding season, respectively (Keck, 1934; Calisi and Bentley, 2007 pers. obs.). Birds were transferred to aviaries and kept under ambient photoperiod for one week before sampling. Using a common capture-handling stress paradigm known to elevate glucocorticoid levels (Wingfield, 1994; Breuner et al., 1999; Wingfield and Romero, 2001), birds were held in small ventilated bags for one hour prior to killing by decapitation (spring: 4 males, 2 females; fall: 4 males, 3 females). Controls were killed within 10 min of researchers entering the aviary in which they were housed (spring: 5 males, 4 females; fall: 6 males, 4 females). After decapitation, brains were fixed in 4% paraformaldehyde and sucrose solutions and later stored in -80ºC until sectioning. Although house sparrows generally do not show sex differences in the glucocorticoid response (Bruener and Orchinik, 2001), we tested for sex differences in both EGR-1 and GnIH cell abundance in control and stressed animals. All procedures were approved by and in compliance with the University of California Office of Lab Animal Care and federal regulations.

Immunocytochemistry

EGR-1. Brains were sectioned coronally at 40μm using a cryostat and collected into phosphate buffered saline (PBS). Every fourth section throughout the hypothalamus was collected for immunocytochemistry. Sections were washed three times in PBS and treated with .01% hydrogen peroxide in methanol for 10 minutes to reduce background immunoreactivity. Sections were again washed three times with PBS and then submerged in 5% normal goat serum (NGS) in 0.2% PBS-T (PBS + Triton X-100) for one hour to block background immunoreactivity. EGR-1 primary antibody (code sc189, Santa Cruz Biotechnology Inc.) was used to incubate sections for 48 hours at a concentration of 1:1000 in 0.2% PBS-T. Three subsequent washes in 0.2% PBS-T were followed by an hour of incubation in biotinylated goat anti-rabbit IgG (1:250 in 0.2% PBS-T), followed by three more washes of 0.2% PBS-T. Sections were incubated in avidin-biotin complex (ABC; Vectastain Elite Kit, Vector Labs) for one hour and visualized in 0.03% 3,3-diaminobenzidine (DAB) intensified with 0.15% nickel chloride for five minutes.

GnIH. Directly following EGR-1 visualization, sections were washed five times in 0.2% PBS-T. Goat anti-rabbit affinity-purified GnIH primary antibody (code PAC 123/124) was used to incubate sections for 24 hours at a concentration of 1:5000 in 0.2% PBS-T. Three subsequent washes in 0.2% PBS-T were followed by an hour of incubation in Alexa Fluor 568 (Invitrogen Labs) at a concentration of 1:500 in 0.2% PBS-T for visualization.

Quantification of EGR-1 and GnIH Immunoreactivity

Using a Zeiss Axio Imager A1 microscope and AxioVision 4.5 software, photographs were taken of the PVN, which was defined by the presence of GnIH-immunoreactive (GnIH-ir) neurons. We counted the total number of EGR-1-ir nuclei within the field of vision of the GnIH-ir cell bodies and the total number of GnIH-ir cell bodies throughout the PVN. We noted any colocalization between EGR-1 nuclei and GnIH neurons. The average number of cells per section was used for analysis.

Statistical Analysis

An analysis of variance (ANOVA) was performed on the response variables of EGR-1 and GnIH positive cells, with sex, breeding season and stress response as explanatory variables. Significance was determined based on sequential Bonferroni adjustments using the Dunn-Sidák method. Independent t-tests were then preformed to examine breeding season inter- and intra-differences in the EGR-1 and GnIH stress response. Because there was no effect of sex, male and female data were combined for analysis. Significance, after Bonferroni adjustment, was determined at $P < 0.0125$.

Results

EGR-1 *response to stress*. The ANOVA on EGR-1 showed a significant effect of breeding season ($F_{1,24} = 12.96$, $P = 0.001$) and stress ($F_{1,24} = 24.83$, $P < 0.001$). The individual t-tests showed an increase in EGR-1 positive cells in stressed birds as opposed to controls (spring: $P = 0.002$; fall: $P = 0.002$), and stressed birds had higher EGR-1 positive neuron numbers in the spring compared to the fall ($P < 0.001$). EGR-1 showed minimal co-localization with GnIH positive neurons.

GnIH response to stress. The ANOVA on GnIH showed a significant effect of breeding season ($F_{1,24} = 10.04$, $P = 0.004$), but not stress, most likely due to a difference in the GnIH stress response seen during the spring versus the fall. A significant increase in GnIH positive neurons was seen in stressed birds only in the spring ($P = 0.007$) as compared to the fall, and GnIH positive neuron numbers in controls were higher in birds in the fall as compared to the spring ($P = 0.001$). See Table 1 for complete statistical results, figures 1 and 2 for EGR-1 and GnIH cell number means and standard errors, respectively, and figure 3 for representative photographs.

Discussion

There is strong evidence that GnIH acts in direct opposition to GnRH and it is thus a key regulator of the reproductive axis and associated behaviors. We hypothesized that the inhibitory effects of stress on reproduction may be mediated via the GnIH system. Because physiological responses to stress can vary seasonally (Wingfield et al., 1982; Wingfield et al., 1992; Astheimer et al., 1994; Wingfield et al., 1994; Romero et al., 1997; Romero and Remage-Healey, 2000), we examined the stress response both at the start and termination of breeding in a seasonally breeding songbird. Elevated levels of glucocorticoids often occur in stressed and control animals during breeding as compared to non-breeding time-periods (Dawson and Howe, 1983; Romero et al., 1998 a,b,c; Breuner and Orchinik, 2001; Romero, 2002; but see Breuner et al., 1999; Romero and Remage-Healy, 2000; Romero, 2002). We localized and quantified neurons expressing the protein product of *EGR-1* to assess the cellular response of the PVN to capture-handling stress. In addition, we measured the numbers of GnIH-ir neurons in control and stressed birds in spring and fall.

EGR-1 response to stress. EGR-1 positive cells were more abundant in stressed birds compared to controls, independent of sampling time. Cell numbers were not significantly different in control animals at the start or at the end of the breeding season, but stressed animals at the start of the season possessed a significantly higher number of EGR-1- positive cells as opposed to stressed animals at the end of the breeding season. A capture-handling stress paradigm is known to elevate glucocorticoid levels in house sparrows and other species (Wingfield, 1994; Breuner et al., 1999; Wingfield and Romero, 2001), many–fold, with baseline and stress response glucocorticoid levels being significantly higher in breeding versus non-breeding animals (Dawson and Howe, 1983; Romero et al., 1998 a,b,c; Breuner and Orchinik, 2001; Romero, 2002). Our EGR-1 data confirm this stress response pattern and extend it to a cellular response in the PVN.

GnIH cells show little to no activation of EGR-1, suggesting that EGR-1 is not involved in transcribing the resulting GnIH protein. Another immediate early gene, *FOS*, co-localizes with

GnRH cells in mammals in response to various stimuli but may not be directly responsible for its release (Moenter et al., 1993; Witkin et al., 1994). Even after stimulation by long days or *N*-methyl-D-aspartate (NMDA) and a resulting release of LH (presumably as a result of increased GnRH release), co-localization of FOS and GnRH has not been observed in birds, although FOS activation in the hypothalamus and GnRH release were positively correlated (Meddle and Follett, 1997; Meddle et al., 1999). Mechanisms regulating GnIH secretion within this experimental paradigm are not yet known and require further study.

GnIH response to stress. Baseline (control) GnIH cell abundance was greater in the fall than the spring, and a response of the GnIH system to stress was only observed in the spring. Perhaps the lack of observable response of the GnIH system to stress was a result of maximal GnIH production during the fall, so no further increase could be induced. In the absence of data to the contrary and in light of Bentley et al. (2003), we assume that GnIH is maximal at this time of year. A similar increase in GnIH content at the termination of breeding occurs in song sparrows (*Melospiza melodia*; Bentley et al., 2003). It is possible that this increase in GnIH facilitates the fine-tuning of termination of reproduction in these species. Another possible explanation for the lack of change in GnIH in the fall is that animals have an attenuated stress response at the end of the breeding season (Romero, 2002; Breuner and Orchinik, 2001), and the GnIH stress response may also follow this pattern.

The GnIH stress response seen at the beginning of the breeding season may reflect a mechanism by which reproduction is slowed or halted in stressful conditions. This response could prove adaptive because animals experiencing a stressor may immediately and transiently be able to shunt physiological resources away from reproductive effort and towards metabolic demands associated with stress. The involvement of GnIH would allow for rapid changes in behavior without the need for long-term inactivation of the reproductive axis (GnRH system), as occurs at the end of the breeding season. Along these lines, GnIH and its receptor are present in avian gonads and reproductive tract (Bentley et al., 2007; McGuire et al., 2008), and rapid activation of the gonadal GnIH system during the breeding season might influence gonadal steroid release and reproductive behaviors.

By using the method of immunocytochemistry to measure EGR-1 and GnIH cell abundance, we are unable to elucidate the temporal dynamics of GnIH synthesis and release in response to our stimulus. An increase in cell number could signify the increased production of the EGR-1 and GnIH protein, but it may also be indicative of the decreased release of stored protein. However, data on GnIH mRNA in rats suggest the former (Kirby et al., 2007). Recently, GnIH was cloned in house sparrow (McGuire et al., 2008), which will permit us further study of this matter. By performing *in situ* hybridization, we will be able to examine GnIH mRNA and thus GnIH production.

In sum, our data show an effect of stress at the cellular level in the PVN of house sparrows. EGR-1 neuron number is elevated in stressed birds relative to respective controls in both the spring and fall seasons. The EGR-1 response to acute stress also shows seasonal variation, with higher cell number in stressed birds in the spring than in fall. EGR-1 showed little to no co-localization with GnIH cells, suggesting that EGR-1 most likely does not directly transcribe the GnIH peptide in this paradigm. GnIH neuron number exhibited a differential seasonal response to stress, with GnIH being more abundant in the fall, but only showing a significant increase in stressed birds in the spring. These data imply that GnIH might act as a gating system for the effects of stress on the reproductive axis at different times of year. Before

we can conclude this, we must use this seasonal approach to examine GnIH mRNA in stressed birds as well as differential affects of stress on gonadotropin synthesis and secretion.

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Table 1. Comparisons of EGR-1 and GnIH cell numbers between sexes, stress and control groups, and seasons. Because there were no sex differences, males and female data were pooled to examine differences between the fall and the spring. Asterisks denote statistical significance.

	EGR-1			GnIH		
	F	df	*P*	*F*	df	*P*
ANOVA						
Sex	0.10	1,24	0.76	0.48	1,24	0.83
Season	12.96	1,24	0.001*	10.04	1,24	0.004*
Stress	24.83	1,24	<0.001*	2.18	1,24	0.15
Sex × Season	0.03	1,24	0.87	0.03	1,24	0.86
Sex × Stress	0.41	1,24	0.53	0.05	1,24	0.82
Season × Stress	2.56	1,24	0.12	2.45	1,24	0.13
Sex × Stress × Season	0.00	1,24	0.99	0.20	1,24	0.66
t-Test						
Stressed vs. Control						
Spring	−3.85-	13	0.002*	−3.178	13	0.007*
Fall	−3.739	15	0.002*	−0.025	15	0.98
Spring vs. Fall						
Stressed	−5.035	11	<0.001*	1.227	11	0.25
Control	−1.519	17	0.15	3.913	17	0.001*

Fig. 1. EGR-1 is expressed in more cells of stressed birds at start and end of breeding season compared to control group. However, EGR-1 stress response is attenuated at end of breeding season, as opposed to start. There is no difference between males and females within groups. Numbers within bars indicate sample sizes.

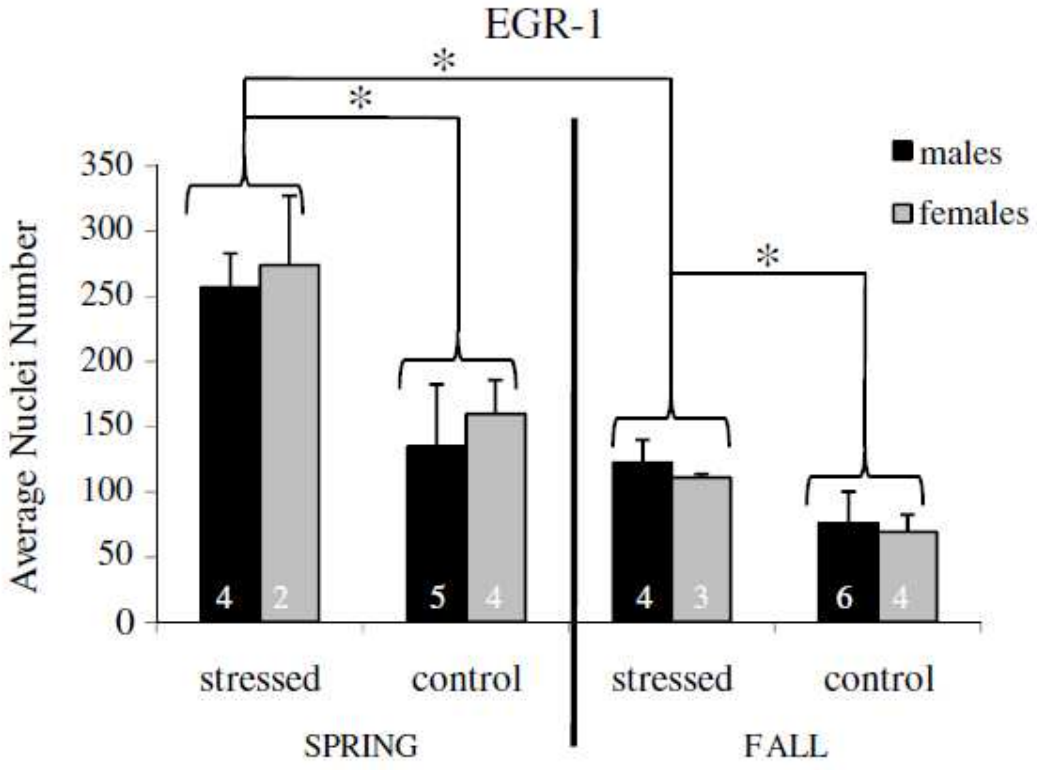


Fig. 2. More GnIH cells were observed in stressed birds at start of breeding season compared to control group. More GnIH cells were present overall at the end of the breeding season, as opposed to the start. There is no difference in number of cells expressing GnIH at the end of breeding season between stressed and control birds. There is no difference between males and females within groups. Numbers within bars indicate sample sizes.

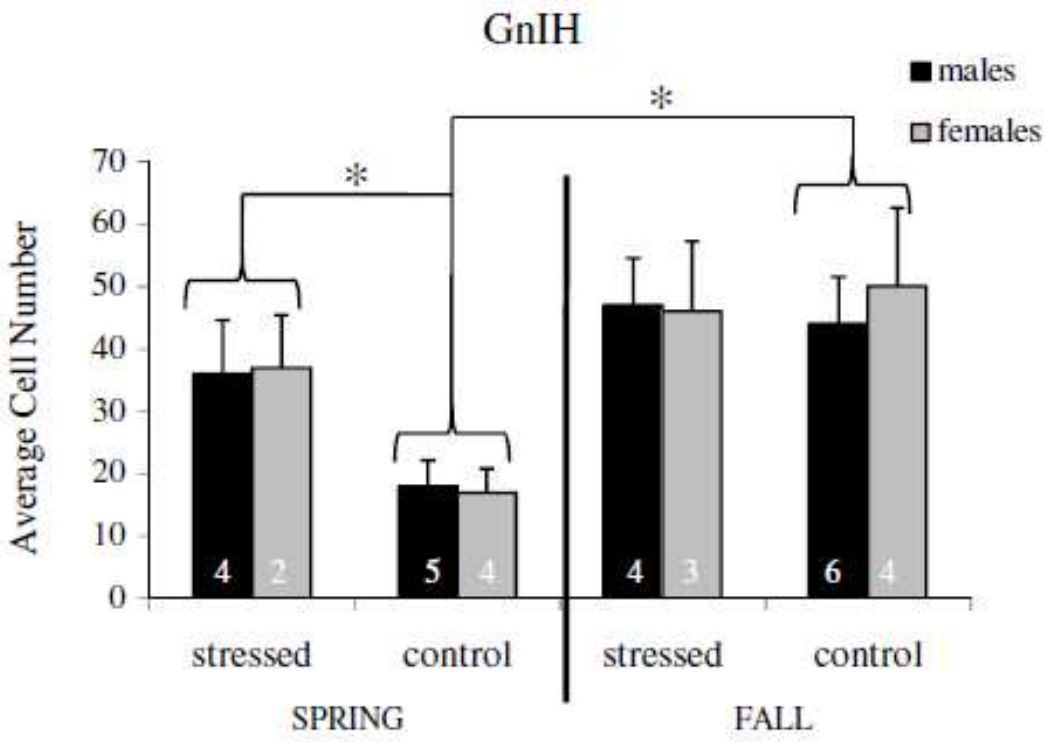
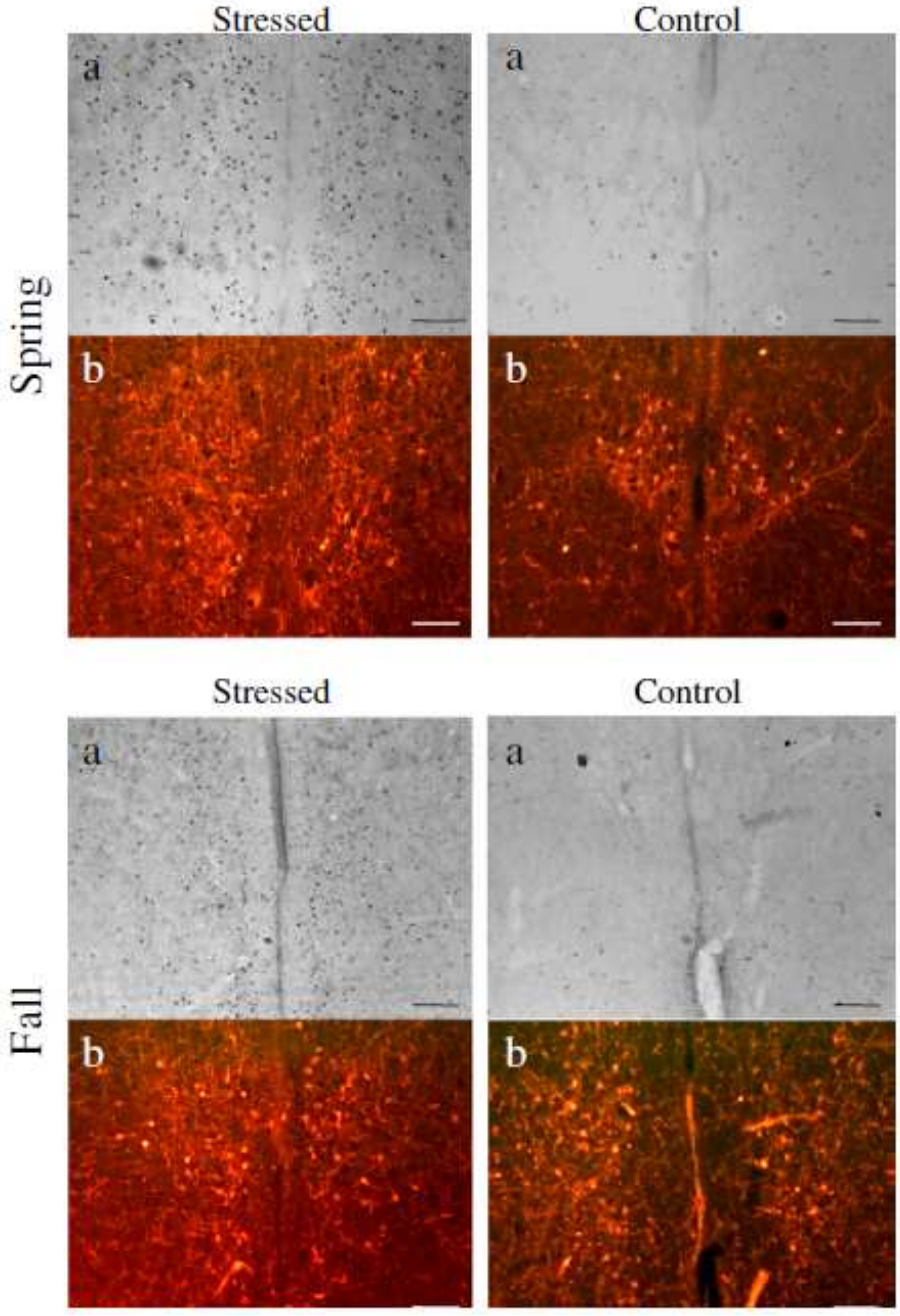


Fig. 3. Representative photographs of EGR-1 (a) and GnIH (b) positive cells in the PVN of the hypothalamus in stressed vs. control animals at the start and end of the breeding season. Scale bars are 100 μm.



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Chapter 3
Social environment influences gonadotropin inhibitory hormone (GnIH) in the brain

Social environment influences gonadotropin inhibitory hormone (GnIH) in the brain

Abstract

Social status can affect hormones circulating in the body and ultimately reproduction, but how social environment affects hormones in the brain is not well understood. Discoveries of how social behavior can influence the plasticity of the gonadotropin releasing hormone (GnRH) system have revolutionized the field of behavioral neuroendocrinology by providing new insights into the neural and environmental mechanisms controlling behavior. A more recently discovered neuropeptide, gonadotropin inhibitory hormone (GnIH), inhibits the activity of GnRH neurons in the brain in addition to reducing synthesis and release of the gonadotropins luteinizing hormone and follicle-stimulating hormone from the pituitary gland. GnIH also reduces testosterone release from the gonads. Central administration of GnIH can decrease copulation solicitations in birds and sexual behaviors in rodents, but how GnIH fluctuates naturally in response to social environment is unknown. Studies of the effects of social behavior on the reproductive axis are seminal to understanding behavioral neuroendocrinology. In this paper we examine how the outcome of competition for limited resources needed for reproduction affects the GnIH system. We experimentally manipulated nesting opportunities for pairs of European starlings (*Sturnus vulgaris*) and examined brain GnIH mRNA and peptide content, as well as GnRH content and plasma testosterone and corticosterone. By limiting the number of nestboxes per enclosure and thus the number of social pairing and nesting opportunities, we observed that birds which outcompeted others for nestboxes had significantly different numbers of GnIH peptide-producing cells than those without nestboxes, and this relationship varied with breeding stage. GnRH content, testosterone and corticosterone did not vary with nestbox ownership. Thus, while the reproductive axis appeared to remain functional across treatments, our data indicate that GnIH has the ability to serve as a modulator of reproductive behaviors in response to social environment. Additionally, we provide evidence of the adaptive value of this mechanism. Because GnIH presence and function are conserved throughout all vertebrates studied, including humans, our findings in birds can create new avenues into studies of vertebrate reproductive physiology and the neural mechanisms that may control reproductive and sexual behavior.

Introduction

Differences in social status can have profound effects on circulating hormones (reviewed in Creel 2001), but the effects of social environment on the brain are less understood. For all vertebrates studied, gonadotropin releasing hormone (GnRH) is a key regulator of the reproductive, or hypothalamic-pituitary-gonadal (HPG), axis. GnRH released from the hypothalamus signals the anterior pituitary gland to release the gonadotropic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the bloodstream. LH and FSH then promote testicular spermatogenesis, follicular growth, and estradiol and testosterone release which feedback to and modulate all levels of the HPG axis. Several studies on how social behavior can influence the plasticity of the GnRH system have revolutionized the field of behavioral and reproductive neuroendocrinology by expanding our understanding of how social interactions can influence neural mechanisms associated with reproduction (Burmeister 2005, White et al. 2002; Fox et al. 1997; White et al. 1995; Francis et al. 1993; Davis and Fernald 1990; Fraley and Fernald 1982). The recent discovery of the novel neurohormone gonadotropin inhibitory hormone (GnIH) is once again changing the way we view how reproduction and sexual behavior are regulated by the brain. Until now, nothing was known about whether GnIH could be influenced by social circumstance. Here, we report that social environment, reproductive stage and seasonal environment all combine to influence GnIH content and we provide evidence as to the functional significance of this phenomenon.

Gonadotropin inhibitory hormone (GnIH), first discovered in birds (Tsutsui et al. 2000), inhibits pituitary gonadotropin release *in vitro* and *in vivo* (Tsutsui et al. 2000, Osugi et al. 2004, Ciccone et al. 2004) in both birds and mammals (Kriegsfeld et al. 2006, Johnson et al. 2007, Murakami et al 2008, Clarke et al 2008), indicating the conserved nature of its actions (reviewed in Bentley et al. 2006). GnIH also inhibits gonadotropin synthesis in birds (Ubuka et al. 2008, Ciccone et al. 2004). Located in the hypothalamus, GnIH-producing neurons appear to make direct contact with GnRH- producing neurons in both birds and mammals (birds: Ubuka et al. 2008, sheep: Smith et al. 2008, rodents: Johnson et al. 2007, Kriegsfeld et al. 2006, primate: Ubuka et al. 2009, humans: Ubuka et al 2010). GnIH receptor mRNA is expressed by GnRH neurons (Ubuka et al. 2008), suggesting that GnIH can directly affect or regulate GnRH and thus the reproductive axis. In mice, direct application of mammalian GnIH (RFRP) to GnRH cells in cultured brain slices decreases firing rate in a subpopulation of cells, further suggesting a direct action of GnIH on GnRH neurons (Ducret et al. 2009; Wu et al. 2009). Thus, the discovery of GnIH and its actions provides us with an exciting and novel tool with which to study the mechanisms controlling reproduction.

Social environment can alter reproductive and stress hormones circulating throughout the body. For example, social dominance, subordinance or instability in general can affect the production of hormones such as testosterone or cortisol (Rubenstein 2007, Goymann and Wingfield 2004, Creel 2001, Wingfield et al. 1990). However, how these social circumstances affect neuropeptides responsible for reproduction is not well understood. In a previous study, we reported that GnIH content could be affected by the external environment. Capture-handling stress in house sparrows caused an increased abundance of hypothalamic cells immunoreactive for the GnIH peptide only during the breeding season (Calisi et al. 2008). Intracerebroventricular administration of GnIH can decrease sexual displays in captive birds (Bentley et al. 2006) and mammals (Johnson et al. 2007), hinting at a possible function for increased GnIH in response to stress - but how does the relationship between GnIH and reproductive behaviors play out in an

animal's natural environment? In this study we ask, can social environment influence GnIH content? If so, then GnIH may serve as a neural modulator of reproductive function and sexual behaviors by acting as a physiological integrator of social interactions.

Most efforts to understand socially mediated reproductive opportunities have examined highly cooperative singular breeding societies, rather than the less derived effects of simple competition for reproductively important resources. European starlings (*Sturnus vulgaris*) are obligate cavity-dwelling species that are dependent upon cavities created by other species for successful reproduction. Thus, they can experience limited and unpredictable nesting site availability from year to year (Newton 1994; Aitken and Martin 2004). We simulated the natural environment of social instability and nesting site competition by limiting the number of nestboxes per semi-natural enclosure. By limiting the number of nestboxes available, birds competed to obtain a nestbox and thus a breeding opportunity. In this way, we forced our population to divide into two separate breeding cohorts – breeders and non-breeders – during the same time of year. We compared GnIH mRNA and peptide content accordingly. We sampled at 1) the beginning of the breeding season, after nest box acquisition, 2) during the middle of the breeding season, when egg laying and incubation were occurring, and 3) the beginning of the non-breeding season (post molt), when the activity of the reproductive axis is attenuated.

Our goal was to investigate how socially mediated differences in breeding opportunity (having a nestbox versus not), can influence the GnIH system. Because of the inhibitory nature of GnIH on reproductive physiology and sexual behavior, we hypothesized that GnIH content would be associated with nestbox status. We tested our hypothesis in both male and female European starlings and report on GnIH mRNA and peptide content during periods of social competition and parental care. In addition, we measured GnRH-I peptide abundance and plasma testosterone and corticosterone concentrations. We report GnIH content, GnRH-I content, testosterone and corticosterone plasma concentrations in European starlings that obtained and maintained nestboxes versus those that did not.

Materials and Methods

Housing

Thirty nine (22 male and 17 female) European starlings were caught as juveniles in the fall of 2007 in Lodi, California, and housed in large, naturalistic outdoor aviaries. This semi-natural experimental set-up is a powerful way to understand the physiological and ecological relevance of the question at hand (Calisi and Bentley 2009). The aviaries were constructed of chain link and chicken wire fencing with natural earth floor and measured 12 x 6 x 3.5m (Fig. 1). Birds were exposed to natural light, weather, hetero- and homo-specific interactions, food sources (invertebrates could be foraged from the air and natural earth floor, in addition to being supplement with *ad libitum* chicken pellet feed) and *ad libitum* water while group size and nestbox number were manipulated. The type of wooden nestboxes used (Fig. 1) was the same type in which European starlings from the previous year had hatched and raised chicks (Calisi and Bentley, *pers. obs*.). Under these conditions, European starlings exhibited a full range of natural breeding behaviors, including singing, copulation solicitations, breeding, social monogamy, nest construction and defense, and care of hatchlings (Calisi and Bentley, pers. obs.; suppl. video of behaviors).

Nestbox manipulation

Males and females were housed together and randomly assigned to each aviary. The number of nestboxes placed in each aviary depended on the group size. Nestboxes were placed in all three aviaries at the beginning of the breeding season during the first week of February. In Aviary 1, we allotted 3 nestboxes, and 5 birds obtained a nestbox while 5 did not. In Aviary 2, we allotted 6 nestboxes, and 12 birds obtained a nestbox and 5 did not. In Aviary 3, we allotted 3 nestboxes, though none of the 12 birds showed a preference for a particular nestbox during this time (Table 1).

Different birds were sampled at each collecting point; the same individuals could not be sampled repeatedly due to the need to sacrifice the animals to examine patterns of GnIH and GnRH-I activity. We sampled at 1) the beginning of the breeding season, after nest box acquisition, 2) during the middle of the breeding season, when egg laying and incubation were occurring, and 3) the beginning of the non-breeding season (post molt), when the activity of the reproductive axis is attenuated. Aviary 1, sampled at the beginning of the breeding season (18 Feb 08), housed 7 males and 3 females, of which 3 males and 3 females obtained nestboxes. However, one of the males that obtained a nestbox escaped before sampling, which is why our total sample size for birds that obtained a nestbox during this time is 5. Aviary 2, sampled during the middle of the breeding season (24 Ap 08), housed 7 males and 10 females, of which 6 males and 6 females obtained nestboxes. Aviary 3, sampled at the beginning of the non breeding season (24 Sept 08), housed 8 males and 4 females, and none of the birds showed preference for a nest box during the beginning of this non-breeding period (Table 1).

Behavioral observations

We placed colored leg bands on birds to identify individuals throughout the study. Behavioral observations were taken during the first five days after nestboxes were placed in aviaries and the last five days before sampling; additional observations were taken periodically in between those time points. Observations were taken at approximately 0900 – 1300 hrs. We used focal sampling of nestboxes and noted which birds came in direct contact with the nestbox (perching on roof, on perch, or entering nestbox). During the beginning and middle of the breeding season, the same male and female pairs would visit a particular nestbox over 95% of the time, and thus were assigned nestbox ownership. Birds showed no preference for a nestbox at the beginning of the non-breeding season.

Brain and blood sampling

Aviary-housed birds were collected by mistnet and handnet and sacrificed by decapitation following rapid terminal anesthesia using isoflurane. Immediately following decapitation, brains were extracted and frozen on dry ice and then stored at -80°C until sectioning. Approximately 1-2 ml of trunk blood was collected and refrigerated immediately for parentage analysis and radioimmunoassay of plasma corticosterone and testosterone. Blood was centrifuged within an hour of collection to separate plasma and solid fractions. The plasma was drawn off with a pipette and then frozen for later hormone analyses. Brains were sectioned coronally at 20 μm using a cryostat and mounted directly onto silane coated slides. Every eighth section throughout the hypothalamus was collected 1) for immunocytochemistry (ICC) to visualize cells labeled for the GnIH and GnRH-I peptides, and 2) *in situ* hybridization (ISH) to visualize cells labeled for GnIH mRNA. The density of the latter label was used to estimate the amount of GnIH mRNA being produced (see Quantification of GnIH and GnRH-I content and

statistical analysis). All procedures were approved by and in compliance with the University of California Office of Lab Animal Care and federal regulations.

Parentage analysis

European starlings are socially but not always genetically monogamous. To determine whether a pair that obtained a nestbox and incubated eggs was in fact the genetic parents, we conducted a parentage analysis. Genomic DNA was extracted from blood samples of all adults in the aviaries using a Sigma Blood Extraction Kit (Sigma-Aldrich, St. Louis, MO). Genomic DNA of offspring was extracted from developing embryos removed from eggs collected in nest boxes using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA).

Genomic DNA was amplified at 5 microsatellite loci previously developed for *Sturnus unicolor* (Celis et al. 2006). Products were fluorescently labeled during amplification by attaching an M13 sequence to the forward primer and adding a universal fluorescent labeled primer (M13 sequence with a 5' FAM or HEX modification) following the procedure of Schuelke (2000). PCR reactions were performed in a Bio-Rad iCycler thermal cycler (Bio-Rad, Hercules, CA) with the following cycling conditions: 94ºC for 4 min; 94ºC for 1 min, T_a (see Table 2) for 1 min, 72ºC for 75 s, repeated 27 times; 94ºC for 1 min, 50ºC for 1 min, 72ºC for 75 s, repeated 8 times; 72ºC for 10 min. PCR volume was 10 µL with: 40 ng of genomic DNA, 1µL of 10X PCR Buffer (ABI, Foster City, CA), 2.5mM $MgCl_2$, 0.8µL 10 mg/mL BSA, 0.4 mM of each DNTP, 3pm of R primer, 3pm of the universal fluorescent primer, 1pm of M13-labeled F primer and 0.5 U of *Taq* polymerase (Invitrogen, Carlsbad, CA).

1µL of PCR product was added to 8.8 µL of formamide with 0.2 µL of GeneScan 500-LIZ size standard (ABI, Foster City, CA) and genotyped samples in an ABI 3730 automated sequencer (ABI, Foster City, CA). Genotypes were scored manually using Genemapper 4.0 (ABI, Foster City, CA).

Parentage analysis was conducted in Cervus 3.0 (Kalinowski et al. 2007). We considered a parent pair to be the correct parents when offspring were assigned at the "strict" confidence level (95%) and had no more than 1 allelic mismatch with the parental pair.

Radioimmunoassay (RIA)

Testosterone and corticosterone can vary with stress and social status (Rubenstein 2007, Goymann and Wingfield 2004, Creel 2001, Wingfield et al. 1990) To determine if capture time affected corticosterone and if social status affected testosterone and corticosterone and their association with GnIH and GnRH-I content, we measured plasma testosterone and corticosterone in a radioimmunoassay (RIA) using the methods of Wingfield and Farner (1975), modified by Ball and Wingfield (1987). We were not able to assay for corticosterone during the beginning of the breeding season due to the low quantity of blood collected. After extracting steroids from the plasma using dichloromethane, testosterone and corticosterone were separated from other steroids using diatomaceous earth/glycol column chromatography. Samples were assessed in duplicate and measured in a single RIA to avoid interassay variation.

In situ hybridization (ISH)

To visualize cells labeled for GnIH mRNA, we performed *in situ* hybridization (ISH) according to Ubuka et al. (2008). We produced a DIG-labeled antisense RNA probe using a standard RNA labeling kit (Roche Diagnostics), and used partial starling GnIH precursor cDNA as a template. After hybridization, brain sections were incubated with alkaline phosphatase-

labeled sheep anti-DIG antibody (Roche Diagnostics), and the immunoreactive product was visualized by immersing the sections in a substrate solution (Nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt). We controlled for the specificity of the ISH using a DIG-labeled sense RNA probe, the sequence of which was complementary to that of the antisense probe.

Immunocytochemistry (ICC)

GnRH-I

We conducted ICC to visualize cells labeled for GnRH-I peptide. Sections were fixed in 4% paraformaldehyde for 1 h. Sections were then washed three times in phosphate buffered saline (PBS, 0.1M) and treated with 0.01% hydrogen peroxide in PBS for 10 min to reduce background immunoreactivity. Sections were again washed three times with PBS and then submerged in 2% normal goat serum (NGS) in 0.2% PBS-T (PBS + Triton X-100) for 1 h to block background immunoreactivity. GnRH primary antibody (code HU60, generous gift from Henryk Urbanski) was used to incubate sections for 48 h at a concentration of 1:5000 in 0.2% PBS-T. Three more washes in 0.2% PBS-T were followed by 1 h of incubation in biotinylated goat anti-rabbit IgG (1:250 in 0.2% PBS-T), followed by three more washes of 0.2% PBS-T. Sections were incubated in avidin-biotin complex (ABC: Vectastain Elite Kit, Vector Labs) for 1 h and visualized using 0.03% 3,3'-diaminobenzidine (DAB) as the chromogen.

GnIH

Directly following GnRH-I visualization, we conducted ICC to visualize cells labeled for the GnIH peptide. Sections were washed five times in 0.2% PBS-T. Goat anti-rabbit affinity-purified GnIH primary antibody (code: PAC 123/124, Bentley) was used to incubate sections for 48 h at a concentration of 1:5000 in 0.2% PBS-T. Three subsequent washes in 0.2% PBS-T were followed by incubation in avidin–biotin complex (ABC; Vectastain Elite Kit, Vector Labs) for 1h and visualized in Vector VIP (Vector Labs). This protocol has been used successfully in previous studies on this and other species (Bentley et al., 2003, Ubuka et al., 2008, Ubuka et al., 2009, 2010).

Quantification of GnIH and GnRH-I content

All hypothalamic GnIH peptide- and mRNA-immunoreactive (ir) and GnRH-I peptide-ir cells were counted using a Zeiss Axio Imager A1 microscope in a double-blind fashion in which an arbitrary number was assigned to each sample. We measured GnIH in three ways: 1) we counted the number of hypothalamic cells containing GnIH mRNA, indicating the number of cells producing the hormone, 2) we counted the number of hypothalamic cells immunoreactive for the GnIH peptide, indicating the number of cells containing the hormone, and 3) we determined the optical density of the former cells to measure the upregulation of the gene for the GnIH precursor. We assumed that increased optical density signified increased transcription and translation of the gene, but with the caveat that there was no way of telling whether all of the mRNA was translated into mature polypeptide and then cleaved to mature peptide. GnIH mRNA optical density was measured using ImageJ software (Rasband 1997-2009). An average pixelation score was taken from the twenty most darkly labeled cells per individual and subtracted from an average of equal background measurements taken from tissue surrounding cell areas.

Statistical analyses

Statistical analyses were performed using PASW v18 (formerly SPSS, Chicago, IL, USA). All data were tested for deviations from normality using a Shapiro-Wilk test and transformed when necessary to achieve normality at $P>0.05$. We modeled the relationship between capture time and corticosterone concentrations using linear regression analysis. Because of the dependent nature of the variables studied, we conducted multivariate analysis of variance (MANOVA) to examine 1) the effect of capture time and corticosterone concentrations on GnIH and GnRH content and testosterone concentrations and 2) the effect of the three seasonal collection times on GnIH and GnRH-I content. Within nestbox treatments per collecting period, we used Pearson's correlation analysis to examine the relationship between cells containing the GnIH peptide, cells producing GnIH mRNA and the amount of GnIH mRNA production.

Rather than conduct a repeated measures design (because we sampled different birds at each collecting point, and not the same birds throughout the study) and because of the multiple dependent variables involved, we conducted a MANOVA to examine the effect of nestbox treatment on GnIH and GnRH-I peptide cell abundance, GnIH mRNA cell abundance, GnIH mRNA optical density, testosterone and corticosterone concentrations. Although our correlational analysis revealed only some of the variables were correlated during time of sampling (see Results), we know all our variables of reproductive function are biologically correlated (as discussed in the Introduction) and we thus chose to err on the side of caution and to conduct conservative MANOVAs to examine data and their interactions.

Birds that outcompete others for nestboxes gain a reproductive opportunity, and this behavioral outcome has adaptive implications. Therefore, in order to look for what differences may be present to promote future studies, we conducted univariate F-tests on variables within models that had a $P<0.05$ and report statistically significant relationships at $P<0.05$.

Results

Effects of social status on GnIH

Beginning of the breeding season

Birds with a nestbox appeared to have fewer GnIH cells than birds without a nestbox. The model examining the effect of nestbox treatment on cells immunoreactive for the GnIH and GnRH-I peptide and GnIH mRNA, GnIH mRNA optical density, circulating testosterone and corticosterone plasma concentrations was not significant (Pillai's Trace=0.637, $F_{5,4}=1.403$, $P>0.05$). However, visual inspection of the data revealed an apparent difference between GnIH peptide cell abundances from the different nestbox treatments (Fig. 2). A post-hoc analysis of these data suggested a difference in GnIH peptide cell abundances between treatments ($P<0.05$; Fig. 1,2) in which birds with a nestbox had fewer cells immunoreactive for the GnIH peptide than birds without a nestbox. Because our original model incorporated numerous non-statistically significant variables, this particular relationship may have been masked. However, we wish to remain conservative in our interpretation and make clear that a relationship *may* exist between GnIH peptide and nestbox obtainment during this time. We discuss the implications of such a relationship in the Discussion and call for further examination.

Middle of the breeding season

In contrast to the the beginning of the breeding season, in the middle of the breeding season birds with a nestbox had more GnIH cells than birds without a nestbox. The model examining the effect of nestbox treatment on cells expressing the GnIH peptide, GnIH mRNA, GnRH-I peptide, GnIH mRNA optical density and circulating testosterone and corticosterone plasma concentrations was significant (Pillai's Trace=0.650, $F_{6,10}$=3.101, P=0.05). Post-hoc univariate F-tests revealed that this outcome was due to a difference between GnIH peptide cell abundance between treatments (P<0.05; Fig. 2, 3), in which birds with a nestbox had a greater GnIH peptide cell abundance than birds without a nestbox. Comparison between sampling periods revealed that birds with a nestbox during this time had a 100.1% increase in GnIH peptide cell abundance over birds with a nestbox at the beginning of the breeding season (*P*<0.01).

Beginning of non- breeding season

Birds did not show a preference for a nestbox during this period and thus comparisons of neuroendocrine activity as a function of nest box status could not be undertaken for this sampling period.

Neuroendocrine seasonal profiles

GnIH and GnRH-I peptide and GnIH mRNA were present at all sampling points. There was a seasonal effect on GnIH and GnRH-I content (Pillai's Trace=0.557, $F_{6,19}$=3.981, P<0.05; post-hoc univariate F-tests : GnIH peptide: *P*<0.05, GnIH mRNA: *P*<0.05, GnIH mRNA optical density: *P*<0.05; GnRH-I: *P*<0.001; Fig. 4). Further post-hoc univariate F-tests revealed that GnIH peptide cell abundance did not differ between the beginning and the middle of the breeding season (*P*=0.08), nor did average GnIH mRNA cell abundance (*P*=0.54) or GnRH-I peptide cell abundance (*P*=0.33). GnIH peptide and mRNA cell abundance did not differ between the beginning of the breeding season and during the non-breeding season (*P*=0.28, *P*=0.17, respectively).

Parentage Analysis of Nest Eggs

During the middle of the breeding season, 12 birds obtained 6 nests. All pairs except one were in the process of laying or incubating eggs when sacrificed. The female of the non-laying pair had large, yolky follicles suggesting she was about to lay. Two of the pairs were incubating eggs in which embryos had not yet formed. Thus, a total of 12 offspring in 3 nests were assigned parentage. All offspring in each nest were assigned to a single parent pair. For all nests the parent pair assigned using genetic data coincides with the nestbox occupants.

Correlational analysis between GnIH peptide, GnIH mRNA and GnIH mRNA density

During the beginning of the breeding season, GnIH mRNA cell abundance was negatively correlated with the mRNA optical density (r=-0.672, *P*<0.05). In birds with a nestbox, GnIH peptide cell abundance was positively correlated with the mRNA production (r=0.912, *P*<0.05). In birds without a nestbox, GnIH peptide and GnIH mRNA cell abundance were positively correlated (r=0.975, *P*<0.01; Fig. 5). During the middle of the breeding season and at the beginning of non-breeding season, no significant correlations between GnIH peptide or GnIH mRNA cell abundance or GnIH mRNA optical density were apparent.

Testosterone and corticosterone concentrations

Recoveries were 52.02% and 75.96% and intra-assay variation was 5.42% and 1.36% for testosterone and corticosterone, respectively, and the assay detection limit was ~ 0.1 ng/ml. The average testosterone and corticosterone concentrations per season, sex and nestbox treatment are reported in Table 3.

Birds were collected by mistnet and handnet within 13.077 min +/- 6.603 min of us entering the aviary. Because the peak average corticosterone concentration was below 5ng/ml, we believe these concentrations (Table 3) are in general indicative of basal, or non-stressed, circulating corticosterone concentrations. Rich and Romero (2005) have reported basal corticosterone levels of captive European starlings at about 5ng/ml, with a peak of 30-40ng/ml after restraint stress.

The model examining the effects of sex on GnIH and GnRH-I content, testosterone and corticosterone was significant (Pillai's Trace=0.617, $F_{7,19}$=4.366, P<0.01). Univariate F-tests revealed testosterone concentrations only differed between the sexes during the middle of the breeding season (P<0.05), with males having higher concentrations. However, no differences in GnIH or GnRH content or corticosterone concentrations were evident between the sexes, and thus data from males and females were combined to increase sample size. In this combined data set, plasma corticosterone concentration was positively correlated with capture time (r=0.63, P<0.01), negatively correlated with GnIH peptide cell abundance at the middle of the breeding season (r=-0.679, P<0.05) and positively correlated with GnIH mRNA cell abundance at the middle of the breeding season (r=0.670, P<0.05). To control for effects of capture time, residuals were taken from these relationships and used for further examination of the effects of the nestbox treatment.

Discussion

Our goal was to investigate how socially mediated differences in breeding opportunity (having a nestbox versus not), can influence the GnIH system. Our data show effects of social status, as measured by obtainment of a nestbox, and season on GnIH production and release. We do not know whether these differences seen in GnIH occurred prior to or after nestbox obtainment. However, this is the first report of a social effect on GnIH in any organism. We interpret data in the following parsimonious fashion: presence of the peptide is indicative of cells containing GnIH. An increase in number of GnIH mRNA cells or optical density is indicative of an increase in GnIH production. A decrease in number of GnIH mRNA cells or optical density is indicative of inhibition or a reduction of synthesis of GnIH, and we discuss the implications of such activity, including alternate interpretations.

Social environment affects GnIH in the brain differentially at different breeding stages

Beginning of the breeding season

The number of GnIH peptide cells appeared to be lower in birds that outcompeted others for a nestbox versus those that did not, though GnRH-I peptide cell abundance, testosterone and corticosterone concentrations did not differ. The model we ran to explore the interrelationships between all variables and nestbox treatment was not statistically significant, but a post-hoc test

revealed GnIH peptide cell abundance was lower in birds with nestboxes, implicating further study of effects of social status on the GnIH system.

While the number of GnIH peptide cells may have been lower in birds with nestboxes, GnRH-I peptide cell abundance, testosterone and corticosterone concentrations did not differ. This lack of change in GnRH-I and testosterone suggests the animals' endocrine reproductive capabilities remained intact, no matter the reproductive opportunity. Having a constantly functioning reproductive axis could lessen the time between the loss of a resource and the obtainment of a replacement. This would permit optimization of reproductive output, as nesting sites can be lost to predators and need to be re-established throughout the breeding season. An adaptive response to obtaining a nestbox and mate would be to have little to no inhibition of the reproductive axis to allow for initiation of breeding. In support of this, males and females that obtained a nestbox had fewer cells containing the GnIH peptide than birds that did not obtain a nestbox. However, we do not know whether these differences seen in GnIH cell abundance occurred prior to or after nestbox obtainment. If the differences were prior, birds with fewer GnIH cells and less inhibition to their reproductive axis may have been better competitors for nestboxes and mates, yielding the same data.

The number of cells containing the GnIH peptide was negatively correlated with the amount of cells producing the hormone mRNA. We consider that having more cells containing the GnIH peptide and fewer producing mRNA may signify slow production and decreased release of the mature peptide during this time, allowing content to build up in the cells. Thus, birds without nestboxes would experience less inhibition to their reproductive axis and sexual behavior, and might have responded by increasing their efforts to obtain them. This response would be adaptive if it increased the chances of birds reproducing, although no birds were usurped from their nestboxes in this study.

A difference in GnIH peptide cell abundance between nestbox treatments suggests GnIH is responsive to social status. Because we did not observe a change in testosterone between treatment groups, we assume the changes in GnIH content could result in (or be the result of) changes in action in the brain to affect behavior. In a previous study, we reported that GnIH is responsive to capture-handling stress in house sparrows (*Passer domesticus*) and peptide cell abundance increases in a period of one hour (Calisi et al. 2008). Receptors for glucocorticoids produced during a time of stress are expressed by mammalian GnIH cells (Kirby et al. 2009), and this is also true for European starling GnIH cells (Calisi et al. 2010). Because of the known inhibitory effect of GnIH on sexual behavior and the reproductive axis (Tsutsui et al. 2000, Osugi et al. 2004, Ciccone et al. 2004, Bentley et al. 2006, Johnson et al. 2007), this evidence suggests a mechanism by which GnIH could be working as a situational reproductive neural inhibitor, or "pause button" to avoid costly sexual behaviors without entirely terminating reproductive activity.

Middle of the breeding season

The number of GnIH peptide cells was higher in birds with a nestbox laying and incubating eggs versus 1) birds without a nestbox and 2) all birds from the beginning of the breeding season, with or without a nestbox. Only one reproductive pair was not laying/incubating eggs during this sampling period, though the female of this pair had yolky follicles, indicating she was about to lay. This pair had the smallest number of GnIH peptide cells compared to the other pairs laying/incubating eggs (as seen in Fig. 1). GnRH-I peptide cell abundance, testosterone and corticosterone concentrations did not differ between treatments,

again suggesting endocrine reproductive capabilities remained intact, and again implying changes in function of GnIH within the brain, rather than on the HPG axis here.

The increase in the number of GnIH peptide cells in birds laying and incubating eggs may be related to the neural inhibition of aggressive and sexual behaviors during incubation. Testosterone in many birds, including European starlings (Pinxten et al. 2007), can decrease during a time of parental care to facilitate parental behaviors (Ketterson et al., 1996, Magrath and Komdeur 2003). European starling testosterone peaks during nest building, remains high during the fertile and incubation period and then decreases after nestlings hatch (Pinxten et al. 2007). As in Pinxten et al. (2007), testosterone concentrations from our study remained high during the incubation period. Thus, our data imply that the change in GnIH (and lack of change in testosterone) may be related to changes in function/action within the brain that facilitate the switch from aggressive and sexual behaviors to incubation behaviors. In support of this hypothesis, receptors for GnIH are expressed on two avian hypothalamic populations of GnRH cells: GnRH-I and GnRH-II (Ubuka et al. 2007). GnRH-II is thought to play a role in sexual behaviors (Maney et al. 1997, Kauffman and Rissman 2004, Bentley et al, 2006), and thus GnIH may be having an affect on behaviors during this time by influencing GnRH-II.

The hormone prolactin plays a role in the stimulation and facilitation of incubation/parental behaviors in both birds and mammals (reviewed in Angelier and Chastel 2009). Prolactin concentrations are generally high during the incubation of eggs and during care/feeding of chicks after they hatch. In our study, testosterone concentrations also were high during incubation and may have gone on to decrease in parents when chicks had hatched (as in Pinxten et al. 2007). In a study conducted on cultured sheep pituitary cells, administration of GnRH and RF-amide-related peptide (RFRP)-3 (a mammalian GnIH homolog), decreased LH and FSH secretion but did not affect secretion of the pituitary hormone prolactin (Sari et al. 2009). Thus, the increase we observed in the number cells containing the GnIH peptide during incubation may be indicative of a build-up of GnIH during this time to prepare for release when chicks hatch, causing the decrease in testosterone to facilitate parental care. If the actions of GnIH can selectively affect the actions of hormones associated with mating and aggression but not interfere with hormones associated with incubation, such as prolactin, we posit the GnIH system as an entirely new mechanism mediating the hormonal shift in the brain from an aggressive life history stage to one of parental care.

Parentage Analysis

The results of parentage analyses show no evidence for extra pair paternity (EPP) or intraspecific brood parasitism (IBP). While rates of IBP and EPP in natural populations reported in the literature vary, these are generally relatively low (EPP: 9-17% of offspring and 29-45% of nests; IBP: 0-27% of chicks and 0-45% of nests) and it has been suggested that these may reflect local ecological conditions (Loyau et al. 2005). Thus, it is possible that the reduced space and increased food provisioning lowered the energetic costs of nest and mate guarding leading to the absence of EPP and IBP, at least in the nests that were examined.

The parentage results provide additional evidence that the differences we observed in GnIH content may impact the fitness of starlings. Comparative analyses have shown that neuroendocrine mechanisms are under selection to facilitate the timing of reproduction (MacDougall-Shackleton et al. 2009). Our results suggest, at an intraspecific level, that GnIH regulation influences fitness of individual starlings. This finding paves the way for future studies

examining fitness differences among individuals that differentially regulate the physiological mechanisms of reproduction.

Neuroendocrine seasonal profiles

GnIH peptide-, GnIH mRNA-producing cells and the optical density of cells expressing GnIH mRNA were more abundant during the breeding season as compared to the beginning of the non-breeding season. This may at first glance seem counter-intuitive (a reproductive inhibitory hormone increasing during the breeding season) but these data are consistent with other studies on songbirds (Bentley et al., 2003) and mammals (Mason et al. 2007, Tsutsui et al. 2010). An increase in cells containing and producing GnIH may be beneficial during the breeding season not to stop, but to modulate or "pause" the reproductive axis in response to environmental conditions. Furthermore in agreement with the current study, GnIH cell number decreased after the end of the breeding cycle. Once birds enter the non-breeding life-history stage, there is no need for further regulation, and cells containing and producing GnIH become downregulated during the non-breeding season.

As with other studies on European starlings, our data show greater GnRH peptide cell abundance during the breeding versus non-breeding season (Dawson et al. 1985, 2001, Ball and Hahn 1997; Ubuka et al., 2009)

Conclusions

In this paper we examined how socially mediated differences in breeding opportunity (having a nestbox versus not) can influence the GnIH system. Because of the inhibitory nature of GnIH on reproductive physiology and behavior during the breeding season, we hypothesized that GnIH could be affected by differences in social environment that influence reproductive opportunity. This study is first to test the social effect on GnIH in any organism. By experimentally manipulating nesting opportunities in both male and female European starlings, we observed that birds which outcompeted others for nestboxes had significantly different numbers of GnIH peptide-producing cells than those without nestboxes, and this relationship changed when birds were exhibiting incubating behaviors. We posit GnIH may play a key role in the switch from mating and aggressive behaviors to those of parental care and call for further studies.

Results from the parentage analysis revealed the social parents also to be the genetic parents. This finding suggests differences in GnIH content may impact reproductive fitness, paving the way for studies examining the adaptive value, and thus evolution, of mechanisms underlying physiological preparedness for breeding.

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Table 1. Breakdown of sex and number of individuals per nestbox treatment during each collection period.

Sampled	Aviary 1 Beginning of breeding season (18-Feb-08)	Aviary 2 Middle of Breeding season (24-Ap-08)	Aviary 3 Non-breeding season (24-Sept-08)
males	7	7	8
females	3	10	4
total	10	17	12
obtained nestbox	5	12	no preference
did not obtain nestbox	5	5	no preference
pairs incubating eggs	none laid	5	no longer breeding

Table 2. Locus specific information for genotypic data used in parentage analysis including: number of alleles (Na), number of individuals typed (N), annealing temperature (Ta), observed heterozygosity (Ho), expected heterozygosity (He) and Hardy-Weinberg equilibrium probabilities.

Locus	Na	N	Size Range	Ta (ºC)	Ho	He	HWE	Null Frequency
Sta70	11	29	242-282	52	0.724	0.873	0.591	+0.0865
Sta97	6	27	263-273	52	0.926	0.795	0.000	+0.4614
Sta213	11	29	172-216	54	0.862	0.862	0.312	-0.0087
Sta294	6	30	309-321	54	0.833	0.762	0.151	-0.0582
Sta308	9	25	151-171	54	1.00	0.869	0.580	-0.0831

Table 3: The average European starling testosterone and corticosterone concentrations per season, sex and nestbox treatment are reported in ng/ml ± stdev.

		Beginning of breeding season		Middle of breeding season		Beginning of non-breeding season
		Nest	No Nest	Nest	No Nest	No nest preference
Testosterone	M	0.48±0.01	0.87±0.45	2.12±1.08	1.22±0.00	0.19±0.20
	F	1.20±1.24	no females	0.33±0.17	0.21±0.17	0.16±0.10
Corticosterone	M	not collected		4.27±2.02	4.63±0.00	1.27±0.55
	F			4.83±3.89	3.73±2.31	3.00±1.25

Fig. 1. Photo of European starling semi-natural environment.

Fig. 2. Nest box treatment and GnIH peptide cell abundance at the beginning and middle of the breeding season. All bird pairs with nests were laying or incubating eggs during the middle of the breeding season, except one pair, seen here as the two bottom outliers.

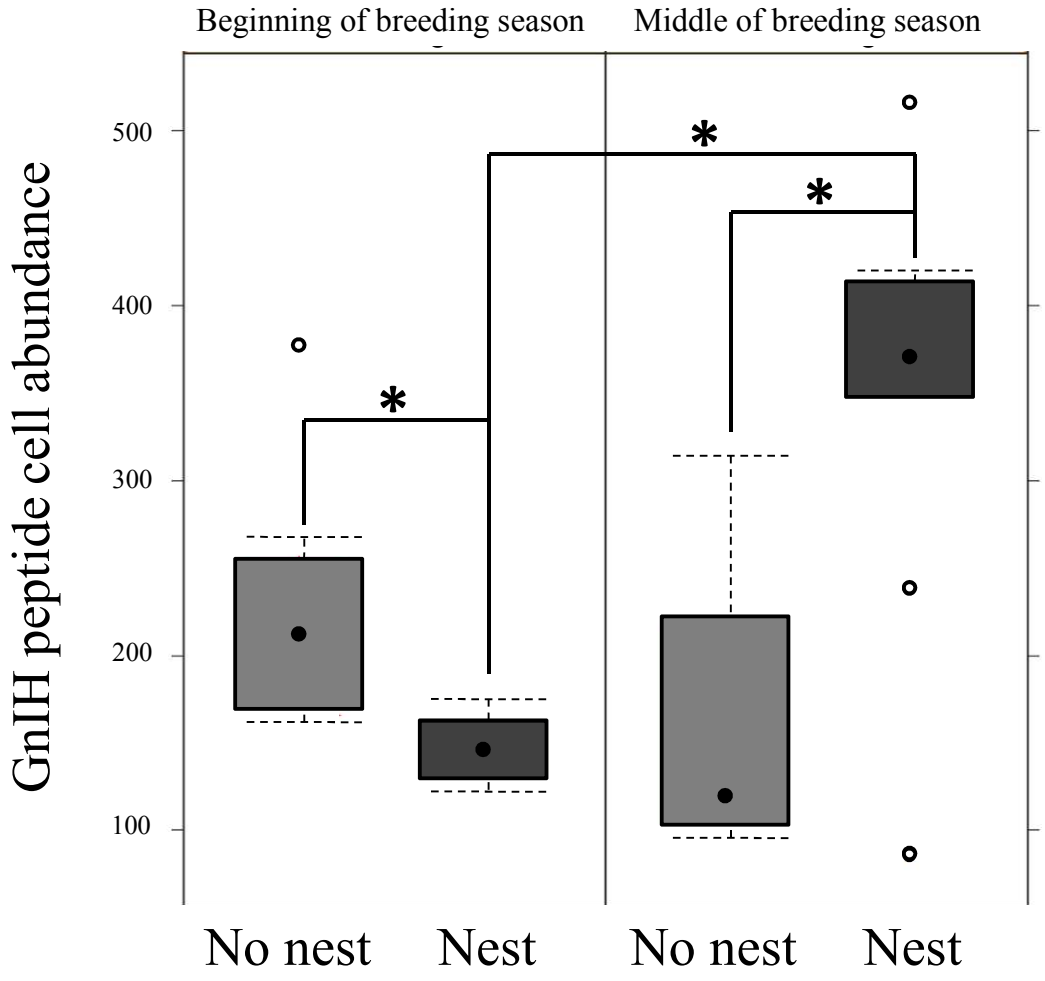


Fig. 3. GnIH peptide cell abundance and nestbox treatment during the breeding season. Cells were visualized using Vector VIP, resulting in a brownish-purple label. Photos were taken at 20X, and scale bars are set at 100µm.

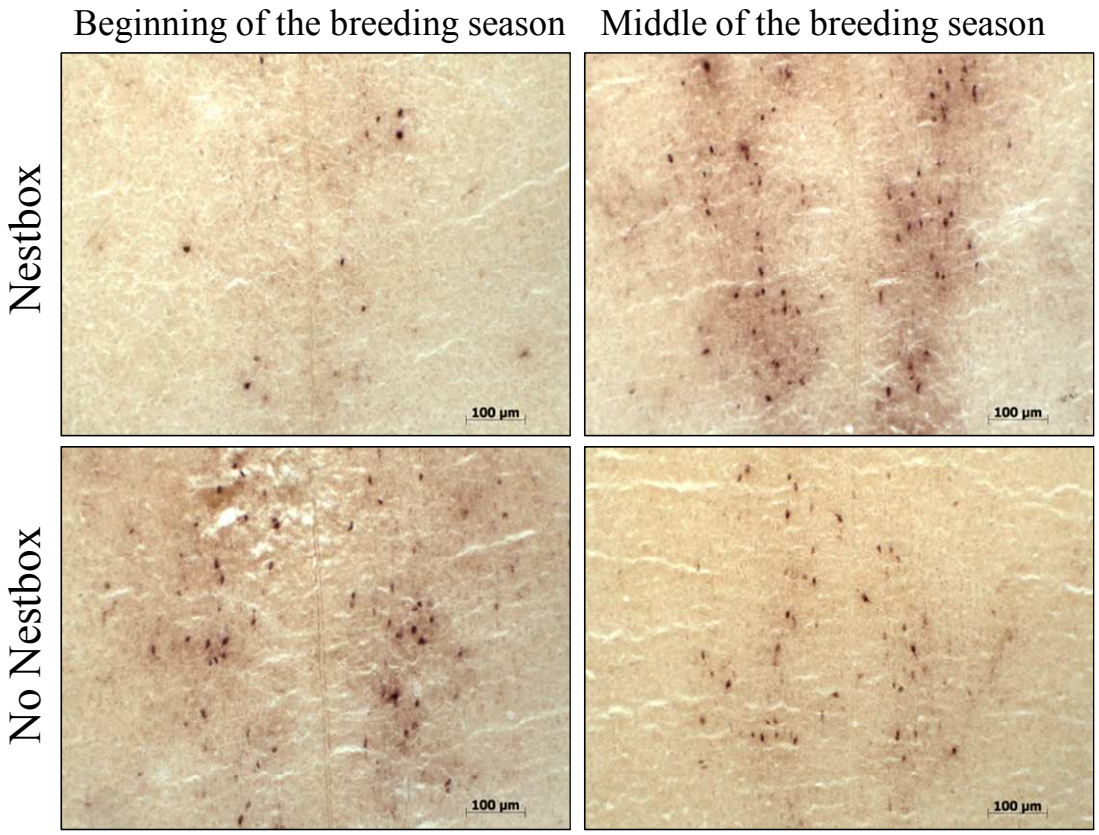
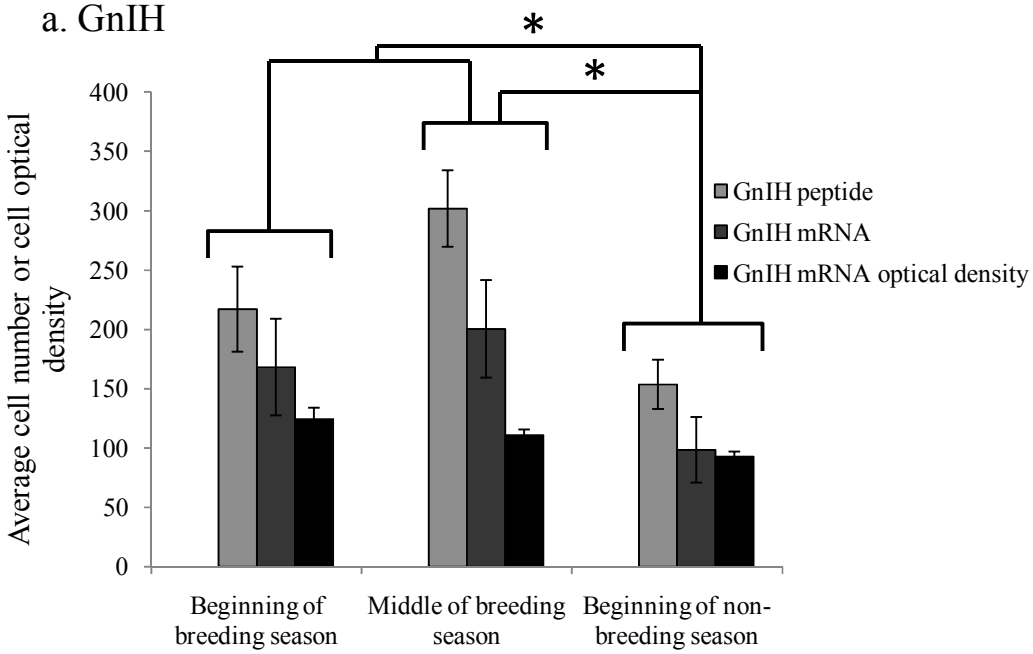


Fig. 4. Seasonal distribution of a) GnIH peptide and GnIH mRNA cell number, and GnIH cell optical density, and b) GnRH-I peptide cell number. The average cell number and optical density, and the standard error of all birds per treatment were plotted. Male and female cell numbers and optical density were not significantly different within treatments and thus data were combined to increase sample size. During both sample points in the breeding season, a) cells immunoreactive for GnIH peptide, GnIH mRNA and the optical density of GnIH mRNA cells and b) cells immunoreactive for GnRH-I peptide were more abundant, or had a higher GnIH mRNA cell optical density, than during the beginning of the non-breeding season; however, GnIH peptide and GnIH mRNA cell abundance and GnIH mRNA optical density were not different at the beginning of the breeding season as compared to the beginning of the non-breeding season.



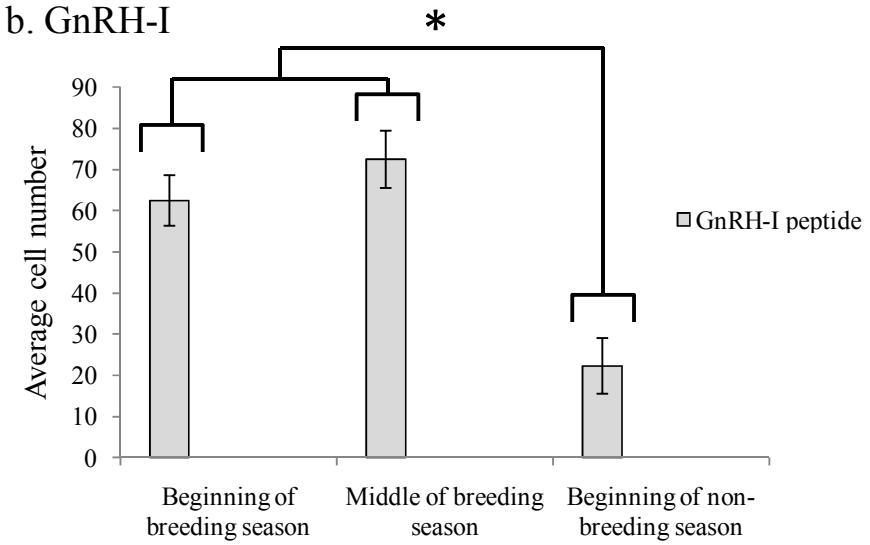
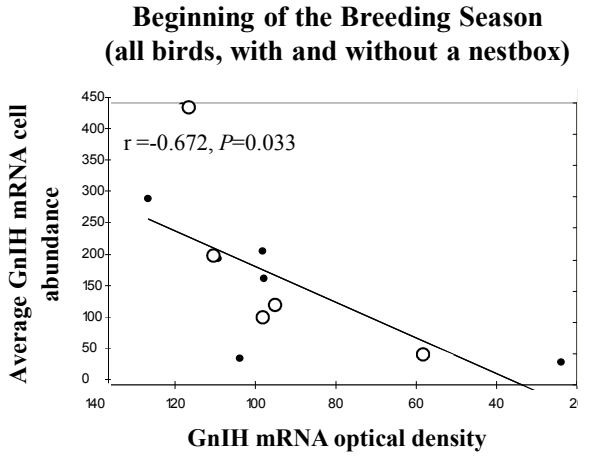
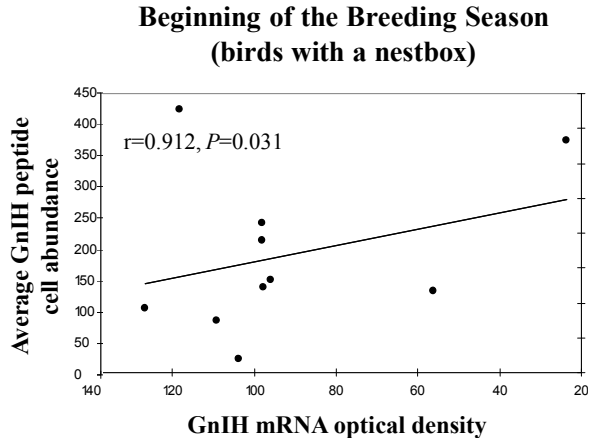
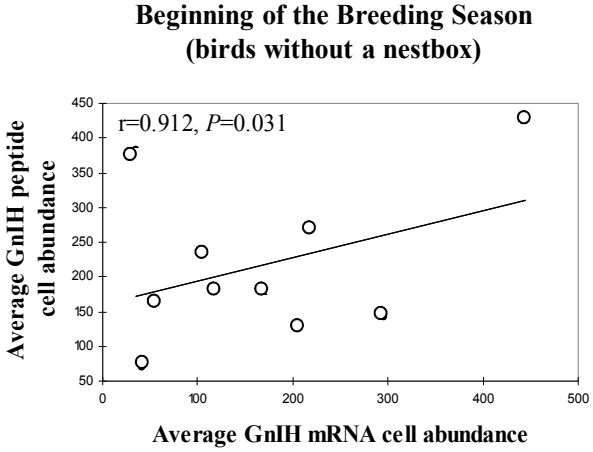


Figure 5. Statistically significant correlations between hypothalamic GnIH peptide and GnIH mRNA cell abundance and GnIH mRNA optical density. Closed circles indicate individuals with a nestbox. Open circles indicate individuals without a nestbox.







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Chapter 4

Stress, the brain and reproduction: implications for conservation neuroendocrinology

Stress, the brain and reproduction: implications for conservation neuroendocrinology

Abstract

Certain types of stress inhibit reproduction. In order to minimize or alleviate the negative effects of stress on the reproductive system, we need to understand better the neural control of reproduction and sexual behaviors. Examination of the brain is largely neglected because of the challenges of studying it in endangered species. However, the use of non-endangered models can greatly increase our knowledge of important reproductive neuroendocrine processes. This type of information has profound conservation implications as many species, endangered or otherwise, exhibit a decrease or termination of reproductive activity and behaviors under captive conditions, stressful or otherwise. Gonadotropin releasing hormone (GnRH) is a key hormone in activating the reproductive system. Two more recently discovered neurohormones, kisspeptin (KISS) and gonadotropin inhibitory hormone (GnIH), are involved in the regulation of GnRH. Evidence suggests that both KISS and GnIH play important complementary roles in precisely driving reproductive function, with KISS serving as an accelerator and GnIH serving as a restraint on GnRH and the reproductive system. Both KISS and GnIH belong to the RFamide peptide family and are affected by stress, and we discuss their roles in the stress response and conservation implications. By understanding the role of these RFamide peptides in reproduction and sexual behaviors in response to stress, we can develop pharmacological tools with which to improve the success of captive breeding programs.

Introduction

In this paper, we highlight some of the major conservation issues we are faced with today and why understanding the interplay between brain and endocrinology is important. Examination of the brain is largely neglected because of the challenges of studying it in endangered species. However, understanding how the brain responds to the environmental perturbations or specific housing situations is key to understanding the mechanisms underlying the resulting reproductive dysfunction. By examining the neural and peripheral endocrine responses of non-endangered models to particular stimuli and housing conditions, we can identify physiological targets for manipulation to minimize inhibition of reproduction by situations that might be perceived as stressful – either acutely or chronically so.

Here we define stress as the response of an individual to an unpredictable event in attempt to maintain allostasis, or stability through change (McEwen and Wingfield 2003). Acute stress can entail an initial fight or flight response, physiologically characterized by acetylcholine released from preganglionic sympathetic nerves, triggering the release of epinephrine and norepinephrine from the adenal glands. We will be focusing on the effects of more chronic stressors which require a constant state of response. This chronic stress response, whether it be "high" or "low" level stress, can incur allostatic load, or cumulative costs to the body which can result in serious pathophysiologies (McEwen and Wingfield 2003), such as reproductive dysfunction and infertility (Siegel, 1980; Moberg, 1991; Moore and Jessop, 2003; Boonstra, 2004; Breuner et al. 2008). Physiologically, when the stress axis is stimulated, a hormonal cascade is set into motion. Corticotropin releasing hormone is released from the brain (specifically, the hypothalamus) and ultimately glucocorticoids, or "stress" hormones (cortisol, corticosterone), are released from the adrenal glands. Glucocorticoids promote the metabolism of fat stores for energetic demands and inhibit temporarily 'unnecessary' activities, such as digestion and reproduction, until the stressor has passed (Sapolsky 1992, Wingfield and Sapolsky 2003). However, constant high concentrations of adrenal glucocorticoids are often correlated with reduced reproductive activity (Moberg, 1985; Sapolsky, 1987, 1992; Romero and Sapolsky, 1996; Wingfield and Sapolsky, 2003). Chronic moderate stress can also suppress reproductive function (Williams et al. 1997, Cameron 2000, Ominski et al. 2003), though we have very little understanding of this phenomenon. We propose that these more subtle chronic stress levels may be responsible for the reproductive disruption in many captive wild species (Fig. 1).

Stress and reproduction: conservation problems

Human disturbance

Many species are faced with overwhelming habitat destruction, fragmentation, contamination and increased competition for resources, and thus are likely to be incurring increased allostatic load. Characterizing interactions among human disturbance, stress and reproductive physiology are important for conservation efforts. For example, female African elephants (*Loxodonta africana*) in areas with historically high poaching risk have higher fecal glucocorticoid concentrations than those in areas with low poaching risk, and females from disturbed groups in which older matriarchs had been poached have a lower reproductive output than females from intact groups, despite many being in their reproductive prime (Gobush et al.

2008). In another example from Larson et al. (2009), sea otters (*Enhydra lutris*) which once ranged freely throughout the north Pacific have since been broken up into smaller scattered populations due to the fur trade. These populations experienced bottlenecks, reducing their genetic variation and resulting in a general reduction of fitness, or inbreeding depression. Larson et al. (2009) found a negative correlation between sea otter (*Enhydra lutris*) genetic diversity and both mean population-level and individual-level glucocorticoid concentrations, suggesting this increase in glucocorticoids may have negative long-term consequences on the overall health of the population. Yellow-eyed penguins (*Megadyptes antipodes*) in highly frequented tourist areas have higher stress-induced glucocorticoid concentrations and lower reproductive success than birds in less trafficked areas (Ellenberg et al. 2007). In a final example, human-engineered chemical contaminants, such as pesticides, can increase glucocorticoid concentrations and decrease immune function, development, survival and reproduction in many species, particularly amphibians (Hayes et al. 2006).

However, some species have baseline and stress induced glucocorticoid concentrations that are unchanged or lower when faced with human disturbance (Schoech et al. 2004, Partecke et al. 2006, Walker et al. 2006, French et al.), and others seem to thrive in urbanized areas (Partecke et al. 2006, Schoech et al 2004). Are these species "less stressed", or have they become physiologically habituated to their captive surroundings – and how do these changes affect reproduction? While peripheral hormone concentrations are relatively easy to measure and yield insightful data, we cannot fully understand these discrepancies without understanding all levels of the stress axis. Many negative effects from human disturbance appear to be mediated by increases in glucocorticoid concentrations, but the mechanisms for these effects remain to be identified and we call for their further examination at the level of the brain.

Breeding in captivity

The *ex situ* genetic management of small captive populations of endangered species is important for many reasons, such as the preservation of species genetic variation, education of the general public and/or ultimately re-introduction into the wild. However, getting certain species to breed and exhibit parental behaviors in captivity is not an easy task (Bronson 1998, Wei et al. 2009, reviewed in Calisi and Bentley 2009; Stead-Richardson et al. 2010). Baseline and stress-induced glucocorticoid concentrations can change when a wild animal is brought into captivity. For example, captive white-throated sparrows (*Zonotrichia albicollis*) and white-crowned sparrows (*Zonotrichia leucophrys*) experience a 2-3 fold increase in glucocorticoid concentrations when brought into a captive setting, and this elevation lasts even after 35 days of acclimation (Marra et al. 1995). However, some species experience a decrease in glucocorticoid concentrations as compared to their wild counterparts (Kunzl and Sachser 1999; Cyr and Romero 2008; Woodruff et al. *in press*). Why do these differences in response exist, how are they mediated and how are they manifested reproductively? For captive breeding conservation efforts to be successful, we need to understand better how certain aspects of captivity affect the neurobiology of stress and reproduction. For example, in 2000, Wingfield and colleagues reported that the population of the Toki, or Japanese Crested Ibis (*Nipponia Nippon*) was reduced to a single female in Japan. Conservation efforts to breed this remaining bird and others before it were unsuccessful. Authors state, "Given the critically low numbers of Toki it is clearly important to determine as accurately as possible the reasons for reproductive failure."

Climate change

Species declines and extinctions are becoming more widespread in response to global climate change (Parmesan and Yohe 2003, Thomas et al. 2004, Thuiller et al. 2005, Pounds et al. 2006; Devictor et al. 2008, Sekercioglu et al. 2008, Wingfield 2008). Climate change can result in the mis-timing of seasonal activities, such as the failure to adjust breeding to peaks in food availability (Visser and Both 2005, Both et al. 2006). Variation in the timing of these activities can influence the expression of sexually-selected characters, mating systems and patterns of paternity (Quin et al. 1996, Storz et al. 2001, Isaac and Johnson 2003, Moller and Szep 2005, Weatherhead 2005a,b, reviewed in Isaac 2009). For example, Moller and Szep (2005) report that male barn swallows (*Hirundo rusica*), in association with rapid change in environmental conditions, are experiencing rapid micro-evolutionary change in the length of their outermost tail feathers, feathers which can provide a mating advantage (Moller and Szep 2005).

In light of the issue of allostatic load and reproduction – the increase in global warming is associated with increased prevalence and, critically, longevity of associated unpredictable events that occur in the environment, such as storms, droughts and fire. Because chronic activation of the stress response by unpredictable events can incur allostatic load and result in reproductive dysfunction and infertility (Siegel, 1980; Moberg, 1991; Moore and Jessop, 2003; Boonstra, 2004; Breuner et al. 2008), understanding how the reproductive neuroendocrinology and physiology of animals respond to these events is extremely timely in the face of a global warming crisis. For example, baseline and stress-induced glucocorticoid concentrations in the superb starling (*Lamprotornis superbus*) changed across years as a function of pre-breeding rainfall, and dominant and subordinate individuals responded differently (Rubenstein 2007). In another example, incubating male Wilson's Phalaropes (*Phalaropus tricolor*) had low circulating concentrations of the pituitary hormone prolactin during a drought year (Delehanty et al. 1997). Clutch size during non-drought years was related to circulating prolactin; however, concentrations were variable, and additional factors could not account for the variation in prolactin explained by clutch size and year (Delehanty et al. 1997). While both examples highlight our knowledge of environmental change on the circulation of peripheral hormones, we still know very little about how the brains, particularly the reproductive neurohormones, of these organisms respond. Examination of upstream neuroendocrine responses to climate change may elucidate these unexplainable variations in circulating peripheral hormones.

Contributions of endocrine physiology to conservation

Conservation physiology is an emerging discipline that has the potential to contribute significantly to conservation efforts, both in captive and wild settings. Wikelski and Cooke (2006) argue that for conservation strategies to be successful, we must understand the physiological responses of organisms to changing environments. Techniques that measure physiological responses of individuals to a whole host of environmental challenges continue to evolve. Research has laudably focused on non-invasive methods to measure endocrine responses of species in both captive and human-impacted wild settings. Methods of stress assessment via glucocorticoid metabolite concentrations in urine and feces have been used to investigate stress physiology in the wild (Wasser et al. 1988, 1997, 2000; Creel et al. 1991, 1996; Monfort et al. 1997; Palme et al. 1998; Creel et al. 2002, Mashburn and Atkinson 2004) and captivity (Wells et

al. 2003, Cyr and Romero 2008, Franceshini et al. 2008, Nilsson et al. 2008, Petrauskas et al. 2008). Urine and fecal samples can be representative of circulating glucocorticoids over an extended time period, rather than a point sample. This can be advantageous, as measurement reflects an average value of long-term glucocorticoid concentrations. For example, in 2002, Grevy's zebras (*Equus grevvi*) were translocated from a privately-owned park to Meru National Park as part of a Kenya Wildlife Service re-population initiative. Franceshini et al. (2008) used fecal glucocorticoid metabolite concentrations as a tool to measure pre-capture, captive and post-release stress. Metabolite concentrations were significantly higher during time spent in captivity and took as long as 18 weeks post-release into Meru Park to return to baseline, suggesting final acclimation into the new environment (Franceshini et al. 2008).

An animal can encounter various stressors (social, predation, nutrition, etc.) and fluctuations in normal and seasonal daily rhythms over an extended period of time. The utility of fecal assay techniques can be confounded by the various events and circumstances that can occur before the time of collection, masking specific stressors that may be a risk to conservation efforts (reviewed in Millspaugh and Washburn 2004). By directly examining timely responses to stressors, particularly at the level of the brain, we can identify specific threats to reproduction and ultimately conservation. However, our understanding of how the brain processes the impacted environment in which wild species live/are housed is very limited.

Much of our understanding of stress effects on the brain has come from highly inbred rodent strains (reviewed in Seyle 1976, Kim and Diamond 2002, Moralik et al. 2005, Luine et al. 2007). The data these inbred models have yielded are invaluable and have played major roles in the advancement of our knowledge of neuroendocrinology in general. However, caution must be taken when interpreting data from such models, as lab rodent strains are far-removed from species that have adapted to survive and reproduce in a natural environment. For example, thalidomide, first tested on lab rodents and shown not to have substantial negative effects on embryonic development, was prescribed as a sedative for pregnant women during 1957-1961, resulting in 10,000-20,000 birth defects around the world (reviewed in Brent 1964). Though this is an extreme example of differential species responses to a drug, it serves as a cautionary tale for the interpretation of physiological responses deduced from inbred lab rodent strains. Thus, in addition to lab rodent models, we call for examination of neuropeptide systems of non-endangered wild species whose neuroendocrine structure, function and modulation by other hormones are conserved across species.

The neuroendocrinology of stress and reproduction

The brain is the central processing point for the perception of stressors as well as for the activation of reproduction and its associated behaviors. Thus, in order to minimize or alleviate the negative effects of stress on the reproductive system, we should understand the neuroendocrinology of stress and reproduction. We have gained much insight about the ways in which stress acts to influence the reproductive axis (see reviews: Breuner et al. 2008; Busch and Hayward 2009, Schoech et al. 2009), though we still do not fully understand the physiological underpinnings involved, particularly within the brain – especially in wild species. When the reproductive axis is activated, the hypothalamus produces gonadotropin releasing hormone (GnRH), a neuropeptide responsible for regulating reproduction and its associated behaviors. GnRH is released from the hypothalamus and causes the anterior pituitary gland to release the gonadotropic hormones (luteinizing hormone [LH] and follicle stimulating hormone [FSH]) into

the bloodstream. Gonadotropins act on the gonads and cause them to develop and increase in activity, and gonadal steroids (androgens and estrogens) are synthesized, released and feedback onto the reproductive axis to regulate it, along with modulating physical and behavioral secondary sexual characteristics.

Many animals have trouble breeding in captivity, and this may be due to low circulating concentrations of reproductive hormones (reviewed in Calisi et al. 2009). For example, during the breeding season, male kestrels (*Falco tinnunculus*) have 3-fold lower concentrations of androgen hormones in captivity as compared to the wild (Meijer and Schwabl 1989). This difference may be due to in part to the influence of social interactions on the physiology and resulting behavior (or vice-versa; Wingfield et al. 1990; Dufty and Wingfield 1986), changes from a natural to a captive diet (Kudwa et al. 2007, Guan et al. 2008), and/ or range restrictions in captivity (Bronson 1998). Of late, administration of GnRH is being used to counter the negative effects of captivity on reproduction. For example, a GnRH agonist (binds to and activates the GnRH receptor) given to captive Asian elephants (*Elephas maximus*) at a specific time of the pre-ovulatory phase will induce the ovulatory LH surge responsible for ovulation (Thitaram et al. 2009). In another example, Mylonas and Zohar (2001) report that captive striped bass (*Morone saxatilis*) and other bass of the *Morone* genus treated with a GnRH agonist experienced increases in circulating LH, production of spermatozoa, an induction of ovulation and spawning. Similarly, a new slow-release implant of a GnRH analogue was tested on Budgerigars (*Melopsittacus undulatus*) as a model for captive-bred endangered birds, and implanted birds showed higher rates of egg fertilization and laying compared to controls (Costantini 2009). Cockrem et al. (2004) have been using Japanese quail (*Coturnix coturnix*) to develop a hormonal method involving GnRH and pregnant mare serum gonadotropin (PMSG) to stimulate breeding in the endangered kakapo (*Strigops habroptilus*). In sum, knowledge of the GnRH system and its effects on reproduction have led to the successful development of GnRH agonists and the like as direct tools for inducing reproduction in captive endangered species. Using non-endangered models as proxies for their related counterparts may yield valuable ways in which to manage reproduction in endangered species.

Certain types of stress can have a negative effect on GnRH expression in the brain and subsequent LH pulse amplitude (reviewed in Tilbrook et al. 2000; Calogero et al. 1999; Ciechanowska et al. 2007; Wagenmaker et al. 2009), but the actual mechanism(s) underlying how stress affects GnRH and thus reproduction is not understood. Two more recently discovered hypothalamic neuropeptides, kisspeptin (KISS) and gonadotropin inhibitory hormone (GnIH), are involved in the regulation of GnRH. KISS can act on GnRH to activate the reproductive axis. It has been implicated in puberty onset, ovulation and metabolic regulation of fertility (Irwig et al. 2004, Han et al. 2005, Castellano et al. 2006, reviewed in Tena-Sempere, 2010). GnIH can also act on GnRH, but in this case, as its name implies, to inhibit gonadotropin release and reproduction (reviewed in Bentley et al. 2009 and Tsutsui 2009). Evidence suggests both KISS and GnIH play important complementary roles in precisely driving reproductive function, with KISS serving as an accelerator and GnIH serving as a restraint on GnRH and the reproductive system (reviewed in Kriegsfeld 2006 and Smith et al. 2008; Fig. 2). While GnIH has been identified in all species examined (fish, birds, rodents, ungulates and primates; Bentley et al. 2009, Tsutsui 2010), KISS has been identified in mammals, fish and amphibians, but does not appear to be present in birds (Felip et al. 2009, Tena-Sempere 2010). Both KISS and GnIH are affected by stress (reviewed below), though currently, clear evidence exists only for the direct

effect of stress on reproduction at the level of the brain via GnIH, and we focus our discussion on the role of this neurohormone in the stress response and its conservation implications.

Stress and reproduction: a role for KISS

Kinsey-Jones et al. (2009) report administration of corticotropin releasing hormone or restraint stress resulted in decreased expression of KISS and its receptor in the rat brain as well as decreased LH pulsatile secretion or increased time between LH pulses. Both acute and chronic administration of corticosterone decreased KISS expression, though it increased expression of its receptor and had no effect on LH interpulse interval. The authors posit that the downregulation of KISS and the upregulation of its receptor in the latter two experiments may somehow serve as a buffer against the negative effects of corticosterone on LH secretion (Kinsey-Jones t al. 2009).

With the growing human population, increasing environmental contamination is a concern, as it could serve as a stressor affecting development and reproduction. Environmental contaminants such as pesticides can be immunocompromising and disrupt endocrine processes involved in development and reproduction. These effects have been reported in association with amphibian population declines, as amphibians are highly susceptible to chemicals in the environment due to their permeable skin and tendency to live and reproduce in the water (Hayes et al. 2006). To study the effect of immunological challenges experienced in early life on the KISS system, Knox et al. (2009) injected female rats with lipopolysaccharide (LPS), an agent known to activate the immune response, or saline as a control. They found that KISS expression, though not its receptor, is downregulated in pre-pubertal rats, and this downregulation is related to the delay of the onset of puberty. These results support the role of KISS in the onset of puberty and that its function is influenced by immunological stress. Similarly, Iwasa et al. (2008) found that LPS injection decreased KISS expression in the brain as well as circulating LH concentrations, and injection of KISS increased LH concentrations. These studies demonstrate the sensitivity of KISS to immune challenges and its effects on the reproductive axis. Thus, we ask how environmental contaminants, such as those that negatively affect the immunology and endocrinology of amphibians (Hayes et al. 2006), are affecting KISS and its influence on puberty onset and reproduction?

Stress and reproduction: a role for GnIH

While the direct effects of stress on KISS and GnRH are unknown, recent studies have revealed 1) an influence of stress upon the GnIH system in the brain, and 2) a direct mechanism for glucocorticoids, and thus the stress response, to be communicated to GnRH and the reproductive axis via GnIH. Gonadotropin inhibitory hormone (GnIH), first discovered in birds (Tsutsui et al. 2000), inhibits gonadotropin (LH and FSH) release from the pituitary gland (Tsutsui et al. 2000, Osugi et al. 2004, Ciccone et al. 2004, Ubuka et al. 2008) in both birds and mammals (Kriegsfeld et al. 2006, Johnson et al. 2007, Murakami et al 2008, Clarke et al 2008), indicating the conserved nature of its actions across taxa. Two important pieces of evidence suggest that GnIH neurons can directly influence GnRH neurons. First GnIH fibers appear to make direct contact with GnRH neurons allowing for direct communication (birds: Ubuka et al. 2008, sheep: Smith et al. 2008, rodents: Johnson et al. 2007, Kriegsfeld et al. 2006, primate: Ubuka et al. 2009, humans: Ubuka et al 2010) and second, receptors for GnIH are expressed by avian GnRH neurons (Ubuka et al. 2008) suggesting that the GnIH peptide can bind to GnRH

neurons and influence them and the reproductive axis directly. In mice, direct application of GnIH to GnRH cells in cultured brain slices decreases firing rate of KISS-sensitive GnRH neurons (Ducret et al. 2009; Wu et al. 2009), further suggesting a direct action of GnIH on GnRH neurons and an interaction of GnIH with KISS.

Administration of GnIH directly into the brain of passerine birds and rodents decreases sexual behaviors (Bentley et al. 2006, Johnson et al. 2007). Natural changes in GnIH content are correlated with specific stages of reproduction (e.g. nest establishment, egg-laying and incubation) and are modified in response to social environment (Calisi et al. 2010, *submitted*). Calisi and colleagues propose that individual differences in GnIH content may impact reproductive fitness, paving the way for conservation studies examining the adaptive value of mechanisms underlying physiological responses for breeding.

Capture-handling stress is associated with an increase in GnIH neurons in male and female house sparrows (*Passer domesticus*) during the breeding season, though there is no association during the non-breeding season (Calisi et al. 2008). These data were the first to imply an influence of stress upon the GnIH system that changes over the annual cycle of reproduction (Calisi et al. 2008). In 2009, Kirby et al. reported a similar stress effect on the GnIH system in mammals. GnIH (also referred to by the mammalian ortholog, RFamide-related peptide) was up-regulated in the adult male rat brain in response to both acute and chronic immobilization stress. This increase in GnIH was associated with the inhibition of reproductive hormone activity. Adrenalectomy blocked the stress-induced increase in GnIH expression, suggesting GnIH cells in the brain require adrenal glucocorticoids to respond to stress. Kirby et al. (2009) were the first to report in mammals that GnIH cells express glucocorticoid receptors, providing a mechanism by which "stress" hormones can directly affect GnIH cells and thus reproduction and its associated behaviors (Fig. 2). We partially cloned glucocorticoid receptor in European starling (*Sturnus vulgaris*) and report receptors are expressed also by avian GnIH cells in the brain (Fig. 3, *supplemental methods in Appendix I*). The presence of glucocorticoid receptors in the GnIH cells of both mammals (Kirby et al. 2009) and birds suggests the conserved nature of this mechanism, with implications for the reproductive endocrinology of vertebrates as a whole.

Applications for KISS and GnIH and neuroendocrine research for conservation

Both KISS and GnIH are affected by stress and changes in the environment. However, the GnIH system provides the only known (as of yet) mechanism by which certain types of stress or changes in environment can affect sexual behaviors and reproduction at the level of the brain. Understanding this neural mechanism enables us to more accurately assess the effects of captivity stress and its resulting reproductive dysfunction. While measurements of plasma and fecal glucocorticoids are less invasive and indeed useful to assess overall stress responses, they can also be variable and inconclusive (Millspaugh and Washburn 2004). Thus, more basic research is needed to fully understand how the social, environmental and stressful events affect the reproductive system at the level of the brain. In the example of the Toki, Ishii (1983, 1984 via Wingfield et al. 2000) and Ishii et al. (1994) examined reproductive hormone concentrations in a non-endangered ibis species (*Threskiornis melanocephalus*) to use as a template for reproductive cycle of the Toki. Similarly, non-endangered species (i.e. deer mice, European starlings, cichlids, etc.) can serve as models in the examination of stress and reproductive

function within the brain. With this more complete knowledge of stress and reproductive physiology can we enhance strategies to assuage the actual stressor and/or its biological effects.

Curtis et al. (2010) have developed a potent agonist (binds to a receptor and triggers a biological response) to the KISS receptor from an analog of KISS-10, the smallest known endogenous form of KISS. Administration of this agonist, [dY](KP)-K-1-10, into the body cavity of lab mice dramatically increased circulating concentrations of LH and testosterone which lasted for at least an hour post-injection (Curtis et al. 2010). The use of [dY](KP)-K-1-10 to stimulate reproductive activity could potentially be extended to breeding endangered captive species, and further studies are needed to understand the effects of this agonist in full.

Pineda and colleagues (2010) characterized a selective mammalian antagonist (binds to and blocks a specific receptor without triggering a biological response itself) of GnIH called RF9. The central administration of RF9 to male and female rats resulted in an increase in gonadotropin secretion (Pineda et al. 2010), lending even more support for a role of the GnIH system in the central control of gonadotropin secretion in mammals. This exciting discovery presents an entirely new tool with which to study the role of GnIH in reproduction and sexual behaviors in response to stress. If this peptide can be used to counter some of the effects of stress-induced reproductive dysfunction in captivity, it could offer new solutions for captive breeding in some species. However, Pineda et al. (2010) administered RF9 intracerebroventricularly, and novel, less-invasive delivery methods need to be developed and optimized if this approach is to be widely adopted.

Conclusion

Having a fundamental understanding of the neural basis of reproduction and behavior can have important conservation implications. While we have long observed the negative reproductive consequences of human encroachment, we do not fully understand how these environmental stressors are expressed physiologically. How can endangered species be managed to breed more successfully in captivity? How do certain species or individuals abstain from reproducing in the face of urbanization while others thrive? Characterization of the novel reproductive neurohormones KISS and GnIH and their effects on the reproductive system under simulated or real environmental perturbation may be key to answering these questions.

The discoveries of KISS and GnIH have provided a paradigm shift for work in stress and reproductive physiology, with implications for conservation physiology. By understanding the roles of these neuropeptides in reproduction and sexual behaviors in response to stress, we gain valuable insight into solving stress- and reproductive- related conservation problems. Of course, we are not suggesting that the underlying causes of stress in captivity or due to human disturbance should be ignored, but that even when these causes have been addressed, some species fail to breed. We call for more basic and behavioral research of the KISS and GnIH systems in all organisms. By examining multiple taxa, we can identify what mechanisms are conserved, though variations between species and individuals could yield valuable insight into what neuroendocrinology is associated with greater reproductive fitness when faced with certain conservation issues. We also call for more studies examining the role of KISS agonists and GnIH antagonists in response to stress and environment, as these data can provide new tools and immediate applications for conservation physiology.

Finally, we call for the development of a central brain bank of endangered species so as to understand their neuroendocrine profiles and how their neuroendocrine stress and reproduction physiology compares with non-endangered model species. Upon the death of captive or wild individual, attempts should be made to salvage all tissue, in this case, specifically the brain, in a timely fashion. Immediate extraction of the brain from the skull upon the death of the animal and freezing/storing it in foil on dry ice or at -20° to -80°C is ideal. However, when these conditions for brain storage are not possible, the following methods may still be used to harvest tissue for later analyses: 1) storing the extracted brain in a 4% paraformaldehyde solution, 2) freezing the entire head of the animal (though this is not optimal), 3) approximating the time between the death of the animal and freezing or post-fixing of the brain to assess tissue degeneration. Organizing a brain bank of this nature would provide a bridge for collaboration between conservationists in the field/zoo and neurobiological specialists. We welcome contact with other researchers who would be willing to collaborate with us in coordinating this effort to benefit the scientific community as a whole.

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Fig. 1. Diagrammatic representation of various stress levels and their associated glucocorticoid concentrations over time. While the body responds to acute stress with an increase of glucocorticoids, concentrations return to baseline shortly after the stressor has passed. Chronic exposure to stress can result in a constant increase of glucocorticoid concentrations, negatively affecting multiple biological systems, such as immune function, development, survival and reproduction. However, little is know of how moderate to low levels of stress and glucocorticoid concentrations affect reproduction, and further research is needed.

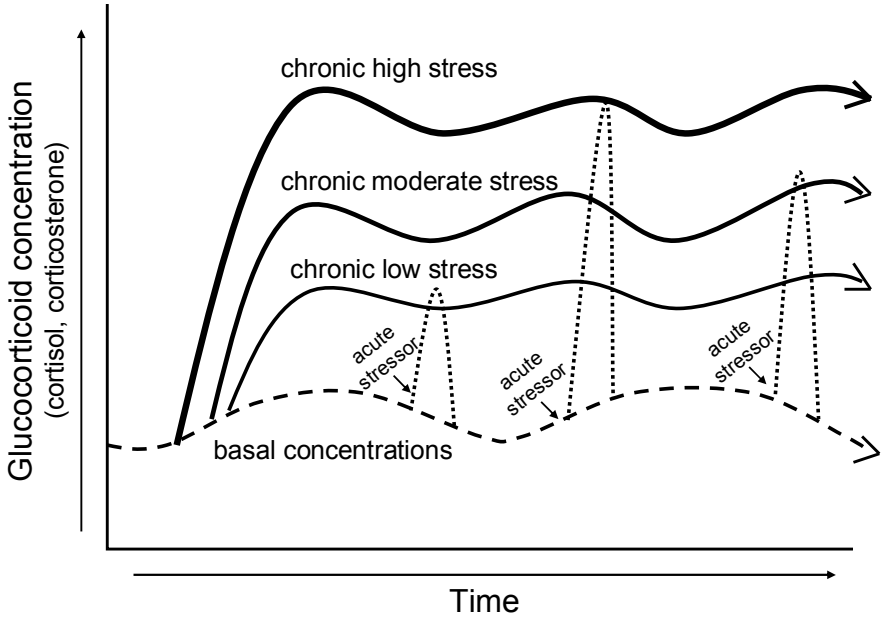


Fig. 2. The reproductive (hypothalamic-pituitary-gonadal [HPG]) axis and the stress (hypothalamic-pituitary-adrenal [HPA]) axis. When the reproductive axis is activated, gonadotropin releasing hormone (GnRH) in the hypothalamus is released, causing the anterior pituitary gland to release the gonadotropin hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the bloodstream. Gonadotropins act on the gonads and cause them to develop and increase in activity, and gonadal steroids (such as testosterone and estradiol) are synthesized, released and feedback onto the reproductive axis to regulate it. Evidence suggests both hypothalamic hormones KISS and GnIH play important complementary roles in precisely driving reproductive function, with KISS serving as an accelerator and GnIH serving as a restraint on GnRH and the reproductive axis. When the stress axis is activated, corticotropin releasing hormone (CRH) is released from the hypothalamus, causing the anterior pituitary gland to release adrenocorticotropin hormone (ACTH) into the bloodstream. ACTH acts on the adrenal glands to synthesize and secrete glucocorticoids (cortisol, corticosterone) which feedback onto the stress axis to regulate it. Currently, only clear evidence exists for the direct effect of the stress axis on the reproductive axis at the level of the brain via glucocorticoid receptors expressed in GnIH cells.

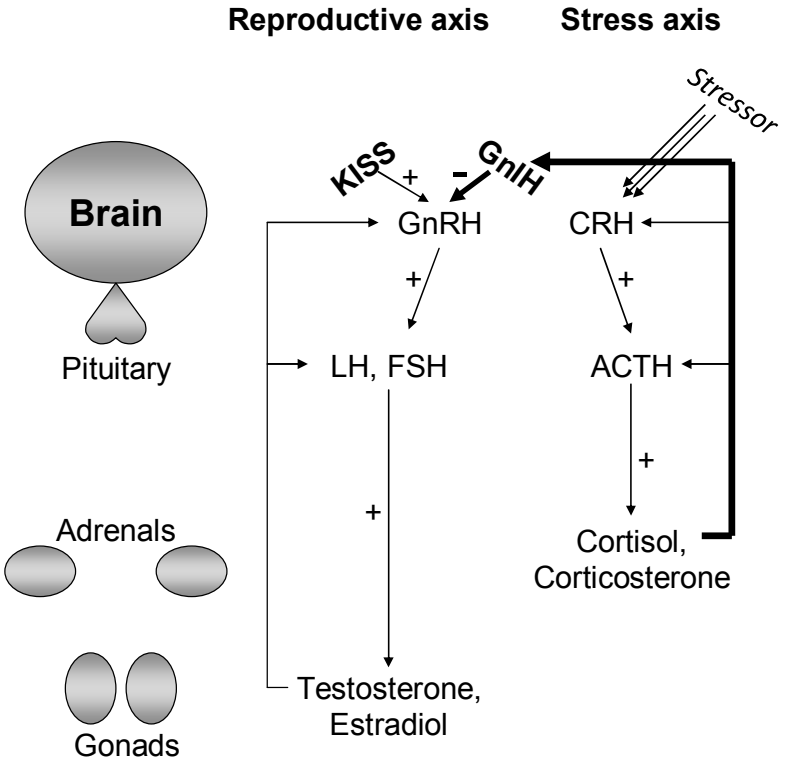
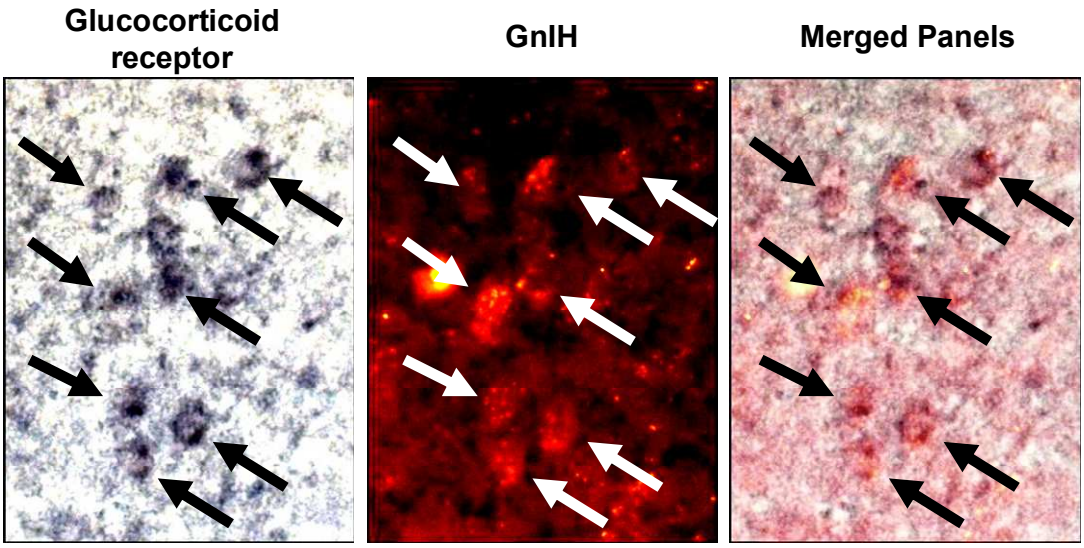


Fig. 3. Co-localization of glucocorticoid receptor with GnIH cells in the European starling paraventricular nucleus of the hypothalamus. First panel: *in situ* hybridization (ISH) labeled cells expressing glucocorticoid receptor mRNA. Second panel: immunocytochemistry (ICC) red-fluorescently labeled cells immunoreactive for GnIH peptide. Third panel: The first and second panels merged to show the same cells expressing glucocorticoid receptor mRNA are also immunoreactive for GnIH peptide.



Appendix I.

Supplemental Methods

Partial cloning of glucocorticoid receptor (GR) and labeling for GnIH:

We identified a cDNA encoding European starling GR precursor following the methods of Ubuka et al. (2009). Total RNA (including rRNA and mRNA) was isolated by using TRIZOL (Invitrogen, Carlsbad, CA). Total RNA was reverse transcribed by using an oligo (deoxythymidine)15 primer (Promega, Madison, WI) and reverse transcriptase (M-MLV Reverse Transcriptase; Invitrogen). Partial European starling GR precursor cDNA was amplified with polymerase chain reaction (PCR) by using various primers (Table 1) based on zebra finch GR precursor cDNA sequence (*Taeniopygia guttata*; Hodgson et al. 2007). All PCR amplifications were performed in a reaction mixture containing Taq polymerase (TaKaRa Ex Taq; Takara Bio, Shiga, Japan). PCR products were subcloned into a pGEM-T Easy vector (Promega), and the DNA inserts of the positive clones were amplified by PCR with universal M13 primers. Amplified DNA was sequenced at the University of California Berkeley DNA sequencing facility by using 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA), and a fragment of European starling GR precursor cDNA was determined. To identify the 3_ end of the European starling GR precursor cDNA, first-strand cDNA was reverse transcribed with the oligo(dT)-anchor primer (5_/3_ RACE Kit, 2nd Generation; Roche Diagnostics, Mannheim, Germany) and poly(A)_ RNA, which was isolated by using an mRNA isolation kit (Roche Diagnostics, Indianapolis, IN). Gene-specific forward primers and PCR anchor primer (Roche Diagnostics) were used to amplify the 3_ end of the European starling GR precursor cDNA. The PCR products were subcloned and sequenced as described above. To identify the 5_ end of the European starling GR precursor cDNA, the template cDNA was reverse transcribed with a genespecific reverse primer followed by poly(A) tailing of the cDNA with dATP and terminal transferase (Roche Diagnostics). The tailed cDNA was amplified with the oligo(dT)-anchor primer and a nested gene-specific reverse primer. A second PCR was performed by using PCR anchor primer and a further nested gene-specific reverse primer. The second PCR products were subcloned and sequenced as described above.

We performed *in situ* hybridization (ISH) according to Ubuka et al. (2008) to visualize cells expressing glucocorticoid receptor in the hypothalamus. We produced a DIG-labeled antisense RNA probe using a standard RNA labeling kit (Roche Diagnostics), and used the partial European starling GR precursor cDNA as a template. After hybridization, sections were incubated with alkaline phosphatase-labeled sheep anti-DIG antibody (Roche Diagnostics), and the immunoreactive product was visualized by immersing the sections in a substrate solution (Nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt). We controlled for the specificity of the ISH using a DIG-labeled sense RNA probe, the sequence of which was complementary to that of the antisense probe.

Directly following ISH, sections were washed five times in 0.2% PBS-T and we performed immunocytochemistry (ICC) to visualize cells expressing the GnIH peptide in the hypothalamus. Goat anti-rabbit affinity-purified GnIH primary antibody (code: PAC 123/124, Bentley) was used to incubate sections for 48 h at a concentration of 1:5000 in 0.2% PBS-T.

Three subsequent washes in 0.2% PBS-T were followed by an hour of incubation in Alexa Fluor 568 (Invitrogen Labs) at a concentration of 1:500 in 0.2% PBS-T for visualization.

Table 1. PCR primers used to partially clone European starling GR precursor cDNA

Forward 1	5' -CTTCTGCAGTACTCCTGGAT - 3'
Forward 2	5' - GTTCCTGATGGCTTTTGCT - 3'
Forward 3	5' - GCAGTCCAACGGCAACCT - 3'
Reverse 1	5' - CAGCAGTTTAGTCAGTTGAT - 3'
Reverse 2	5' - CCACATCATGCATAGAGT - 3'
Reverse 3	5' - TGTCTGGAAGCAAAAGCT - 3'

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Bibliography

Rebecca M. Calisi was born and raised in Dallas, Texas. She received her B.A. in Psychology from Boston College and became an elementary school teacher for the Dallas Independent School District (2001-2002). In late 2002, she was employed by the Dallas Zoo and Aqaurium, Department of Conservation Education and Research, and developed a love for reseach while working with Zoo biologists. She was awarded her M.S. in Biology from the University of Texas at Arlington in 2006. Her M.S. thesis explored the endocrinology of Mexican boulder spiny lizards, *Sceloporus pyrocephalus*, a species in which females are brightly colored and exhibit aggressive behavior during the breeding season. In 2006, she was accepted into the doctoral program (Integrative Biology, lab of Professor George E. Bentley) at the University of California, Berkeley, to study how hormones in the brain responsible for reproduction and sexual behavior are affected by stress and social environment. She received her Ph.D. in 2010 and was awarded a post-doctoral fellowship from the National Science Foundation. Advised by Professor John C. Wingfield at U.C. Davis, her post-doctoral reseach seeks to understand how the brain mediates transitions in reproductive behaviors.