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By

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Abstract

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Reproduction is a critical component of an animal's fitness, but it is also energetically intensive. In order to maximize reproductive fitness, organisms modulate their reproductive investment or timing in response to environmental cues that provide information about resource availability or other indicators of reproductive success. Physiologic systems that monitor these cues then influence both reproductive readiness and more scaled measures of investment, including number of offspring or offspring provisioning effort. This kind of physiologic modulation is especially salient to mammals, for whom reproduction (gestation and lactation) is exceptionally intensive, and there is extensive documentation of variation in reproductive effort and success under specific environmental and physiological challenges. Still, the underlying mechanisms that coordinate these shifts in investment remain poorly defined and understood.

This dissertation identifies novel endocrine and paracrine mechanisms that regulate ovarian and uterine function in the context of reproductive success. Chapter 1 summarizes our current understanding of female reproductive function and modulation, ultimately identifying 4 key areas of modulation for which the molecular mechanisms are poorly understood or defined: (1) evaluating autonomic nervous system regulation of reproductive organs in ecophysiological contexts, (2) identifying new signals regulating ovary and uterine function; (3) determining the extent to which reproductive stage (e.g., pregnancy) alters regulatory networks and sensitivity; and (4) scaling function of specific cell types or organs to organism-level reproductive outcomes. Chapters 2-5 address areas 2-4 by identifying new mechanisms of mammalian reproductive system control in the ovary and uterus and by evaluating the function of established signaling networks in other reproductive states.

Chapter 2 interrogates the direct sensitivity of the ovary to physiological cues using mouse ovarian explants. By using pharmacologic inhibition of glucose metabolism,

these experiments identify novel glucose sensitivity in the ovary. Chapter 3 examines the effect of neuropeptide gonadotropin inhibitory hormone (GnIH) on ovarian follicle growth, survival, and steroidogenesis in the domestic cat. As has been shown in other mammals to-date, GnIH promotes follicle degradation *in vitro*. These findings support a conserved role for GnIH in vertebrate ovarian folliculogenesis. Together, these chapters provide new paracrine and endocrine mechanisms of ovarian regulation that adds to our molecular understanding of how reproductive effort and investment are modulated.

Chapter 4 explores the sensitivity of feline uterine endometrial epithelial cells to sex steroids. We demonstrate that sex steroids stimulate morphological changes to endometrial epithelial cell 3D growth *in vitro*, and that morphological changes are associated with functional changes in gene expression. Though often manipulated as part of normal fertility treatment, we do not have a good understanding of how these hormones facilitate uterine preparedness for implantation and pregnancy in felines. Chapter 4 thus identifies specific mechanisms of reproductive modulation in the feline uterus and also beings to flesh out molecular mechanisms underlying reproductive failure at the organism level. Our results have direct application to endangered felid breeding programs, which utilize *in vitro* follicle maturation and *in vivo* hormones treatments to promote breeding success of genetically-important individuals.

Finally, chapter 5 uses a classic stress physiology paradigm to identify the mechanism(s) by which psychological stress inhibits ovarian steroid synthesis during pregnancy in mice. These experiments demonstrate that stress-dependent inhibition of ovarian function does not occur through classical, hypothalamic inhibition, emphasizing the dynamic shifts in endocrine function across mammalian pregnancy.

These studies add to our comparative understanding of reproductive function, which is critical because of its fundamental connection to organismal fitness. Taken together, these studies also highlight the utility of *in vitro* approaches for experimentally approaching the challenge of connecting molecular variation to organismal function. We summarize the results and their ultimate importance to female reproductive physiology research in Chapter 6.

Dedication

For my grandmother, Patricia Louise Strachan,
Who has inspired me with her stubborn and self-assured approach to life

For my mother, Ellen Patricia Wilsterman,
Who supported me with fierce support and deep love at every stage of this journey

and

For my first mentor, Z Morgan Benowitz-Fredericks
Who recognized things I did not see in myself at the earliest stages of my career

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1. Introduction

1.1 Reproductive flexibility and fitness

An organism's fitness is determined in part by its ability to reproduce successfully. While some organisms will only get a single opportunity to reproduce (semelparous), most vertebrates are iteroparous, having the potential to reproduce multiple times in a lifetime [1–3]. For semelparous organisms, optimizing reproductive fitness is a function of an animal's ability to allocate all available resources towards their reproduction irrespective of environmental conditions or individual condition. For iteroparous organisms, the math is somewhat more complex. If an organism's access to resources is low in one season, but it is likely to have future opportunities to reproduce in subsequent seasons, it is probably advantageous to skip breeding that year and conserve energy to ensure individual survival, increasing the likelihood of reproductive success in the next season. Alternatively, if the organism is unlikely to have future reproductive opportunities, it should still reproduce now even though it is in poor condition [4,5]. On a finer scale, it may also be advantageous for organisms to vary their effort or investment within a given reproductive bout in response to environmental and endogenous conditions [6]. An organism's ability to "calculate" and "re-calculate" investment strategy using information on its own physiological condition and that of its environment is thus a core mechanism by which iteroparous organisms optimize their fitness.

Though we still do not understand how or whether animals can accurately quantify their future reproductive potential, the past 50 years have seen huge advances in identifying the mechanisms by which animals modify their reproductive investment. Animals do so by flexibly adjusting both their behavior and their physiology, and often adjustments are necessarily coordinated between these systems. Neuroendocrine circuits have emerged as master regulators of these processes, integrating cues and coordinating a response across behavioral and physiological systems. We therefore often treat these systems as performing the proximate "calculation" about the likelihood of reproductive success and the perceived cost to the individual, and thus driving allocation within the bounds of the trade-offs that underlie life history theory [7–9].

Such calculations are especially complex in species where reproduction requires multiple, continuous stages to complete. The neuroendocrine "calculation" about appropriate investment and energy allocation will therefore not only vary with environmental or individual condition leading up to a given reproductive attempt, but also as an animal moves through stages of their reproductive attempt. As an example, a bird just beginning to nest-build that suddenly experiences persistent storms should probably abandon the reproductive attempt because the chicks (and parents!) are unlikely to thrive under the environmental conditions. In contrast, if a mouse has already proceeded through gestation and birth in good condition and is now lactating, it may not be advantageous for the individual to modify investment

in response to degradation in the environment. Although considerable theoretical and experimental work has focused thought around how reproductive stage should influence investment decisions [10–12], ecophysiologists have spent less time experimentally defining and integrating the neuroendocrine systems that control this dynamic flexibility.

1.2 The Female Reproductive Axis

The ability to modify investment through this physiological recalculation may be most relevant to female vertebrates because they tend to be obligate participants in more costly reproductive functions than are males (of course, interesting exceptions to this rule exist). In many bird species, females produce energetically-costly eggs in addition to participating in mating and nest-building, incubation, and chick-rearing; some may even provide chick care following fledge. In mammals, females are physiologically obligated to perform gestation and lactation, and they often also provide additional care after weaning young. We therefore focus on female reproductive flexibility when considering the potential diversity of mechanisms by which recalculation could occur.

In females, the basic system on which modulation must act is the canonical hypothalamic-pituitary-ovary (HPO) axis; this axis is principally responsible for core reproductive functions. The basic set-up and endocrine interactions that control the HPO axis have been the focus of intense study for over a century (e.g. [13]). These three primary nodes, the hypothalamus, the pituitary, and the ovary, communicate via both endocrine and neural signals.

1.2.1 Anatomy of the HPO axis.

The hypothalamus lies within the forebrain and controls a wide variety of behaviors and homeostatic processes via the production and release of neuromodulatory hormones and neuropeptides. Networks within the hypothalamus and connections to other brain regions allow the hypothalamus to transduce physiological and environmental cues and regulate a variety of peripheral physiological systems, including reproduction. The hypothalamus is physiologically connected to the anterior pituitary gland, which lies beneath it, by the median eminence and hypophyseal portal system. Neuroendocrine signals released by the hypothalamus into the median eminence will travel directly and in a concentrated form to the pituitary through the hypophyseal portal system. This portal system allows the neuropeptides to remain at high concentration because it is separate from the peripheral blood supply. In this way, hypothalamic neuropeptides released into the portal system control various secretory cell types in the anterior pituitary gland. The anterior pituitary gland thus releases hormones into general circulation that exert control over peripheral organs, including the ovaries. The ovaries contain several cell types within the ovarian follicles that are sensitive to hormones from the anterior pituitary gland. The ovarian follicle is the functional unit that controls the maturation and development of oocytes for eventual ovulation and fertilization. Thecal cells, which form the outermost layer of the follicle, are recruited from

stromal cells during early follicle development, and they produce steroid hormones (progestins, androgens) that are further processed into estrogens by a second steroidogenic cell type in the follicle, the granulosa cell. Granulosa cells can be found with the oocyte in its most juvenile state (primordial follicles), and they mature and proliferate to form the majority of the follicle as the oocyte matures. Specialized granulosa cells called cumulus cells perform some metabolic functions for the oocyte, whereas granulosa cell layers adjacent to the thecal cells synthesize hormones. Finally, following ovulation, the granulosa cells and thecal cells transform to form corpora lutea, steroidogenic structures critical for producing hormones that facilitate implantation and early pregnancy maintenance in most mammals.

1.2.3 Endocrine signals coordinating the HPO axis

The HPO axis coordinates predictable cycles of reproductive activity in females. Cycling females first go through a phase of ovarian follicular growth, followed by ovulation of mature oocytes from mature follicles. The event of ovulation produces the corpora lutea for which the luteal phase of the cycle is named. This series of events is dependent on hypothalamic production of the decapeptide gonadotropin-releasing hormone (GnRH). GnRH exerts positive control over the pituitary gland. GnRH release into the fenestrated capillary network of the portal system in the median eminence stimulates release of two gonadotropins from the pituitary: Follicle stimulating hormone (FSH) and luteinizing hormone (LH). Follicle-stimulating hormone principally supports ovarian follicle growth, whereas a bolus release of luteinizing hormone stimulates ovulation of mature oocytes from follicles and luteinizes the granulosa and thecal cells to form the corpora lutea. FSH and LH also promote the production of peptide and steroid hormones from the ovary. The peptide hormone inhibin is synthesized by granulosa cells and exerts negative feedback on pituitary gonadotropin secretion. Ovaries produce sex steroids in response to gonadotropins, including progestogens, estrogens, and – to a lesser extent – androgens. Estrogens and progestogens are most well-recognized for their role in regulating feedback on the hypothalamus and pituitary. While both steroids can exert negative feedback, the estrogen estradiol also plays a critical role in positive feedback necessary for triggering the bolus of LH required for ovulation. As follicles mature and grow, their estradiol secretion increases. Once the circulating estradiol concentration reaches a threshold, it switches from exerting negative feedback on hypothalamic circuits to positive, and the effect of the positive feedback is to stimulate release of a large bolus of LH, which triggers ovulation in the ovary. The LH surge also luteinizes the granulosa cells and thecal cells from ovulated follicles, causing a rapid drop in circulating estrogens, thereby switching the system back to negative feedback. Furthermore, the luteinized cells in the corpus luteum begin to secrete predominantly progestogens , which will support early pregnancy maintenance. However, the corpus luteum has a limited shelf-life, and its degradation causes a decrease in progestogens that triggers menstruation in humans and signals the beginning of a new reproductive cycle in animals.

1.3. Completing the canonical HPO system: stress as a case study for failed expansion

Most physiology textbooks lay out the female reproductive system as we have here – a cyclic process controlled through a complex yet closed feedback system. However, for animals to exhibit flexibility in reproductive function, the system must integrate information about perceived environmental quality, social context, and physiological status.

Great strides have been made in the past 20 years towards identifying neural circuitry that facilitates such integration. We now know that the neural networks that respond to environmental and endogenous cues communicate directly and indirectly with GnRH neurons to influence pituitary gonadotropin release. For example, the hypothalamic hormones gonadotropin-inhibitory hormone (GnIH) and kisspeptin (KISS) communicate daily (circadian) rhythms generated in the suprachiasmatic nucleus and activity of the stress axis (hypothalamic-pituitary-adrenal axis) to the HPO axis [14–18]. Some hypothalamic hormones that integrate signals in the brain are also able to regulate gonadotropin release directly from the anterior pituitary (e.g., GnIH, NPY) [19–21]. Nuance is constantly being added to the hypothalamic control over HPO axis function as new neuropeptides and neurocircuits are still being discovered. In the last 20 years, there has also been renewed interest in the roles other cells in the brain, including glial cells and tanycytes, play in regulating GnRH function and circuit remodeling in the axis [22–24].

To date, research laying out these neuroendocrine circuits has primarily focused on male animals and/or cycling females (or, more often, ovariectomized females). Thus, the insight generated over the past two decades has generally not yet extended to other stages of the female reproductive cycle (e.g., pregnancy). In addition, because most recent research into HPO axis modulation has focused on the brain, complexity and integration that occurs at the level of the ovaries and other peripheral reproductive organs (uterus, placenta) remains poorly defined.

The result of such unintentional bias is most obvious if we consider a specific example: the role glucocorticoids play in regulating HPO axis function. There is ample experimental, physiological evidence that psychosocial stress associated with increased glucocorticoid secretion results in increased rates of reproductive failure[14,15,25,26]. For this reason, researchers interested in individual variation in reproductive outcomes related to environmental and individual variation have been asking for decades how glucocorticoids fit into these reproductive investment decisions.

One established mechanism by which glucocorticoids decrease reproductive system activity (presumably scaling to reproductive effort or success) is through the hypothalamic suppression of GnRH release, which can result in irregular or absent reproductive cycles [14]. In addition to action in the hypothalamus, glucocorticoids influence fetal development when they pass through the placenta; the role of glucocorticoids in fetal programming is an active and exciting area of current

research [27,28]. However, these mechanisms do not explain the well-established fact that psychosocial stress during early pregnancy alters progesterone production by the ovaries. No one has investigated whether this phenomenon is due to classic signaling through the hypothalamus, an alternative mechanism, or both. Because pregnancy involves the addition of a major endocrine organ (the placenta) and suppression of HPO axis activity and/or sensitivity of the HPO axis to stimulatory and inhibitory signals, there is good reason to suspect at the very least that additional or unknown mechanisms regulate ovarian progesterone production during pregnancy.

A popular idea that could explain stress-induced suppression of ovarian progesterone production is that glucocorticoids can inhibit ovarian steroid production by acting directly on the ovary. This idea is broadly applied in non-reproductive as well as reproductive females, and it is now regularly cited by ecophysiologists as a mechanism by which psychosocial experiences affect HPO axis function (e.g., [29]). However, there are very limited physiological data to support this idea. In mammals, glucocorticoid availability is tightly controlled within ovarian follicles by differential expression of 11βHSD I and II[30], and their effects on steroidogenesis vary among species and stage of the follicle examined [26,31]. In birds, only two *in vitro* studies have examined the effects of glucocorticoids on steroid production, one showing inhibition of steroid secretion[32], and the other showing no effect[33]. This equivocal support for the hypothesis that glucocorticoids directly inhibit ovarian steroidogenesis point to the need for broader, mechanistic studies. However, researchers have instead continued to focus on organism-level function and have broadly generalized these isolated findings to explain their data despite the limited evidence.

1.4. Areas of opportunity

The above are only examples, but the large holes in our understanding over female reproductive flexibility on a molecular level are likely to lead to similar outcomes without attention to understanding underlying mechanisms. Four broad areas of focus in particular have the potential to make major advances in the field of life history theory by examining the mechanisms underlying reproductive flexibility.

1.4.1 What is the importance of autonomic innervation for rapid modulation of female reproductive function?

Both the ovary and the uterus receive autonomic innervation and there is good evidence that these systems meaningfully regulate function of these organs. For example, denervation during proestrous results in a 50% decrease in estradiol release within 4 minutes of denervation [34]. Interestingly, afferent nerve stimulation during estrous rapidly reduces both estradiol and testosterone secretion in rats [35,36], suggestive of dynamic function across the reproductive cycle (for other examples and further discussion, see [37,38]). Though the function is poorly defined, the uterus experiences dramatic remodeling of sympathetic innervation across the reproductive cycle, which is associated with changes to local

norepinephrine and noradrenaline availability [38]. Interestingly, sympathetic nerve fibers that contain neuropeptide NPY specifically associate with endometrial glands in the uterus, suggestive of a secretory role[38].

Despite this substantial histological body of knowledge, the role of autonomic regulation for reproductive flexibility in these organs and its relationship to canonical endocrine regulation of the HPO axis has received very little attention. Autonomic regulation of ovarian function has principally received attention over the past 30 years in relation to polycystic ovarian syndrome development in humans [37,39,40], so there remain major opportunities to explore the role of rapid effects of autonomic regulation in ecophysiological contexts. The same is true for the functional role of cyclic changes in uterine innervation, where our principal body of knowledge surrounds vasculature dilation; dilation is important for contexts where fetal hypoxia is possible (e.g., altitude or sustained dives). Examining a role in endometrial gland function may reveal further implications for implantation and early pregnancy maintenance.

1.4.2 What signals is the HPO axis listening to?

Neuroendocrinologists have adeptly explained how various internal and external cues, such as body condition and mate access, influence HPO axis function in the hypothalamus. Some of these advances have occurred through the identification of novel neuropeptides (e.g., GnIH and Kisspeptins). Interestingly, many of these neuropeptides have also been found within the ovaries and uterus [41]. However, the roles of these neuropeptides in the periphery are poorly understood both in breadth and depth. There are many interesting one-off manuscripts which identify expression or explore one possible function in the periphery, but we rarely see more comprehensive attempts to explore the molecular pathways by which neuropeptides act in these extra-neural tissues. Perhaps more importantly, we also have very little information on the ultimate importance of any impact these neuropeptides may have on reproductive outcomes. The limited number of studies that pursue either identification or function means that conclusions about production and function are often based on only a few findings in one or two species. These limitations are potentially further exacerbated by negative publication bias – experiments that are unable to find a given peptide or hormone or a function for it in the ovary are less likely to be published, and thus we cannot easily evaluate whether there is adequate support for conserved expression or function of neuropeptides in the gonads. Improving both breadth (species diversity) and depth (mechanism and relevance to reproductive outcomes) of studies are greatly needed.

Furthermore, just as the brain is directly sensitive to metabolic signals such as glucose availability, the ovary and other reproductive tissues are also sensitive to a number of cues outside of gonadotropins and a handful of established hormones (see [32]). However, we still have a limited understanding of the breadth to which this is the case. Very basic but well-thought-out experiments are needed to expand

the set of cues we know to be important or relevant to ovarian function and reproductive outcomes.

1.4.3 How does reproduction reorganize the reproductive axis?

A female's reproductive physiology changes throughout the estrous cycle as estrogen and progestogen concentrations cycle as a function of ovarian follicle growth and corpus luteum degradation. However, reproduction itself depends on additional endocrine organs that intimately interact with the canonical HPO and effectively reorganize the way this system functions across a reproductive attempt. The uterus and placenta exert endocrine activity during pregnancy and they influence HPO function and reproductive outcomes during various stages of pregnancy. Other pituitary hormones, in particular prolactin (PRL), are also involved in pregnancy maintenance and mammary gland development. Neurocircuits that have little to do with canonical HPO axis activity thus become critical during pregnancy and lactation. Given that these systems are integral components of reproductive success, we should make further effort to incorporate them into our dynamic view of the HPO axis. We still understand relatively little about how each of these pieces interacts with the canonical HPO axis, which is not truly quiescent at any stage of the cycle. We also have a relatively superficial grasp on how these systems respond to environmental and endogenous variation in condition.

1.4.4 How does complexity in an organ scale to organism?

Although the brain is immensely complex, when we focus on neurocircuits, there are tidy connections between one neuron and another which can be mapped. If you stimulate a set of GnRH neurons, you will probably get release of GnRH at the terminals. If you stimulate a set of GnIH neurons that contact GnRH neurons, you expect to see some effect on GnRH neurons as well. However, the ovary, uterus, and placenta rely principally on endocrine and paracrine signaling, which makes their complex underlying structure more difficult to parse. For example, we know that the sensitivity to gonadotropins or steroidogenic potential of follicles varies with stage of development. However, it is not clear that if you know a given follicle class is sensitive to glucocorticoids, you can predict the degree to which circulating estradiol or any other endocrine signal will change in response to an acute change in glucocorticoid production. Similarly, if only one class of follicle is sensitive to glucocorticoids, it does not follow that follicular atresia associated with stress must only occur in that class – the extensive paracrine signaling in the ovary means that any signal can be readily be communicated to other follicles in the hierarchy. Ultimately, we need to develop a better understanding of how a physiological signal received by any of these units results in organism-level effects. This begins with relatively simple questions about how units within each system (e.g., the follicle, the endometrial epithelium in the uterus, the labyrinth zone in the placenta) integrate and response to cues (Fig 1 A) and then carefully increasing the complexity of our study set-up by moving to increasingly complex levels of organization (Fig 1B). We

should expect that the way information moves through these levels of the hierarchy will vary across reproductive states and with other contextual variation. Building knowledge from both directions (organism-down and unit-up Fig 1B,C) will generate a more complete understanding of how these systems function dynamically by connecting meaningful variation in reproductive outcomes at the organismal level to complex integration processes that move cues from the simplest level of organization through to the top level.

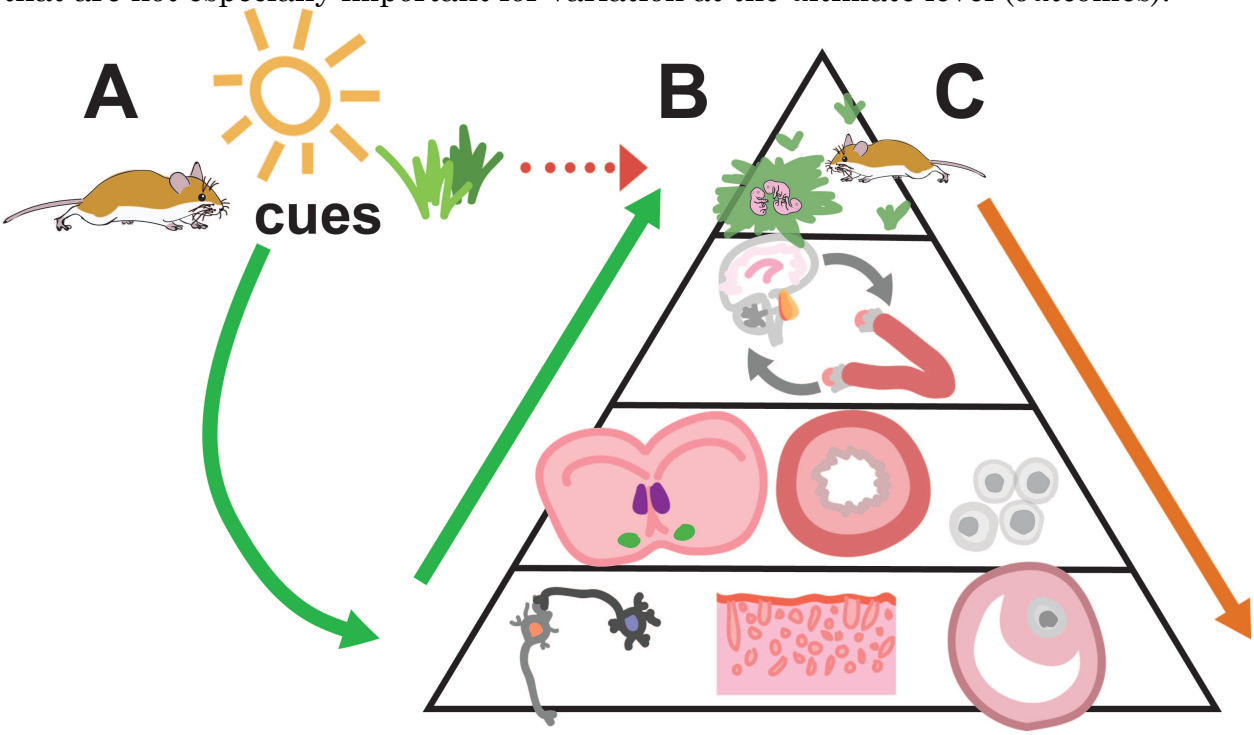
1.5. Goals laid out for the dissertation

This dissertation directly addresses three of these four areas of opportunities by using *in vivo* and *in vitro* experimental approaches. Chapters 2 and 3 identify new signals to which the HPO axis is sensitive (Section 4.1). Chapter 2 uses ovarian explants to determine whether glucose availability in circulation can directly impact ovarian steroidogenesis. Chapter 3 explores the expression and function of the hypothalamic neuropeptide GnIH in the gonads of the domestic cat (*Felis catus*), adding a new node of knowledge to the slow-growing pool of data on function of this peptide in the gonads. By exploring function of GnIH at the level of the ovarian follicle, Chapter 3 also explores a new level of organization at which GnIH acts (Section 4.3). Chapter 4 tests whether ovarian sex steroids regulate complex changes in function and organization of cells that line the uterus, addressing questions about both signaling (Section 4.1) and units of organization (Section 4.3). Finally, Chapter 5 pursues the questions surrounding stress-related regulation of ovarian progesterone secretion during pregnancy. These experiments address reorganization of HPO axis function during pregnancy (Section 4.2). Together, these projects work to illustrate the diverse avenues that can be pursued to expand our framework for non-classical control of the reproductive axis.

1.6. Figures

Figure 1 Biological hierarchies provide targeted directions for experimentally determining how meaningful variation in reproductive function arises. Although we often focus on the associations between cues and reproductive variation (dotted arrow), cues (social and environmental) are usually being translated into biological signals by sensory or other systems in the animal and then altering function at the cellular level within organs (A, solid arrows). In order to generate variation in reproductive outcomes, the cue information must therefore be transmitted across many complex levels of biological organization within the reproductive system (B). Neurons, stromal and epithelial cells in the uterus, and specialized cells within individual ovarian follicles must coordinate the activity of neural networks within the brain, endometrial and myometrial function the uterus, and intrafollicular activity in the ovary, respectively. These discrete organs communicate via endocrine networks to regulate reproductive outcomes, such as number and quality of offspring. These top two layers have received the most attention in comparative reproductive physiology. Because the level of variation which matters for selection will be at this ultimate level (reproductive outcomes), identifying systems that are

interesting to explore must also use a top-down approach (C) – focusing only on bottom-up regulation could easily result in focus on mechanisms of cue integration that are not especially important for variation at the ultimate level (outcomes).



2. Low glucose availability stimulates progesterone production by mouse ovaries in vitro

2.1 Abstract

Steroid production by the ovary is primarily stimulated by gonadotropins but can also be affected by biological cues that provide information about energy status and environmental stress. To further understand which metabolic cues the ovary can respond to, we exposed gonadotropin-stimulated mouse ovaries *in vitro* to glucose metabolism inhibitors and measured steroid accumulation in media. Gonadotropin-stimulated ovaries exposed to 2-deoxy-D-glucose increased progesterone production and steroidogenic acute regulatory protein mRNA levels. However, oocytes and granulosa cells in antral follicles must not independently mediate this response, because targeted treatment of these cell types with a different inhibitor of glucose metabolism (bromopyruvic acid) did not affect progesterone production. Elevating progesterone production is consistent with the homeostatic role of progesterone in glucose regulation in mammals. It also may regulate follicle growth and/or atresia within the ovary. These results suggest that ovaries can regulate glucose homeostasis in addition to their primary role in reproductive activity.

2.2 Introduction

Reproduction is carefully regulated in most species to enable maximal reproductive success. Many of the environmental and physiological cues that animals use to time reproduction appropriately, including photoperiod, food availability, and fat stores, are integrated by hypothalamic neural circuits that modulate gonadotropin production and release from the anterior pituitary gland. The gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) likewise control activity and function of the gonads. In the ovary, LH stimulates steroid production by theca cells in the follicle (including progesterone [P_4] and other precursor steroids necessary for estradiol production by granulosa cells). FSH promotes estradiol (E_2) production from granulosa cells of the follicle and stimulates follicle growth. Progesterone and estradiol exert paracrine action within the ovary to control follicle development [42]. These sex steroids also facilitate reproduction through action on peripheral tissues (e.g. stimulating hypertrophy in the uterine epithelium or the oviduct; [43–45]. They can also act in the brain to regulate other components of reproductive activity, including reproductive behaviors. Sex steroid production from the ovaries can therefore be indirectly controlled by physiological cues through central modulation of LH and FSH release.

Data collected over the last two decades demonstrates that physiological signals other than gonadotropins also meaningfully impact ovarian sex steroid

production and follicle development. For example, the hormone leptin, which reflects fat stores, modulates estradiol and progesterone production by granulosa cells from human ovaries *in vitro* [46,47]. Similarly, avian ovaries decrease estradiol production when treated *in vitro* with pharmacological agents that mimic low glucose and fatty acid availability [32]. However, we still know relatively little about the extent to which stimuli other than gonadotropins can modulate ovarian steroid production and/or follicular growth.

In light of data from avian ovaries [32], we tested whether glucose availability could directly influence gonadotrophin-induced sex steroid production from the mouse ovary. Glucose exhibits ultradian (<24 hr) oscillations across a day in humans [48,49], rodents[50], and horses [51], and more extreme decreases in blood glucose can occur naturally during acute fasting [52] and exercise [53]. Thus, low glucose is a salient biological cue to which the mammalian ovary could be sensitive. To test whether ovarian steroidogenesis in mice responds to low glucose, we utilized two different pharmacological agents that inhibit glucose metabolism: 2-deoxy-D-glucose (2-DG) and bromopyruvic acid (3BR). 2-DG enters cells via glucose transporters (e.g. [54], and therefore can be taken up by most cells in the ovary. In contrast, 3BR must be transported across the cell membrane by monocarboxylate transporter 1 (MCT1) [55], and expression of MCT1 is largely limited to granulosa cells and oocytes in antral follicles in the mouse ovary [56,57]. Thus, 2-DG can inhibit glucose utilization throughout the entire ovary, whereas 3BR targets a subset of cell types in antral follicles. Oocytes and cumulus granulosa cells, the targets of 3BR, employ extensive paracrine signaling that controls development and maturation of the follicle and oocyte, in part by regulating glucose metabolism and steroid production [58–61]. Oocytes uptake relatively little glucose directly; they therefore rely on pyruvate produced by cumulus granulosa cells for metabolic substrate. Because maturation of follicles and oocytes is sensitive to glucose [59], we hypothesized that oocytes and/or granulosa cells would mediate any effect of low glucose availability on steroidogenesis.

2.3 Materials and Methods

2.3.1 Animal use

All laboratory protocols were approved by the UC Berkeley Office of Laboratory Animal Care and consistent with NIH Guidelines for the care and use of laboratory animals. Six-week-old, female C57BL/6J mice (*Mus musculus*) purchased from Jackson Laboratory were given a week to acclimate to the laboratory environment before the experiment began. The mice were group-housed under a 17:7 L:D cycle. A vaginal lavage was used to determine stage of estrus for all animals [62]. Animals were provided *ad libitum* food and water. Mice were deeply anaesthetized using isoflurane and rapidly decapitated prior to ovary collection. From each individual, one ovary was assigned to the control group and the second was assigned to the treatment group. Left versus right ovary assignment was randomized (see below).

2.3.2 Culture media and reagents

Culture media consisted of Eagle's MEM with Earle's balanced salts supplemented with 2 mM L-glutamine, 100 UI ml^{-1} penicillin, 100 ug mL^{-1} streptomycin, 50 uM ascorbic acid, 1% v/v insulin-transferrin-selenium (ITS+, Gibco Laboratories), and 0.3% bovine serum albumin. All media (control and treatment media) were supplemented with 2 IU mL^{-1} luteinizing hormone (L5269, Sigma-Aldrich) and 1 IU mL^{-1} follicle-stimulating hormone (F2293, Sigma-Aldrich) immediately prior to use. LH and FSH concentrations used in these experiments are supraphysiological, but are routinely used to stimulate follicle growth and induce ovulation-related changes in gene expression *in vitro* [63–65]. In addition, the LH and FSH concentrations used here stimulated steroid accumulation in media from cultured mouse ovaries in preliminary studies (compared to control media without gonadotropin supplementation). 2-DG (D8375, Sigma-Aldrich) was diluted in culture media to a concentration of 5 mM, and 3BR (16490, Sigma-Aldrich) was similarly diluted in culture media to 5 mM. These concentrations of 2-DG and 3BR reduce ATP production and inhibit glycolytic activity *in vitro* [66–70].

2.3.3 Experimental design and ovarian culture

Data were collected in two experiments using identical tissue collection protocols. In experiment 1 (one), ovaries were treated with 2-deoxy-D-glucose (2-DG). Experiment 1 was independently repeated (n = 8 in the first iteration, n = 7 for the second); similar results were achieved in both replications and data are pooled for presentation and analysis. In experiment 2 (two), ovaries were treated with bromopyruvic acid (3BR; n = 11). 3BR treatments were carried out once.

For both experiments, ovarian culture was carried out immediately after sacrifice. Ovaries cleaned of fat and accessory tissues were paired within an experiment such that one ovary from each mouse was cultured in control medium while the other was cultured in medium containing the experimental treatment. Ovaries were cultured in 24-well plates. Each well contained 500 µl of media (control, 2-DG, or 3BR). Ovaries were placed in a sealed, humidified incubator for 6 h at 37°C with 5% carbon dioxide gas. Similar culture conditions have previously been used to evaluate the effects of other proteins, hormones, and pharmacological agents on steroidogenesis from whole rodent ovaries *in vitro* [64,71,72].

After 4 h of incubation, 100 µl of culture medium was replaced with fresh medium. After 6 h of incubation, all culture media were transferred into clean eppendorfs and heavily vortexed prior to aliquoting for storage. Tissues were snap-frozen on dry ice. Tissues and culture supernatant were stored at -80°C until analysis.

2.3.4 Hormone production measurements

Media collected from ovarian cultures were assayed utilizing ELISAs for progesterone (Item No. 582601, Cayman Chemical) and estradiol (Item No. 582251, Cayman Chemical) in compliance with instructions for use with culture media.

All samples were assayed in triplicate. Intra-assay variation ranged from 1.9% to 6.2% (P_4) and from 4.1% to 5.5% (E_2). Inter-assay variation was 7.9% for P_4, and 9.9% for E_2.

2.3.5 Quantification of mRNA expression

RNA was extracted from frozen ovaries (Bioline Isolate II RNA Mini Kit) and 1 µg RNA was reverse-transcribed for downstream analysis (iScript Reverse Transcription Supermix, Bio-Rad). Quantitative PCR was carried out using SsoAdvanced Universal SYBR Green Supermix with 0.5 uM primers for steroidogenic acture regulator protein [STAR; F: 5'-CTTGGCTGCTCAGTATTGAC; R: 5'-TGGTGGACAGTCCTTAACAC; T_A: 55°C] and cholesterol side-chain cleavage enzyme [SCC; F: 5'-CGATACTCTTCTCATGCGAG; R: 5'-CTTTCTTCCAGGCATCTGAAC; T_A: 55°C]. Primers were validated prior to use and efficiencies for all primer pairs were between 90 and 105%. All data were corrected for efficiency, normalized to the geometric mean of two reference genes (tubulin beta 5 [TTUB5; F: 5'-GGACAGTGTGGCAACCAGAT; R: 5'-CCCCAGACTGACCGAAAACG; T_A: 60°C] and beta-2-microglobulin [B2M; F: 5'-CTGCTACGTAACACAGTTCCACCC; R: 5'-CATGATGCTTGATCACATGTCTCG; T_A: 55°C]) that did not change with treatment, and then analyzed for fold-change using the Pfaffl method [73]. Because there was no difference in P_4 and E_2 production by 3BR-treated ovaries, RNA extraction and gene expression analyses were not carried out for this treatment group.

2.3.6 Statistical Analyses

Statistical analyses were carried out in R 3.1.2. All statistical analyses use paired Wilcoxon rank-sum tests. Differences were considered statistically significant at $p < 0.05$. All values are expressed as mean ± s.e.m.

2.4 Results and Discussion

Accumulation of progesterone (P_4) in media increased in response to 2-DG treatment (Fig. 1A, V = 5, $p < 0.001$), whereas estradiol accumulation in the media did not change (Fig 1A, V = 35, $p = 0.169$). Estrus stage at collection did not affect the qualitative response to 2-DG of hormone accumulation or mRNA levels, though we could not statistically evaluate this response. Preantral and antral follicles are found in the ovary at all stages of the estrous cycle in mature mice (McGee and Hsueh, 2000; Peters et al., 1975), and stimulation with gonadotropins *in vitro* may therefore explain the similar response of all ovaries to treatment. Treatment with 2-DG also increased steroidogenic acute regulatory protein (STAR) mRNA levels in mouse ovaries (Fig. 1B, V = 1, $p < 0.002$), though it had no effect on cholesterol side-chain cleavage enzyme (SCC) mRNA levels (Fig. 1B, V = 24, $p = 0.465$). Taken together, our hormone accumulation and mRNA levels data suggest that gonadotropin-stimulated mouse ovaries can increase steroid hormone production in direct response to pharmacologically-induced hypoglycemia. These findings are

novel because it was not previously known that ovarian steroidogenesis in mammals is directly responsive to glucose availability.

Though SCC acts as the rate-limiting step for steroid production, differential expression of STAR is involved in rapid changes in steroid production [74,75]. STAR expression (mRNA and protein) increases in rodent gonads *in vitro* and *in vivo* within 4 h following gonadotropin stimulation [76], whereas SCC tends to be up-regulated in response to gonadotropins over longer time periods, from 8 h to days following exposure [77,78]. Our data are consistent with acute up-regulation of steroid hormone production (here, progesterone) via increased expression of STAR. Further quantification of mRNA and protein expression of steroidogenic enzymes will help us better understand how STAR modulation affects production of progesterone and other gonadal steroids.

Steroid production by the follicle is regulated, in part, by intra-follicular signaling, especially between oocyte and granulosa cells [79,80]. Moreover, cumulus granulosa cells are responsible for metabolizing glucose into pyruvate for use by the oocyte (which uptakes very little glucose by itself)[61]. We therefore expected cumulus granulosa cells and oocytes were responsible for the increased steroidogenesis that occurred in response to 2-DG. 3BR primarily inhibits glucose utilization by oocytes and granulosa cells within antral mouse follicles. 3BR therefore provides information about whether oocytes, granulosa cells, and their interactions are independently responsible for the increased progesterone accumulation that occurs in response to 2-DG treatment. However, 3BR did not increase progesterone accumulation in the media ($V = 21$, $p = 0.32$), nor did it affect estradiol ($V = 22$, $p = 0.37$) (Fig. 3). Our data consequently suggest that elevated progesterone production in response to 2-DG is not mediated solely by oocyte-granulosa cell interactions. Still, these experiments cannot provide information on the role of paracrine signaling from other sources or between theca cells and oocyte-granulosa cell complexes. Because outputs like steroid production are modulated by interactions among several cell types in the ovary, determining the precise mechanism by which 2-DG stimulates progesterone secretion requires further, cell-specific targeting.

Our findings contrast with McGuire et al.'s study that showed gonadotropin-stimulated avian ovaries decrease steroid hormone production in response to metabolic substrate depletion [32]. McGuire et al. considered inhibition of steroid production consistent with an adaptive suppression of reproductive activity in response to metabolic stress (indicative of poor environmental conditions for breeding). Our data demonstrate an alternative response that is not consistent with an adaptive suppression of reproduction.

The differential response to low substrate availability could reflect differences in breeding strategy in the studied species. McGuire et al. showed an inhibitory effect of low glucose on ovarian steroids in European starlings, which are a strongly photoperiodic (seasonal) breeder. C57BL/6J mice, in contrast, are continuous breeders. Analogous to the Starling work, seasonally breeding mammals, such as deer mice (*Peromyscus maniculatus*), will readily inhibit

reproductive attempts in energetically demanding conditions [81]; the continuously breeding house mouse (*Mus musculus domesticus*) will continue to produce litters under energetically-demanding conditions, but cannibalizes young during lactation [81]. Comparable to these mammalian examples, opportunistically-breeding Zebra finches will maintain a partially-activated reproductive system in spite of low body condition [82]. The ability to breed opportunistically or continuously is therefore thought to reflect weak inhibition and increased sensitivity to positive environmental cues in both mammals and birds. The failure of low glucose availability to inhibit ovarian steroid production, as shown here, may therefore reflect a continuous breeder's lower sensitivity to inhibitory signals.

However, avian and mammalian physiologies differ more broadly in ways that might also explain different ovarian responses to low energy availability. In mammals, progesterone is a well-established modulator of blood glucose (e.g. [83,84], but similar regulation has not been shown in birds. In mammals, progesterone promotes an increase in circulating blood glucose. Though progesterone has primarily been recognized for its chronic role in glucose homeostasis during pregnancy [84,85], progesterone signaling broadly inhibits action of insulin (thus buffering blood glucose) by inhibiting storage and metabolism of glucose in adipocytes and muscle [86–89]. Progesterone can also promote the production of other hormones that increase blood glucose via action in the central nervous system: infusion of progesterone to the forebrain increases plasma norepinephrine, presumably through modulation of sympathetic drive [90]. Elevating progesterone in an acute response to low glucose availability may therefore stimulate known homeostatic processes through multiple mechanisms.

Our finding that the ovary increases progesterone production in response to low glucose availability is also consistent with work demonstrating that, *in vivo*, low glucose is associated with elevated progesterone in circulation. For example, bolus insulin injections used to induce hypoglycemia result in rapid increases in circulating progesterone in women [91], and exercising women exhibit concomitant (<15 min apart) increases in circulating progesterone and blood glucose [92]. In addition, young men infused with 2-DG exhibit a relatively rapid (<1 hr) elevation of serum progesterone [93–95],. The acute increase in progesterone production measured *in vivo* has previously been attributed entirely to adrenal gland activity [96,97], however our data indicate that the ovary acts as an additional source of progesterone following acute decreases in blood glucose concentration.

The contribution of progesterone to homeostatic glucose regulation is thought to depend on its concentration relative to circulating estrogens [98–100]. Elevated progesterone stimulates feeding behavior, but only when estrogens are simultaneously low [101,102]. Despite finding no increase in estradiol production in response to 2-DG treatment, the ratio of P_4:E_2 production did not differ between 2-DG and untreated ovaries (mean change in the ratio of P_4:E_2 = 0.12 ± 0.11; V = 36, p = 0.19). Whether ovarian progesterone production shown here is sufficient to stimulate homeostatic responses *in vivo* will depend on dynamics we were not able to investigate in this context. For example, progesterone and estradiol are released

in pulsatile rhythms [103–105], but surprisingly little is known about the physiological importance of these pulses to their targets. In women, pulsatile estradiol production by the ovary is delayed by about 20 minutes relative to progesterone peaks [103], and thus, the temporal separation of pulsatile progesterone and estradiol may allow ovarian progesterone to positively regulate glucose homeostasis on acute timescales.

In addition to its role in glucose regulation, progesterone exerts paracrine effects within the ovary on follicle development [106]. For example, elevated progesterone in the follicular fluid of early-stage follicles is associated with atresia [107]. In later stage follicles, progesterone may also positively regulate oocyte maturation and ovulation and exert anti-degenerative effects on granulosa cells [42,108]. Glucose availability also impacts follicle maturation and oocyte quality [109,110]. However, the effects of steroids and glucose availability on follicle maturation have been largely studied in the context of chronic exposure, so it is difficult to speculate as to whether acute changes in intra-ovarian glucose and progesterone availability meaningfully impact these outcome measures.

In summary, increasing steroidogenesis in response to pharmacologically-induced hypoglycemia is not consistent with the expected response of the rest of the reproductive system under low energy availability (down-regulation) [111]. Our findings instead are consistent with a positive, homeostatic action of progesterone on blood glucose concentrations in mammals, and perhaps also with the opportunistic breeding strategies in the lab mouse. Though the underlying mechanism and *in vivo* physiological importance of these changes require further investigation, the connection between ovarian steroidogenesis and glucose availability presents an intriguing new aspect of ovarian regulation (Fig. 3). Finally, even if hypoglycemic-induced progesterone production by the ovary plays a limited homeostatic role, dysregulated blood glucose likely impacts ovarian function via modulation of steroidogenic activity. We suggest that evaluating organ function in isolation can provide novel insight to organ interactions *in vivo* and further explain whole-organism responses. This is particularly true when examining hormone systems, which usually exhibit pleiotropic effects across tissues.

2.5 Acknowledgments

We thank L.J. Kriegsfeld for access to facilities.

2.6 Figures

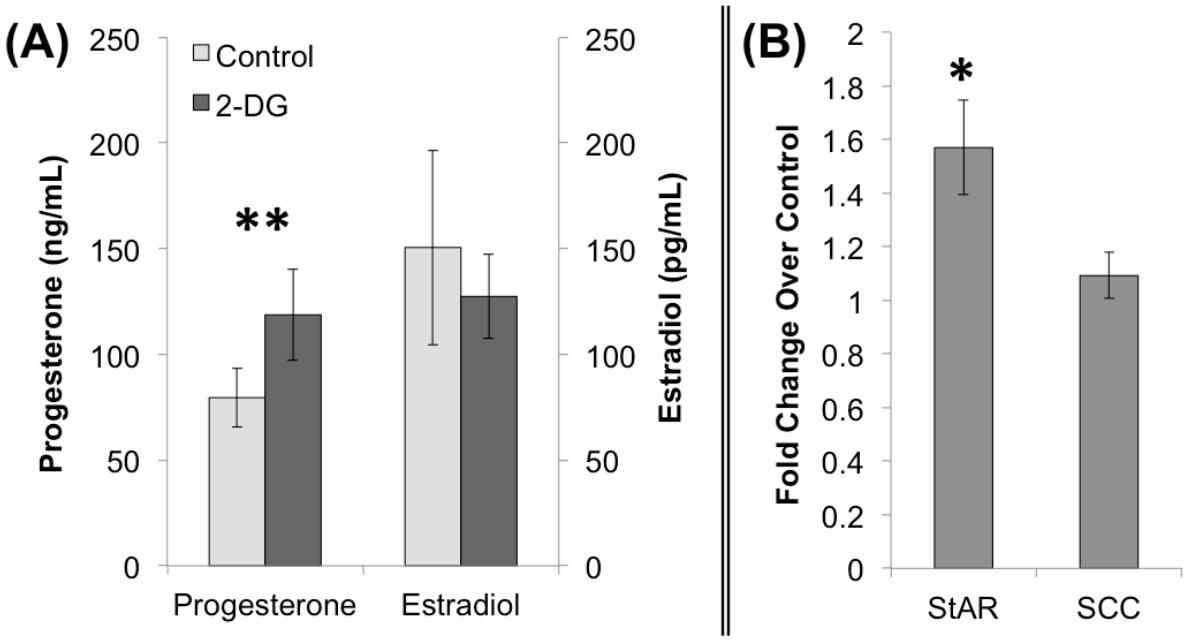


Figure 1 (A) Exposure to 2-deoxy-D-glucose (2-DG) *in vitro* increased progesterone accumulation relative to controls, however estradiol production did not change (N = 15). (B) Exposure to 2-DG *in vitro* also increased mRNA levels of steroidogenic acute regulatory protein (STAR), but did not affect mRNA levels of cholesterol side-chain cleavage enzyme (SCC) (N = 15). Values are expressed as fold-change over controls. Asterisks indicate P<0.002 (*) or P < 0.001 (**).

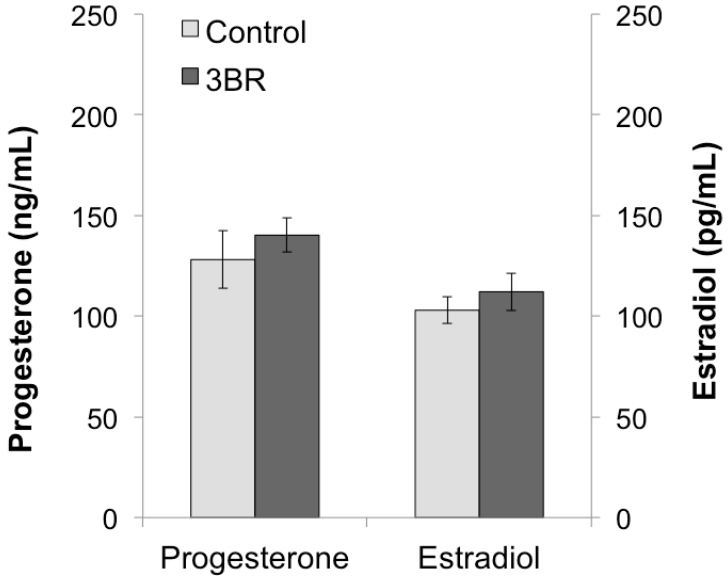


Figure 2 Treatment of mouse ovaries with 3BR had no effect on progesterone or estradiol production (N = 11).

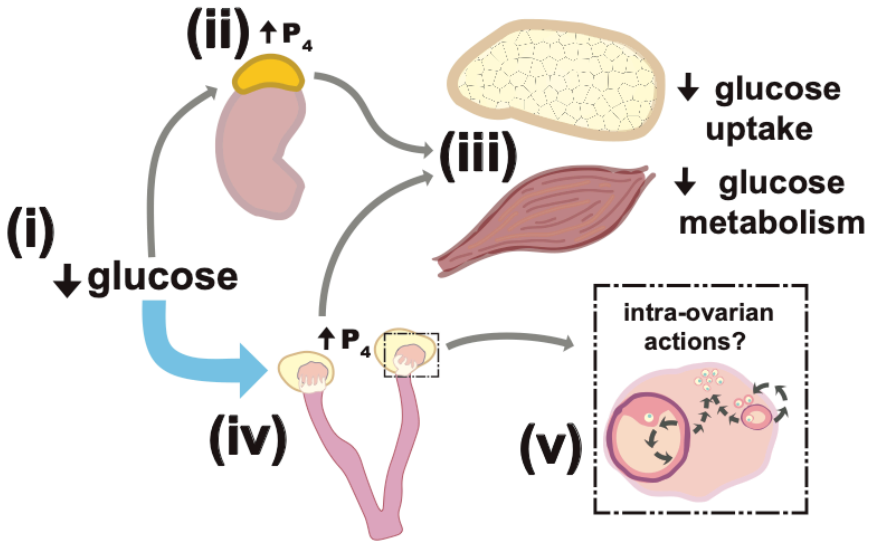


Figure 3 (i) Low blood glucose is already known to stimulate progesterone production from (ii) the adrenal gland, which can relatively rapidly (iii) inhibit glucose uptake and metabolism in adipose tissue and muscle (see text). (iv) We show that low blood glucose also stimulates progesterone production from the ovary (thick, blue arrow). Stimulation of ovarian progesterone production could also contribute to (iii) the inhibition of glucose uptake in adipose tissue and muscle. (v) Progesterone production could also impact follicle metabolism and development through paracrine signaling within the ovary. Thin, grey arrows: Known pathways of control; Thick, blue arrow: Novel pathway.

3. RFRP-3 promotes basal lamina degradation and cellular death while increasing progesterone secretion in cultured cat ovarian follicles

3.1 Abstract

The hypothalamic neuropeptide RFRP-3 can suppress hypothalamic GnRH neuron activation and inhibit gonadotropin release from the anterior pituitary. RFRP-3 is also produced locally in the ovary and can inhibit steroidogenesis and follicle development in many vertebrates. However, almost nothing is known about the presence and regulatory role of RFRP-3 in gonads of any carnivore species. Such knowledge is important for developing captive breeding therapies for endangered carnivores and for inhibiting reproduction in feral species. Using the domestic cat as a model, our objectives were to (1) demonstrate the expression of RFRP-3 and its receptor in the cat ovary and (2) assess the influence of RFRP-3 on ovarian follicle integrity, survival, and steroidogenesis *in vitro*. We first confirmed that RFRP-3 and its receptors (NPFFR1 and NPFFR2) were expressed in cat ovaries by sequencing PCR products from ovarian RNA. We then isolated and cultured preantral ovarian follicles in the presence of 10 or 1 μM RFRP-3 + FSH (1 μg/mL). We recorded the percentage of morphologically viable follicles (basal lamina integrity) over 8 days and calculated percentage survival of follicles on Day 8 (using fluorescent markers for cell survival and death). Last, we quantified progesterone accumulation in media. 10 μ*M* RFRP-3 had no observable effect on viability, survival, or steroid production compared to follicles exposed to only FSH. However, 1 μM RFRP-3 decreased the percentage of morphologically viable follicles and the percentage of surviving follicles on Day 8. At the same time, 1 μM RFRP-3 increased the accumulation of progesterone in media. Our study shows, for the first time, direct action of RFRP-3 on the follicle as a functional unit, and it is the first in a carnivore species. More broadly, our results support a conserved, inhibitory role of RFRP-3 on ovarian follicle development and underscore the importance of comparative functional studies.

3.2 Introduction

In spite of their name, neuropeptides are synthesized in and act on many peripheral tissues in addition to their classical action in the nervous system[41]. In part due to persistent biases in how these peptides are studied, we still have a rudimentary understanding of how these neuropeptides evolved to play these often disparate central and peripheral roles. In many cases we do not even have a comprehensive understanding of their function or importance outside of the brain[112].

While some neuropeptides have peripheral effects that serve distinct functions from their role in the central nervous system (e.g, the neuropeptide gonadotropin-releasing hormone [GnRH][112,113]), other neuropeptides exhibit

putatively concordant function in the central nervous system and periphery. In particular, gonadotropin-inhibitory hormone (GnIH, also known as RFRP-3) inhibits activity of the vertebrate reproductive axis via direct action in the hypothalamus, on the anterior pituitary, and in the gonads[112]. In the hypothalamus, GnIH can inhibit the activity of GnRH cells[114–120]. Hypothalamic GnIH can also directly influence pituitary release of gonadotropins[114,115,121–124]. Furthermore, GnIH is found in ovarian tissues across nearly all vertebrate taxa studied to-date; of 18 species examined from lampreys to humans, only the grass puffer lacks ovarian GnIH (Table 1). However, there are far fewer studies that have assessed function of GnIH in the ovary. Of the 17 species in which ovarian GnIH has been identified, only seven have had any function evaluated (Table 1).

We and others have suggested that the widespread presence of GnIH and its receptor in the ovary is indicative of a conserved regulatory role[21,112,125]. To date, GnIH is known to decrease cell viability and steroidogenic gene expression or steroidogenesis in the ovary of the chicken[126] and four mammal species (domestic pigs[127], Parkes mice[128], Sprague-Dawley rats[129], and humans[130]). Other work in avian systems also indirectly suggests that GnIH expression in the ovary is associated with inhibition of the reproductive axis[32,131]. However, in the one fish species in which function has been examined, ovarian GnIH promoted the transcription of steroidogenic genes (LHR, StAR, 3β-HSD)[132]. Because only 7 studies have interrogated any functional role of the GnIH system in the ovaries (Table 1), the discordant actions of GnIH among taxa could reflect species-specific variation or broader taxonomic patterns. Further comparative experiments that assess function are needed to evaluate whether ovarian GnIH exerts conserved function across species.

The potentially-conserved inhibitory function of GnIH in the ovary is exciting because it provides many potential applications ranging from reproductive medicine[129] to invasive species management[133,134] and endangered species survival programs[135]. By antagonizing the receptor, we may be able to improve follicle quality or maturation *in vitro*, and genetic tools have the potential to use local overexpression of inhibitory peptides like RFRP-3 to provide long-term fertility suppression in feral animals and/or invasive species. Both such applications are particularly pressing for felines: feral cats are an on-going problem for native species[136], and the preservation of endangered species requires continued progress in assisted reproductive technologies[135]. To date, GnIH has not yet been examined in any felid or carnivore species. Although many carnivore species and endangered species are difficult to use for large-scale studies of reproductive function, there are tractable, established techniques for studying ovarian function in the domestic cat (*Felis catus*) as a model. In particular, ovarian follicle isolation and *in vitro* culture are a major focus of reproductive physiology research in the domestic cat[135,137,138], and thus this system is well-optimized to serve as a model for studies of effects on follicular function. Although others have suggested that effects of GnIH on granulosa cell function *in vitro* will ultimately alter viability of the ovarian follicle and/or oocyte maturation[126,139], no one has directly

examined the effect of GnIH on follicle viability. These advantages identify the domestic cat as an important model for both basic and applied research into GnIH function.

We pursued two aims related to understanding RFRP-3 function in the domestic cat. First, we asked whether a functional RFRP-3 signaling system exists in the ovaries of domestic cats. We predicted that RFRP-3 and its receptors, NPFFR1 and NPFFR2, would be expressed in the ovary. Second, we asked whether RFRP-3 impacts cat follicle integrity, survival, and steroid production by using an alginate-embedding system to culture isolated ovarian follicles[137,140,141]. Based on studies in other mammals, we expected RFRP-3 to inhibit dose-dependently any increase in follicle size and to result in decreased cell viability within follicles[126,139]. We also predicted that RFRP-3 would dose-dependently inhibit progesterone production by follicles[127–130]. We evaluated effects of RFRP-3 in the presence of gonadotropins because effects of RFRP-3 have been dependent on gonadotropin presence in primates and rats[129,130].

3.3 Methods

3.3.1 Expression of RFRP-3 and receptors in ovaries of the domestic cat

Whole ovarian tissue was flash-frozen in isopentane on dry ice immediately following surgery from routine spays on domestic cats. Tissues were stored on dry ice for transport and then at -80°C until extraction.

Tissues were sliced at 20 μm using a cryostat, collected into an RNAase-free Eppendorf tube, and stored at -80°C. Sections collected across the entire ovary (N = 2) were represented in each tube. Samples were homogenized and extracted using the Bioline Isolate II RNA Mini Kit (Cat. No. BIO-52073) as per manufacturer instructions. RNA was quality-tested at the University of California, Berkeley Functional Genomics Laboratory using a bioanalyzer. All samples had RIN scores of 8 or higher.

After extraction, 750 ng of RNA from each extraction was reverse-transcribed using the iScript gDNA Clear cDNA Synthesis Kit (Cat. No. 1725035) according to manufacturer instructions. No-RT samples were reverse-transcribed at the same time using 750 ng of RNA and the kit's No-RT control supermix.

Gene targets were amplified using 18.75 ng of cDNA in a 30 μL reaction containing 0.4 μ*M* forward and reverse primers (Table 2). Other reaction components (TaqPolymerase, reaction buffer, Mg SO_4, and dNTPs) were added as per manufacturer instructions (Platinum *Taq* DNA Polymerase, High Fidelity; Cat. No. 11304011, Invitrogen). PCR was run for 45 cycles with a 60°C annealing temperature and 3 min extension. The reaction product was run on a 2% agarose gel and imaged. Amplification of the target gene was confirmed using length of the expected product (See table 2 and Fig. 1 and 2) and sequencing. PCR products were cleaned and sequenced at the University of California, Berkeley DNA Sequencing facility. NPFFR1 sequencing attempts failed to return any sequence, and thus we relied on product length for specificity.

3.3.2 Tissue handling for cell culture

Whole uterine and ovarian tissue were collected into chilled transport media (Table 3) immediately following routine spays. Within 8 h, ovaries were separated from uterine horns and halved longitudinally. Only follicular ovaries were used for these experiments. Ovaries from pregnant or lactating cats were excluded by assessing uterine status, and ovaries from luteal-phase animals were excluded by presence of corpora lutea in the bisected ovary.

Ovary halves were placed in collection medium (Table 3) and minced using a scalpel blade to release follicles. Primary and secondary follicles were collected from the debris using a 10 μL pipette and transferred to clean, warmed collection medium.

Follicles were embedded in alginate beads for culture[142,143]. First, follicles were washed twice in warmed 0.5% purified alginate (W201502, Sigma Aldrich) dissolved in Mg^{2+}- and Ca^{2+}-free Dulbecco's PBS (14190-144, Gibco). Individual follicles were embedded in alginate beads by dropping 5 μL of media containing the follicle into warmed, sterile-filtered solution of 50 mM $CaCl_2$ and 140 mM NaCl (Sigma Aldrich). Alginate beads were crosslinked for 2 minutes before transferring beads to growth media for conditioning.

Follicle culture was carried out in 96-well plates (353077, Corning). Plates were held in an incubator at 38°C and 5% CO_2 throughout experiments except during media changes. Vehicle treatment contained growth medium only (Table 3). FSH-supplemented follicles received 1 μg/mL from aliquoted stock (F2293, Sigma Aldrich). Follicles were exposed to RFRP-3 peptide (Cat. No., 048-46, Phoenix Pharmaceuticals Inc., Burlingame, CA) at two doses: 10 μ*M* and 1 μ*M*. RFRP-3 peptide dissolved in sterile PBS.

Follicles were individually photographed every 48 h throughout the duration of the experiment (days 0, 2, 4, 6, and 8), and half the volume was changed at the same time (75 μL) . All media were conditioned in the incubator prior to addition. Media collected during changes were stored at -80°C in sterile Eppendorf tubes.

3.3.3 Follicle integrity and size measurements

Follicle integrity and size were assessed from brightfield photos using ImageJ. Photos were taken on a stereoscope at fixed magnification. Follicles were classified as intact only when their shape was circular and the basal lamina was smooth (representative images shown in Fig. 1, A & B). Follicles were classed as degraded based on breaks or irregularity in the basal lamina or evidence of the oocyte hatching from the follicle (representative images shown in Fig. 1, C-F). Follicle diameter was measured using two perpendicular line measurements in ImageJ. Follicle size was then calculated as the area of an ellipse using the two diameter measurements. All measurements were collected and recorded by viewers blind to treatment. The average follicle diameter on day 0 was 170.0 ± 40.1 μm (Average ± SD), corresponding to an average area on day 0 of $23.7x10^3 \pm 11.2x10^3$ μm^2 (Average ± SD).

3.3.4 Follicle survival

Follicle viability was assessed qualitatively using the LIVE/DEAD Viability/Cytotoxicity Kit (L3224, Invitrogen). Concentrations of calcein-AM (Ca-AM) and ethidium homodimer-1(EthD) were optimized prior to use as per the kits instructions. On day 8, follicles embedded in alginate beads were removed from wells and placed in warmed Dulbecco's PBS containing 6 mg/mL of alginate lyase (A1603, Sigma Aldrich). Alginate was digested for 30 minutes at 38°C. Follicles were then washed in warm dPBS and incubated in dPBS contain 4 μ*M* EthD and 1 μ*M* Ca-AM for 30 minutes. Follicles were cover-slipped and immediately visualized under a fluorescent microscope. Follicles exhibiting any red fluorescence were categorized as dead or dying, whereas follicles that fluoresced only with green were categorized as alive (See Fig. 1G,H for representative images).

3.3.5 Progesterone extraction and quantification

Media collected across the culture period (days 2, 4, 6 and 8) were pooled for each follicle. Steroid hormones were extracted using an ethyl acetate/water wash extraction (N = 95). Pooled media (~300 μL) were mixed with 1 mL of ethyl acetate (Sigma-Aldrich, 270989) by vigorously vortexing for 5 seconds. The samples were then mixed on an orbital shaker (700 rpm) for 5 minutes. Following mixing, layers were separated for 5 minutes. The organic layer was transferred to 1 mL MilliQ water, which was mixed and separated identical to the ethyl acetate steps described above. The organic layer was then transferred to a borosilicate glass vial. The procedure was repeated (1 mL ethyl acetate, 1 mL MilliQ water) and organic layers were combined. Samples were dried under nitrogen stream and stored at -20°C.

Samples were reconstituted in 150 μL EIA Buffer (Item No. 400060, Cayman Chemical) immediately prior to assay. Samples were assayed using the Cayman Chemical Progesterone ELISA kit (Item No. 582601) according to manufacturer instructions. Samples that fell below the detection limit of the kit (N = 4) were assigned the lowest detectable amount on the standard curve (7.81 pg/mL). Samples above the standard curve were diluted (1:10 or 1:41) and reassayed – those samples that still remained above the standard curve after dilution were then assigned the highest detectable value (N = 23; 1000 pg/mL) and corrected for dilution factor prior to analysis. The median intra-assay variation was 10.1%, and the inter-assay variation was 22%.

3.3.6 Statistical approach and analysis and rationale

In total, we embedded 560 follicles isolated from 70 ovaries as part of six replicate days of experiments. 242 follicles (30 ovaries) were used for basal lamina degradation and progesterone production experiments. 318 follicles (40 ovaries) were used in the follicle survival experiments. For all isolation days, follicles were evenly assigned to treatments on each day such that all treatments were run for each isolation date. Follicles isolated from each ovary were assigned evenly across the four treatment groups such that each ovary were isolated was represented

evenly in each treatment group; note that some ovaries yielded up to 16 follicles used in experiments, whereas others yielded only 4. Only isolated follicles with intact basal lamina were embedded for experiments. Experiments and sample sizes are outlined in Figure 2.

Our predictions and experiments were focused on only a handful of possible comparisons among treatments. We used a vehicle-control and FSH supplementation to determine whether FSH protected follicle viability, as expected based on previous work in this system[137,140]. We therefore were only interested in comparisons between vehicle-controls and FSH-only. We then used two concentrations of RFRP-3 (1 and 10 μM) in combination with FSH to determine whether RFRP-3 has any effect on proposed outcome measures. Our experimental design does not allow us to determine whether gonadotropin treatment affects RFRP-3 action; vehicle-controls cannot be meaningfully compared to RFRP-3 treatments. Consistent with this experimental approach, we use planned pairwise comparisons among treatments throughout our analyses.

All analyses were completed in R 3.5.1. We used general linear models (glmer) for most analyses in order to include a random effect of isolation date. We used QQ plots to assess normality and goodness of fit for all models.

In order to determine whether follicle area varied among treatments on day 0, we transformed the dependent variable, follicle size on day 0, using inverse root-squared and applied a model with a gaussian distribution. We included treatment as a fixed effect. All follicles were included in the analysis.

Because follicle size could only be determined for follicles with intact basal lamina, follicles that degraded had to be excluded from analyses of the effect of treatment on change in size over the culture period. To determine whether size of intact follicles varied with treatment at the end of the culture period, we transformed the dependent variable, follicle size on day 8, using inverse root-squared and applied a model with a Gaussian distribution. Treatment was included as a fixed effect. To test for effects of treatment on change in size over the period of culture, we again transformed the dependent variable, follicle size, using inverse root-squared and applied a model with a Gaussian distribution. We included a day by treatment interaction and day and treatment independently as fixed effects.

We estimated the effect of treatment on likelihood of loss of follicle integrity using a Cox proportional hazards model, with an interaction between treatment and starting size as fixed effects.

We estimated the effect of treatment on follicle survival by applying a model with a binomial distribution and logit link. Treatment was included as a fixed effect.

We estimated the effect of treatment on progesterone production by applying a model with a gamma distribution and log link. Treatment was included as a fixed effect. Analyses of the dataset excluding samples that did not fall within the range of the standard curve (N = 31) or those with CV >40% (N = 9) yielded qualitatively similar results (reported effects remain significant at $P < 0.05$). For this reason, all samples are included in the results presented here.

3.4 Results

3.4.1 Presence of RFRP-3 and receptors in ovarian tissues

RFRP-3 was expressed in ovarian tissue of domestic cats (Fig. 3A). Both primary (NPFFR1) and secondary (NPFFR2) RFRP-3 receptors also were expressed in domestic cat ovaries (Fig. 3C,D).

3.4.2 Influence of RFRP-3 on follicle size and integrity

There were no differences in follicle size among treatments at the time of isolation (day 0; Fig 4A; Vehicle vs. FSH, $t_{247.13} = 0.24$, $P = 0.81$; FSH vs. low, $t_{247.2} = 0.11$, $P = 0.91$; FSH vs. high, $t_{246.9} = 0.74$, $P = 0.46$; low vs. high, $t_{247.3} = 0.58$, $P = 0.56$). There was no effect of culture period on change in follicle size over time ($t_{3096} = 1.05$, $P = 0.29$) or interaction between time and treatment ($P > 0.61$ for all comparisons) for follicles that maintained integrity. However, by Day 8, intact follicles in the low dose of RFRP-3 (1 μ*M*) were significantly smaller than follicles in the FSH-only and the high dose (10 μ*M*) groups (Fig. 4A; FSH vs. low, $t_{496.6} = 2.77$, $P = 0.006$; low vs. high, $t_{484} = -3.09$, $P = 0.002$). There were no differences between FSH-only treatment and Vehicle or high dose of RFRP-3 (Fig. 4A; Vehicle vs. FSH, $t_{465.4} = 1.25$, $P = 0.21$; FSH vs. high, $t_{476.9} = -0.46$, $P = 0.64$).

We were unable to detect an interaction between treatment and starting follicle size ($P > 0.09$ for all comparisons). However, the likelihood of degradation was lower in FSH-treated follicles relative to vehicle-controls (Fig. 4B; FSH vs Vehicle, $z = 2.62$, $P = 0.009$). The low dose of RFRP-3 (1 μ*M*) increased the likelihood of basal lamina degradation relative to FSH-treated follicles (Fig. 3B; FSH vs. low, $z = 2.04$, $P = 0.04$). The likelihood of basal lamination degradation in the high dose (10 μ*M*) did not significantly differ from FSH-only or low RFRP-3 treated follicles (Fig. 4B; FSH vs. high, $z = 1.33$, $P = 0.18$; low vs. high, $z = 0.80$, $P = 0.42$).

3.4.3 Influence of RFRP-3 on follicle survival

There was no effect of FSH supplementation on cell survival relative to vehicle-controls ($z = 0.141$, $P = 0.88$). However, the low dose of RFRP-3 (1 μ*M*) decreased follicle survival ($z = -2.198$, $P = 0.03$, Fig. 4C). There was no difference in survival between high-dose RFRP-3 (10 μ*M*) and FSH-only or low RFRP-3 treated follicles (Fig. 4C; FSH vs. high, $z = 1.69$, $P = 0.09$; low vs. high, $z = -0.46$, $P = 0.65$).

3.4.4 Influence of RFRP-3 on progesterone production

FSH supplementation did not modify progesterone production relative to vehicle-controls (t_{91}=1.492, P = 0.14). However, addition of the low dose of RFRP-3 (1 μ*M*) resulted in increased production of progesterone relative to FSH-only and high dose RFRP-3 treatments (Fig. 5; FSH vs. low, t_{91} = 2.504, P = 0.01; low vs. high, t_{91} = -3.573, P = 0.0003). There was no difference in progesterone production between high-dose RFRP-3 (10 μ*M*) and FSH-only treated follicles (Fig. 5; FSH vs. high, t_{91} = -0.952, P = 0.34).

3.5 Discussion

Our study is the first to examine effects of RFRP-3 on the isolated follicle as a functional unit, and it is the first study of RFRP-3 expression and function in any carnivore species. We demonstrated that the domestic cat ovary produces RFRP-3 and its receptors. Furthermore, we show that RFRP-3 influences the survival, integrity, and steroid production of isolated domestic cat follicles *in vitro*.

We were able to identify transcripts for the RFRP-3 peptide in the domestic cat ovary; our data are thus consistent with the hypothesis that there is conserved expression of the GnIH peptides in the vertebrate ovary. We also found expression of both receptors for RFRP-3, NPFFR1 and NPFFR2, in the ovary of the domestic cat. To our knowledge, no other study has yet examined presence/absence or distribution of the NPFFR2 receptor in the ovaries, thus any suggestions of its functional relevance would be speculative. Both receptors are also found in the testes of Syrian hamsters[144], so we expect that both receptors are likely to be present in the ovaries of other species. In Syrian hamster testes, NPFFR1 was localized to spermatids at all stages of spermatogenesis, whereas NPFFR2 expression was limited to late elongated spermatids[144], leading Zhao et al. to suggest that RFRP-3 may act on these receptors to regulate related but distinct processes in spermatogenesis[144]. To determine whether similar hypotheses can be extended to direct action on oocyte maturation, future studies will need to combine localization and targeted functional (receptor-specific antagonists/agonists[145]) studies.

In order to assess functional effects of RFRP-3 on follicle viability, we quantified morphological integrity, follicle size, and cell survival over the course of treatment. We found no effect of FSH or RFRP-3 exposure on follicle size during the study period. However, because we focused on early-stage follicles, longer periods of culture may be needed to generate measurable changes in follicle size. We did find that exposure to a low concentration (1 μM), but not a high concentration (10 μM), of RFRP-3 increased the proportion of morphologically degraded follicles over time (Fig. 5) and increased the proportion of follicles exhibiting cellular death (Fig. 6). Moreover, the decrease in follicle size in the low-RFRP-3 treatment group suggests that there is selective loss of larger early-stage follicles or decrease in size in response to low-dose RFRP-3. Together, these findings suggest that RFRP-3 inhibits maturation and viability of early-stage follicles *in vitro*. Given that the receptor for RFRP-3 is often found to be more abundant in early-stage follicles[126,146],

whereas the RFRP-3 peptide is more abundant in tertiary or pre-ovulatory follicles[129,130,147,148] (see Table 1 for some exceptions), we speculate that RFRP-3 production by tertiary follicles suppresses the maturation of early-stage follicles in a paracrine fashion by promoting follicle degradation.

More broadly, our finding that RFRP-3 decreases follicle integrity and cell viability is consistent with the inhibitory effect of RFRP-3 on reproductive function of the ovary in other mammals and in birds. These results thus add credence to the conserved role of RFRP-3 within vertebrate ovaries.

We also found that the lower dose RFRP-3 *increased* steroid production from domestic cat follicles, whereas GnIH has been shown to *decrease* steroid production by the ovaries in all other studies to-date (see Table 1). There are several potential explanations for this discrepancy which highlight the need for more comparative, functional experimentation. First, our results may differ from others published to date because we are the first to examine action of RFRP-3 on the follicle as a functional unit; previous work has focused on isolated granulosa cells or whole ovaries (Table 1). Apparent effects of RFRP-3 on the whole follicle may differ from effects measured at the whole ovary or on isolated granulosa cells because of the extensive role endocrine and paracrine signaling play in regulating follicle development, degradation, and ovulation. Studying isolated granulosa cells may obscure important interactions between thecal and granulosa cells in a functional follicle. Similarly, action of RFRP-3 on the whole ovary does not necessarily tell us about action at an individual follicle within the hierarchy. We focus here on primary and secondary follicles, whereas studies examining whole ovaries may be principally measuring steroid secretion by more dominant (hierarchical and pre-ovulatory) follicles. Experiments that explore a broader range of follicle classes will be informative.

Second, the stimulatory action of RFRP-3 on progesterone production by domestic cat follicles could be a result of the environmental conditions that cats experienced prior to spaying; the environmental context may determine the capacity for and/or direction of the ovarian follicles' responses to RFRP-3. RFRP-3 action is context-dependent in seasonal breeders, including Siberian hamsters[149] and European Starlings[150]. In Siberian hamsters, RFRP-3 inhibits gonadotropin release under long-day photoperiods (breeding conditions), but it promotes gonadotropin release under short-day photoperiods (non-breeding)[149]. In European Starlings, the inhibitory action of GnIH on testicular testosterone secretion occurs only prior to the onset of breeding – testicular testosterone production of breeding birds is insensitive to GnIH[150]. Domestic cats are also seasonal breeders [151–153], and feral females do not become pregnant between November and early February, even in mild climates (unpublished data, K Wilsterman). Tissues used in this study were collected from feral cats during the early summer – follicles may respond differently to RFRP-3 if experiments were carried out at other times of the year. Contrasting results from different periods within known breeding cycles is necessary to determine whether ovarian RFRP-3 exerts context-dependent action.

Third and finally, it is also possible that the apparently unique stimulation of steroid production reflects species-specific or carnivore-specific function of the RFRP-3 system. Similar species-specific action of RFRP-3 has been demonstrated in the hypothalamus and ovary in other species. In the Syrian hamster, RFRP-3 stimulates hypothalamic GnRH instead of inhibiting hypothalamic GnRH, as is the case in most vertebrates[154], and in the grouper ovary, GnIH promotes transcription of steroidogenic enzymes and luteinizing hormone receptor (LHR)[132]. Although GnIH does exert remarkably conserved action in the reproductive system of many vertebrates, it is also clear that there is species-specific variation in. Because felids display unique diversity in reproductive function[155], we also might expect to see greater diversity or idiosyncrasy of peptide function. Understanding the evolutionary drivers (e.g., natural selection, genetic drift) of variation in reproductive system function among felids is largely an open but exciting question.

Taken together, our results are broadly consistent with the functional and observational studies conducted to-date, and they support the potential for RFRP-3 to serve as a useful target for development of tools related to feral and invasive species management and endangered species survival plans. Ultimately, effects of RFRP-3 on follicle viability will be more important for species management than will the effects on steroid production alone, and our results support that RFRP-3 significantly decreases follicle viability. For feral and invasive species management, overexpression of RFRP-3 in the ovary may be able to suppress follicular development, thereby reducing fecundity of these individuals. For endangered species breeding programs, antagonists for the RFRP-3 receptor may protect or promote follicle viability for *in vitro* and *in vivo* use[135]. For both, understanding the molecular pathways underlying RFRP-3-mediated degradation of follicle integrity and any potential dose-dependency of these effects is vital. We used concentrations of RFRP-3 at the upper range of those found to be effective in other studies because alginate embedding can inhibit the diffusion of proteins to the follicles in culture[156,157], however our findings suggest that the higher concentrations used here might not be biologically-relevant. More conservative (lower) concentrations are likely to be more effective for future functional studies in felines.

In addition to these applications, the concordance between GnIH action in the hypothalamus and ovary offers a unique opportunity to understand hormone functional evolution across vertebrates. Our findings that similar effects occur in felines as in other mammals and chickens should encourage further comparative research into the function of GnIH across the reproductive axis in vertebrates. Because the conservation of expression does not guarantee conservation of function, functional studies are more likely to rapidly advance our understanding of GnIH functional evolution and conservation.

Though there is interest in the application of RFRP-3 to human reproductive diseases[129], animal control[133,134], and endangered species survival programs[135], its potential in these arenas depends on functional studies that will

determine species-specific dose-dependency, gonadotropin-sensitivity, and efficacy of action at the level of the whole ovary. In addition to focusing on RFRP-3, the regulation and function of the two receptors for the RFRP-3 peptide (NPFFR1 and NPFF2) needs to be elucidated to complete our understanding of this system. RFRP-3 provides a unique opportunity to study the basic evolution of hormone systems in central and peripheral reproductive systems while simultaneously advancing development of applied technologies.

3.6 Acknowledgments

The authors thank Dr. Crystal Heath and the Community Concern for Cats, Inc. Cat Hospital (Concord, CA) for donating tissues from spay/neuter clinics for this study. Technical assistance from KA Conner, CA Limber, and A Pepper was also essential to completion of this project.

3.7 Tables

Table 1 Observational and functional studies of GnIH/RFRP-3 in vertebrate ovaries.

Cit.	*Class*	*Order*	Species/*Strain*	*Approach*	*Summary of findings*
Observational Findings					
[158]	Hyperoartia	Petromyzontiformes	*Petromyzon marinus*	PCR	GnIH expressed in the ovary
[159]	Osteichthyes	Cichliformes	*Oreochromis niloticus*	PCR	GnIH and receptor expressed in the ovary
[146]	Osteichthyes	Cypriniformes	*Carassius auratus*	*in situ*	GnIH receptor expression abundant in early-stage follicles
[160]	Osteichthyes	Cypriniformes	*Danio rerio*	PCR	GnIH and GnIH-R expressed in the ovary
[161]	Osteichthyes	Cypriniformes	*Danio rerio*	PCR/*in situ*	GnIH expression in the ovary highest during primary growth of follicles, and lower during later stages of follicle growth (PCR); GnIH expressed in the granulosa cells of vitellogenic follicles (*in situ*)
[162]	Osteichthyes	Perciformes	*Dicentrarchus labrax*	PCR	GnIH expressed in the ovary (very low)
[163]	Osteichthyes	Pleuronectiformes	*Cynoglossus semilaevis*	PCR	GnIH expressed in ovary; GnIH expression ten-times more intense during previtellogenesis relative to other stages of ovary maturation
[164]	Osteichthyes	Tetraodontiformes	*Takifugu niphobles*	PCR	GnIH expression **absent** in the ovary
[147]	Reptilia	Squamata	*Calotes versicolor*	IHC/slot blot	GnIH-ir highest in the stroma of resting-phase ovaries (IHC); GnIH-ir present in GCs of the dominant follicle during recrudescence and the oocyte of the dominant follicle during folliculogenesis (IHC); GnIH protein abundance increases across vitellogenesis, ovulation, and regression, with abundance highest in resting-phase ovaries (slot blot)
[165]	Aves	Galliformes	*Coturnix japonica*	PCR	GnIH and GnIH-R expressed in the ovary (PCR);
[126]	Aves	Galliformes	*Gallus gallus domesticus*/ White Leghorn	PCR	GnIH-R expression in TCs decreases with follicle maturation; GnIH-R is more abundant in GCs of all follicles than in TCs
[165]	Aves	Passeriformes	*Zonotrichia leucophrys Gambelii*	*in vivo* & *in vitro* receptor fluorography/ IHC	Bindings sites for GnIH found in the GCs of ovarian follicles (*in vivo* & *in vitro* receptor fluorography); GnIH-ir localized to ovarian GCs (IHC)
[165]	Aves	Passeriformes	*Sturnus vulgaris*	*in vivo* receptor fluorography/ PCR/IHC	Bindings sites for GnIH in the GCs of ovarian follicles (*in vivo* receptor fluorography); GnIH and GnIH-R expressed in the ovary (PCR); GnIH-ir localized to ovarian GCs (IHC)
[166]	Mammalia	Artiodactyla	*Ovis aries*/ Dorper×Hu F1	PCR/IHC	GnIH expression in the ovary (PCR); GnIH expressed in oocytes (IHC); GnIH expressed in GCs tertiary follicles only (IHC)
[167]	Mammalia	Artiodactyla	*Sus scrofa domesticus*/ Suzhong (PCR)	PCR/IHC	GnIH expression in ovary most abundant during proestrous and least abundant during estrous

			Large white cross-bred (IHC)		(PCR); GnIH-R expression in the ovary most abundant at estrous and least abundant during diestrous (PCR); Most intense GnIH-ir found in the GCs during estrous; GnIH-ir also found in TCs, and CL (IHC); GnIH-R-ir most intense in the TCs and GCs of mature follicles during estrous; GnIH-R-ir also found in the CL (IHC)
[168]	Mammalia	Artiodactyla	*Sus scrofa domesticus*/ Yorkshire	PCR	GnIH expressed in the pubertal ovary
[148]	Mammalia	Rodentia	*Mus musculus domesticus*/ Parkes	IHC	GnIH-ir greatest in non-luteolytic CLs and the GCs/TCs of mature follicles
[129]	Mammalia	Rodentia	*Rattus norvegicus domesticus*/ Sprague-Dawley	IHC	GnIH-ir present in the interstitial tissues and GCs of antral follicles; low GnIH-ir in the CL
[130]	Mammalia	Primates	*Homo erectus*	IHC	GnIH and GnIH-R present in CLs and GCs/TCs of pre-ovulatory follicles
[41]	Mammalia	Primates	*Macaca mulatta*	*in situ*	GnIH and GnIH-R expressed in GCs and oocytes
Functional Findings					
[132]	Osteichthyes	Perciformes	*Epinephelus coioides*	Ovarian explant	GnIH(I) increases expression of StAR and 3βHSD; GnIH(II) increases expression of LHR
[147]	Reptilia	Squamata	*Calotes versicolor*	Ovarian explant	GnIH decreases expression of GnRH-R in the ovary
[126]	Aves	Galliformes	*Gallus gallus domesticus*/ White Leghorn	Isolated granulosa cells	GnIH decreases cell survival at 10 and 1000 nM, but only in the absence of FSH
[127]	Mammalia	Artiodactyla	*Sus scrofa domesticus*/ Large White	Isolated granulosa cells	GnIH at 1000 and 0.1 nM groups exerts non-dose dependent inhibition of E_2 production; GnIH has no effect on P_4 production; GnIH exerts dose-dependent inhibition of ERK and PCNA expression.
[139]	Mammalia	Artiodactyla	*Sus scrofa domesticus*/ Unknown	Isolated granulosa cells	GnIH dose-dependently inhibits GC proliferation; GnIH induces cell cycle arrest in the G2/M phase
[148]	Mammalia	Rodentia	*Mus musculus domesticus*/ Parkes	Ovarian explant	GnIH dose-dependently inhibits GnRH-1-R expression in the ovary
[128]	Mammalia	Rodentia	*Mus musculus domesticus*/ Parkes	Ovarian explant	GnIH inhibits P_4 production; GnIH decreases the expression of StAR and 3βHSD
[129]	Mammalia	Rodentia	*Rattus norvegicus domesticus*/ Sprague-Dawley	Ovarian explant	GnIH decreases P_4 & T production only under the presence of LH
[129]	Mammalia	Rodentia	*Rattus norvegicus domesticus*/ Sprague-Dawley	Peristaltic pump	GnIH treatment is associated with larger CLs in the ovary
[130]	Mammalia	Primates	*Homo erectus*	Isolated granulosa cells	GnIH dose-dependently inhibits P_4 accumulation in the presence of gonadotropins; GnIH inhibits StAR expression in the presence of

					gonadotropins; GnIH has no effect on steroid accumulation or steroidogenic gene expression when gonadotropins are not present

Abbreviations used in table: ***3βHSD:*** *3-beta-hydroxysteroid dehydrogenase;* ***CL:*** *corpora lutea;* ***E_2 :*** *estradiol;* ***ERK:*** *extracellular regulated kinases;* ***FSH:*** *follicle stimulating hormone;* ***GC:*** *granulosa cell;* ***GnIH:*** *gonadotropin inhibitory hormone; used throughout table for simplicity for all genes in the LPXRFa, GnIH and RFRP-3 gene family;* ***GnIH-ir:*** *GnIH immunoreactivity;* ***GnIH-R:*** *GnIH receptor;* ***GnIH-R-ir:*** *GnIH receptor immunoreactivity;* ***GnRH-1-R:*** *receptor for gonadotropin-inhibitory hormone-1;* ***IHC:*** *Immunohistochemistry;* ***LH:*** *luteinizing hormone;* ***LHR:*** *luteinizing hormone receptor;* ***P_4:*** *Progesterones;* ***PCNA:*** *proliferating cell nuclear antigen;* ***PCR:*** *polymerase chain reaction;* ***StAR:*** *Steroidogenic acute regulatory protein;* ***T:*** *testosterone;* ***TC:*** *thecal cell*

Table 2 Primer sequences for PCR amplification

Target Gene	NCBI Accession Number	Forward	Reverse	Amplicon length
RFRP-3	XM_023242159.1	TGATGTCCGGTTTTCACAG	TTTGGACCCCAGTCTTG	118
NPFFR1	XM_023240516.1	CTGTATGCCCACCACTCTCG	CGGAACCTTTCCACAGCAATG	144
NPFFR2	XM_003985291.5	CGGGAAGACTGGCCAAATCA	GTGGGGCACTGTCATCTTGA	141
	XM_023253059.1			
GAPDH	NM_001009307.1	AGTATGATTCCACCCACGGCA	GATCTCGCTCCTGGAAGATGGT	102
HPRT	XM_006939462.4	ACTGTAATGACCAGTCAACAGGGG	TGTATCCAACACTTCGAGGAGTCC	210

Table 3 Medium composition for follicle culture experiments

Component	**Catalogue number**	**Media Type**		
		Transport media	Collection media	Growth media
Base media		MEM with Hank's Salts [11575-032, Gibco]	MEM with Hank's Salts [11575-032, Gibco]	MEM with Earle's Salts [M5650, Sigma-Aldrich]
HEPES buffer	15630-080, Gibco	-	1%	-
Penicillin	P7794, Sigma-Aldrich	100 IU/mL	100 IU/mL	50 IU/mL
Streptomycin	S1277, Sigma-Aldrich	100 µg/mL	100 µg/mL	50 µg/mL
Ascorbic acid	A61-100, Fisher	0.25 mM	-	0.25 mM
L-glutamine	G8540, Sigma Aldrich	-	2 mM	2 mM
ITS+	41400045, Thermofisher	-	-	1%
Bovine serum albumin	A9418, Sigma-Aldrich	-	0.3% w/v	0.3% w/v

3.8 Figures

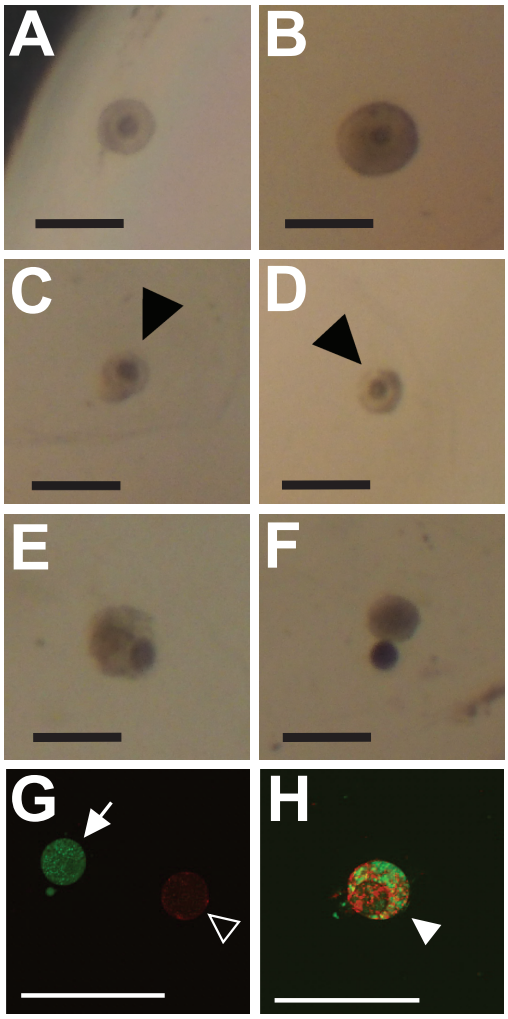


Figure 1 Representative images showing classification of follicles for integrity (A-F) and viability (G,H; cell death) outcome measures. Follicles with intact and relatively even basal lamina were classified as intact (maintaining integrity) (A, B). Follicles with gaps in the basal lamina (arrowheads in C, D), a hatching oocyte (E, F), or having an absent basal lamina were classified as degraded. (G, H) Fluorescent dyes indicate cell survival in follicles (red for dead, green for alive). Follicles exhibiting only green fluorescence were categorized as alive (filled arrow in G). Follicles with any red fluorescence were categorized as dead or dying (empty arrowhead in H indicates mixed staining, whereas the filled arrowhead in G indicates a follicle fluorescing only in red). Scale bars are equal to 250 μm.

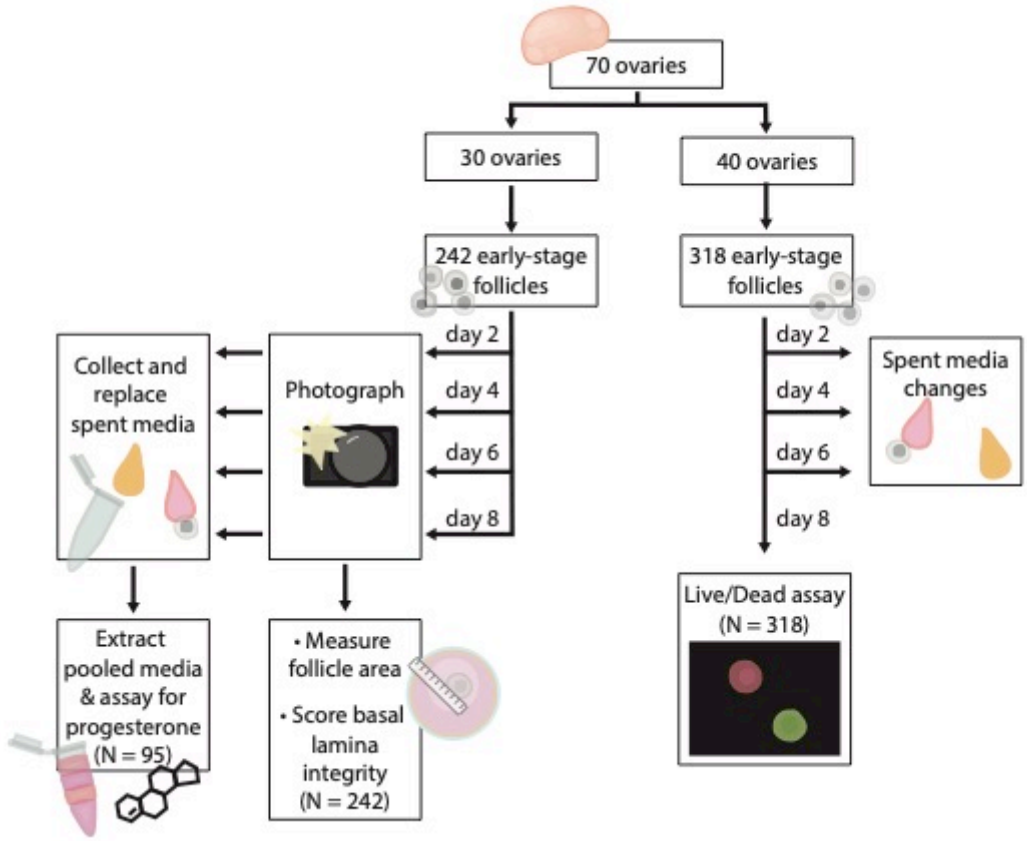


Figure 2 Flow chart summarizing procedures and sample sizes for experiments.

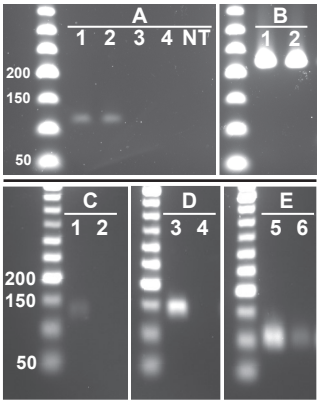


Figure 3 Representative images showing presence of RFRP-3 and receptor transcripts in the domestic cat ovary. RFRP-3 transcripts can be found in RNA isolated from whole ovary (A, lanes 1 and 2), but not in No-RT controls (A, Lane 3 and 4) or no-transcript controls (A, lane NT). HPRT was used as a positive control (B, lanes 1 and 2). The primary RFRP-3 receptor (NPFFR1, C) and the secondary receptor (NPFFR2, D) were also found in ovarian tissues (lane 1 & 3). No-RT controls show no amplification (lanes 2 & 4). GAPDH was used as positive control for both the sample (lane 5) and No-RT (lane 6) (E).

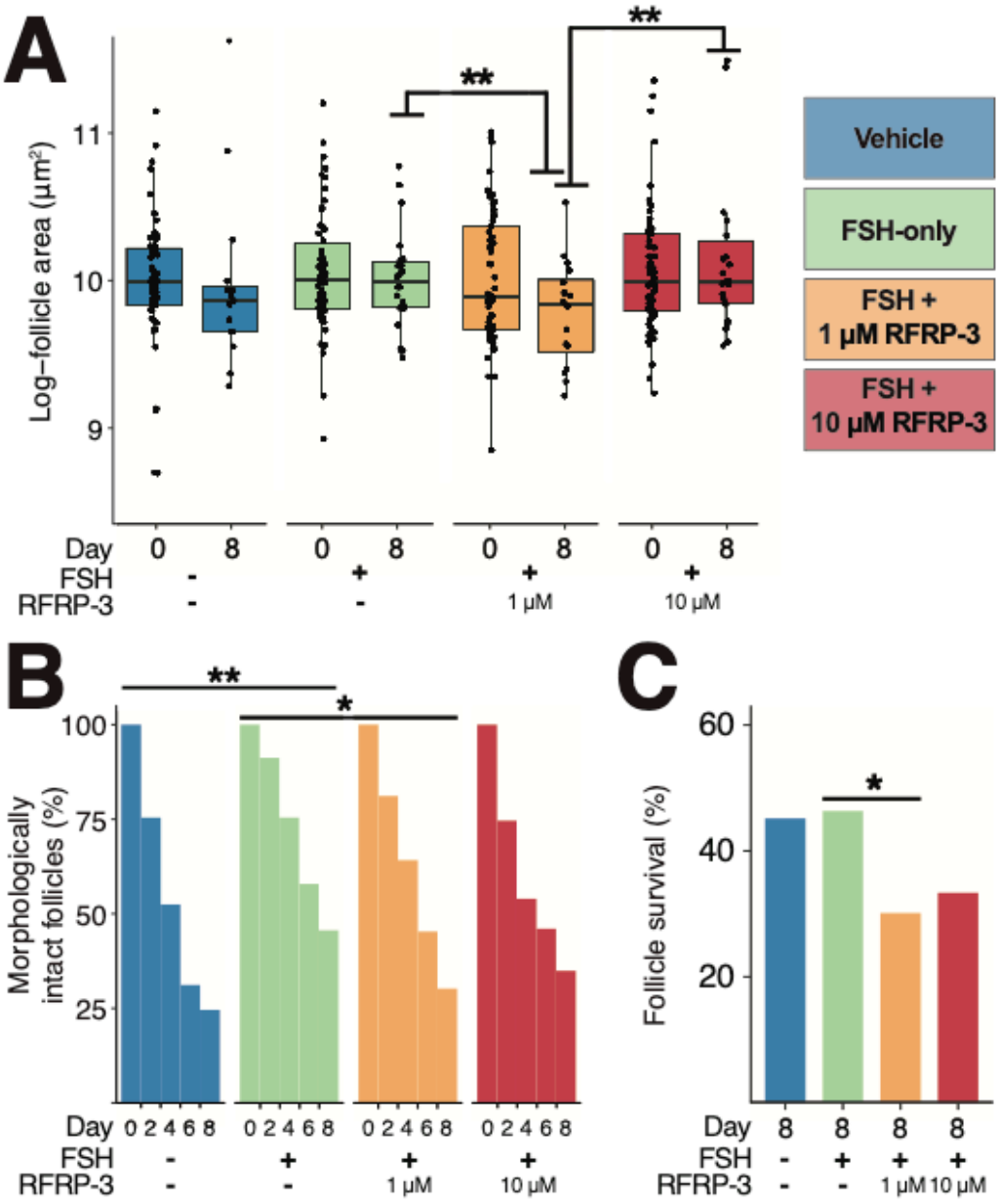


Figure 4 Follicle size, integrity of the basal lamina, and morphology were all affected by 1 µM RFRP-3 treatment. A color-based key to treatments is shown the top right of the figure and along the x-axis in each panel. Isolated follicles were treated with vehicle (blue), 1 µg/mL FSH (green), or 1 µg/mL FSH combined with 1 µM (orange) or 10 µM (red) RFRP-3 throughout the culture period. (A) The size of follicles with intact basal lamina in each experiment for days 0 and 8 of culture. Follicle size (area) was log-transformed for plotting. (B) The percent of ovarian follicles with intact basal lamina every 2 days for the duration of the culture period. (C) The percent of follicles alive on day 8 of treatment based on live/dead fluorescent assay. Asterisks indicate significant differences based on planned comparisons. For all panels, **P < 0.01, *P<0.05.

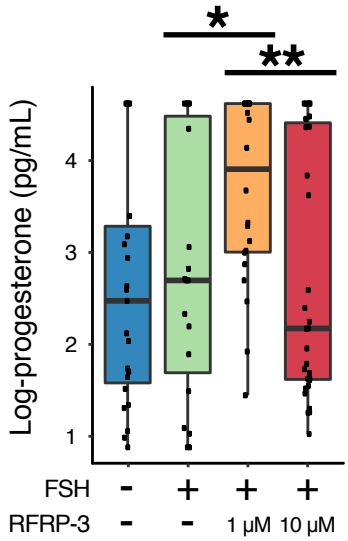


Figure 5 Cumulative progesterone production by individual follicles across the culture period. Progesterone concentration was log-transformed for plotting. Asterisks indicate significant differences based on planned comparisons. **P < 0.01, *P<0.05.

4. Sex steroids coordinate organizational but not functional decidualization of feline endometrial epithelial cells in a 3-dimensional culture system

4.1 Abstract

Successful implantation requires complex signaling between the uterine endometrium and the blastocyst. Prior to the blastocyst reaching the uterus, the endometrium is remodeled by sex steroids and other signals to render the endometrium receptive. *In vitro* models have facilitated major advances in our understanding of endometrium preparation and endometrial-blastocyst communication in mice and humans, but these systems have not been widely adapted for use in other models which might generate deeper understanding of these processes. The objective of our study was to use a recently-developed 3-dimensional (3D) culture system to identify specific roles of female sex steroids in remodeling the organization and function of feline endometrial epithelial cells (EECs). We treated EECs with physiologically-relevant concentrations of estradiol and progesterone, either in isolation or in combination, for one week. We then examined size and density of 3D structures, and we quantified expression of candidate genes known to vary in response to sex steroid treatments and that have functional relevance to the decidualization process. Both estradiol and progesterone influenced the organization and gene expression of primary endometrial cells in culture. Combined sex steroid treatments recapitulated organizational patterns seen *in vivo*, however sex steroid manipulations failed to induce expected changes to expression of decidualization-related genes. Our results identify keys areas where sex steroids may not sufficiently prepare feline EECs for implantation, thereby highlighting key areas of opportunity and suggesting some unique functions of felid uterine tissues.

4.2 Introduction

In mammals, successful pregnancy establishment relies upon interactions between the blastocyst and endometrium (lining of the uterus) during implantation [169–171]. Insufficient or inappropriate endometrial preparation prior to blastocyst arrival is thought to be a major cause of idiopathic pregnancy failure[172]; if the endometrium is unreceptive to the blastocyst due to incomplete or inappropriate preparation, implantation cannot occur. Development of appropriate interventions to prevent blastocyst rejection relies on a thorough understanding of the processes that lead to endometrial preparedness and maintenance of a permissive environment during early pregnancy.

Endometrial preparation involves both morphological and functional changes. Leading up to potential pregnancy, the cells of the endometrium proliferate and reorganize to enlarge the luminal spaces within the endometrium (Figure 1A,

[173]). Regulatory networks within endometrial cells also modify gene expression to promote angiogenesis and glycogen storage. These changes include up-regulation of glucose transporters, receptors for sex steroids (estradiol and progesterone), and angiogenic factors (e.g., VEGF) that will ultimately support placentation. The reorganization of endometrial epithelial cells (EECs) and broad functional changes are superficially conserved among placental mammals [174–179], but the underlying mechanisms that coordinate these changes are only well-understood in rodent and human models.

The sex steroids estradiol and progesterone play a major role in many of the functional and organizational changes[173,180] . For example, elevated progesterone during the late luteal phase and early pregnancy is associated with the elevated transcription of glucose transporter 1 (GLUT1) and inhibition of glucose transporter 3 (GLUT3) transcription in humans, mice and rats[181–184]. However, *in vitro* studies that have focused on the molecular mechanisms suggest that the direct effects of estradiol and progesterone on endometrial function varies among these species. For example, although estradiol and progesterone treatment in combination stimulates GLUT1 expression[185–188], some studies suggest that estradiol alone is responsible for these changes[189], whereas others suggest that progesterone is principally responsible for GLUT1 up-regulation[185]. The same appears to be true for other conserved processes, including angiogenic gene expression. Although estradiol is sufficient to up-regulate vascular endothelial growth factor (VEGF) in human EEC lines[190,191], it has no effect *in vitro* on VEGF expression in EECs isolated from mice[192].

Thus far, these taxon-specific mechanisms have principally been used to evaluate the efficacy of rodent models for use in human health research[193]. While these straight-forward comparisons are useful for such efficacy testing, examining the broader range of mammalian placentation through comparative models may help us identify deeply conserved mechanisms of control, thereby improving likelihood of translational success. Such a broadening of understood models will also aid development of reproductive technologies for agricultural or conservation applications. The development of these *in vitro* endometrial models are the basis for more complex culture systems used to study blastocyst-endometrial interactions during early pregnancy, and thus lead to advances in both basic and applied biology research.

The need for such reproductive tools is especially great for felids. Although much effort has been focused on maintaining the genetic diversity through strategic breeding in captivity, breeding success is very low (<30%) in many endangered species [155,194], and intervention development has been slow[135,155,194]. Low breeding success may be related in part to aberrant endocrine profiles that sometimes follow ovarian stimulation in felids[155,195]. Even if follicular maturation and ovulation are successful, atypical endocrine environments may not sufficiently or appropriately prepare the endometrium for receptivity, thus resulting in failed implantation[155,195]. Identifying the extent to which sex steroids prepare the endometrium for implantation in felids will allow us to identify aspects of an

aberrant hormone profile that might contribute to failed reproduction and determine whether manipulating the hormonal profile might improve outcomes in felines and inform broader comparisons among species.

In order to determine the specific and interactive roles of sex steroids on feline endometrial organization and function, we used a recently-developed three-dimensional (3D), Matrigel-based culture system for primary EECs from the domestic cat[196]. Matrigel-based culture systems have been used for decades to facilitate cell polarization and organoid growth by EECs *in vitro*[197–199], and primary EECs grown in Matrigel-based matrices form 3D structures that are strikingly similar across mammalian taxa[197–200]. This system therefore allows us to evaluate both organizational and functional (gene expression) changes to EECs under tightly controlled conditions.

We hypothesized that estradiol and progesterone would drive distinct processes related to endometrial organization and function in ways that were consistent with cyclic dominance of each steroid across the natural reproductive cycle (Fig. 1A). To test this hypothesis, we chronically treated replicate cultures with either estradiol or progesterone at physiologically-relevant concentrations (Table 1). We specifically expected to see an increase in the number of gland-like structures in response to estradiol, as occurs *in vivo* during the follicular phase in domestic cats[174] (Fig 1B). We expected to see an increase in structure size in response to progesterone, as occurs during the luteal phase and in response to exogenous progesterone treatment[174] (Fig 1D).

Second, we examined the effects of physiologically-relevant combinations of sex steroids on these same outcome measures (Table 1). Many reproductive processes rely on permissive effects of one or more steroids, and we might therefore expect that some changes to EEC organization in the endometrium will depend on presence of both sex steroids. However, if sex steroids control truly distinct processes in the endometrium, the morphological organization of EECs should reflect the dominant sex steroid at any given stage, and thus be consistent with results from our isolated hormone experiments (shown in Fig 1C & E).

Because we were also interested in molecular markers of functional change, we chose to examine expression of genes that are relevant to endometrial preparedness and are regulated by estradiol or progesterone in other species[182,201–205]. For experiment 1, we predicted that estradiol would dose-dependently up-regulate expression of angiogenic genes, including VEGF, but would have no independent effect on glucose transporter 3 (GLUT3), progesterone receptor (PGR), or insulin receptor (IR) expression. We also predicted that progesterone would up-regulate VEGF, PGR, and IR, and down-regulate GLUT3[202]. For the second experiment using combined sex steroid treatments, we predicted that the response of EECs would again reflect the dominant sex steroid.

4.3 Materials and methods

4.3.1 Tissue handling and endometrial epithelial cell (EEC) culture

Whole uterine and ovarian tissues from reproductive queens in the follicular phase were collected into chilled transport media (Table 2) immediately following routine spays at local veterinary clinics. EEC isolation from uterine horns was carried out within 8 h of tissue collection.

Only uteri from cats in the follicular phase were used for experiments. Uterine horns were snipped into ~0.5 cm pieces and washed in ice-cold, sterile Dulbecco's PBS (dPBS, D8537, Sigma-Aldrich). The lumen of each piece was flushed thrice with 100 μL of dissociation solution (1% porcine trypsin [15090046, Gibco] in dPBS) and submerged in dissociation solution. In pilot tests, we found digestion with porcine trypsin resulted in cleaner EECs following isolation compared to digestion with trypsin from bovine pancreas (T9935, Sigma Aldrich). Tissue was incubated in dissociation solution at 10°C for 1 h followed by 45 min at room temperature. The digestion was stopped by washing tissue in 20% fetal calf serum (MT35010CV, Corning) in basal media (Table 2). Tissue pieces were halved to expose the lumen and then scraped using a scalpel blade to release endometrial epithelial cells (EECs) into media. EECs were transferred to a 35 mm plate containing clean basal medium with 10% FCS and placed on a slide warmer at 38°C until all tissue segments had been processed. Isolated EECs in ~2 ml of 10% FBS basal medium were moved in solution to 15 ml sterile conical tubes and washed twice with 13 ml of warmed dPBS. For each wash, EECs were suspended in fresh, warmed dPBS and centrifuged for 3 min at 150 g. Supernatant was poured off as waste. After the second wash, all supernatant was removed and replaced with 10 ml of fresh basal medium containing 10% charcoal-stripped FCS (A3382101, ThermoFisher Scientific). EECs were suspended by pipetting up at down with a 1 ml pipette 25 times and plated in 6-well plates (353046, Corning). If we found that only a very small number of EECs were isolated from an individual or EECs appeared very contaminated (hazy media or excessive red blood cell retention), the sample was not used for experiments.

EECs were allowed to attach in basal medium for 24 h, at which time medium was removed, wells were gently washed with warmed, sterile dPBS, and medium was replaced with matrigel medium along with treatments (see Table 1). Steroid hormones (progesterone [P8783, Sigma-Aldrich], and ß-estradiol [E8875, Sigma-Aldrich]) were dissolved in DMSO (D128-500, Fisher Chemical) to generate stock solution aliquots, which were stored at -20°C until use. Fresh aliquots were thawed and diluted into medium prior to applying treatments to the plates. Vehicle-treated EECs received equivalent amounts of pure DMSO. DMSO concentration was less than 0.01% in culture for all treatments. For experiment 1, we exposed EECs to two concentrations of progesterone or estradiol at physiologically-relevant concentrations. For experiment 2, we simulated interestrous (low concentration of

both steroids), estrous (high estradiol concentration), and pregnancy (high progsesterone) conditions using physiologically-relevant concentrations of hormones in combination based on established *in vivo* measurements in the literature[206].

Thereafter, medium was replaced every 24-48 h with fresh steroid hormones added to each well. All culture was carried out at in a 38°C, 5% CO_2 air-jacket incubator. After 7 days, EECs from confluent wells were collected by adding trypsin (0.25% porcine trypsin in pre-conditioned PBS) for 5 min and scraping EECs from the bottom of the well. Trypsin treatment was halted by addition of 10% charcoal-stripped FCS. EECs were moved to 1.5 ml Eppendorf tubes and washed twice with sterile dPBS. After the final wash, all supernatant was removed and replaced with 100 µl of lysis buffer (RLY Buffer, Bioline). EECs were vortexed at RT in lysis buffer before storage in -80°C until RNA extraction.

4.3.2 Morphological measurements

EECs formed distinct 3-dimensional structures in the Matrigel medium. Prior to EEC collection from wells, four brightfield images of each well were taken using a stereoscope at fixed magnification (Fig. 2) from which we quantified the size and density of the two primary types of structures: gland-like (Fig 2 VEH, arrow heads) and tube-like (Fig 2 VEH, arrows). Images were all analyzed in ImageJ.

The area of gland-like structures was measured in arbitrary units using the freehand tool. Each structure was outlined, and the area was recorded. All structures with an area of larger than 89 μm^2 (100 sq. pixels) were measured and the number of structures in each image was counted. We counted the total number of tube-like structures that connected two or more gland-like structures. We used the segment tool to measure the diameter at the smallest point along the length of all tubes.

For each well, the number of gland- and tube-like structures were summed across the four images analyzed, and then a density was calculated by correcting for the total area photographed (the total area of each well imaged varied, e.g. Fig. 2B,C).

4.3.3 RT-qPCR

RNA was extracted using the Bioline RNA Micro Kit according to manufacturer guidelines (BIO-52075, Bioline).

750 ng of RNA were reverse transcribed to cDNA in a 40 µl reaction using the BioRad gDNA-clear reverse transcription for qPCR kit (BioRad 1725035). Each sample was assayed in triplicate, 12 µl quantitative PCR reactions in 384-well plates on a BioRad CFX 384 Touch Real-Time PCR Detection System. All primers were validated for specificity (using melt-curves and agarose gel) and efficiency (standard dilution curve) prior to use. Primer sequences, concentration used in reactions, annealing temperatures, and efficiencies are listed in Table 3.

4.3.4 Experimental design and sample sizes

For each experiment, EECs from each individual were evenly allocated among 6 wells in one 6-well plate (i.e., EECs isolated from individual X were evenly allocated among 6-well plate A, and EECs from individual Y were evenly allocated among 6-well plate B, etc.). For experiment 1, one of the six wells was not used for further experimentation, and the 5 remaining wells were assigned to each of the five treatment groups (Table 1). For experiment 2, three wells were assigned to treatment groups from experiment 1 (VEH, E_2H, and P_4H), and the other three wells were assigned treatments for experiment 2 (IE, Es, and Pr; see Table 1). Of 111 total wells plated from 20 unique individuals, 3 wells were not included in further analyses due to yeast contamination during the culture period. Morphology of 5 wells were not analyzed because images were not captured prior to collecting EECs from wells. Finally, sample sizes for gene expression data are smaller because EECs were collected only from a subset of plates. Resulting sample sizes are summarized in Table 1.

4.3.5 Statistical analysis

Differences in morphological measures of EEC organization were analyzed using a generalized linear model approach (glmer). Model fit was evaluated using QQ plots. Treatment was included as fixed effects, with a random effect of individual ID nested within isolation date. For gland-like area, an inverse Gaussian distribution with identity link was fit to untransformed data. For tube diameter, a gamma distribution with log link was fit to untransformed data. For gland- and tube-like structure density, a gamma distribution with log link was fit to untransformed data. Significant differences among groups were determined using Tukey's HSD post-hoc testing ($P < 0.05$).

For gland-like structure area, we plotted estimated marginal means, which normalize values among random effects (ID and date of isolation), as generated by the emmeans package.

Relative gene expression for each gene was calculated using the Pfaffl method with single-run efficiency corrections based on a standard curve generated from pooled samples [207]. For experiment 1, all reference genes showed a significant effect of treatment on expression when including the vehicle-control wells ($P < 0.06$ for all). We therefore removed the vehicle control and used the 4 pg/ml estradiol treatment (E_2L) as our reference group for calculations and figures related to gene expression in experiment 1. For experiment 2, the interestrous-like treatment (IE) was used as the reference group. Thus, in figures, gene expression is shown as fold-change over the average expression in E_2L- or IE-treated wells.

Gene expression was normalized relative to the geometric mean of two reference genes (YWHAZ and RPL27) that did not vary with treatment ($P > 0.30$). We rank-

transformed all gene expression measures prior to analysis to ensure residual normality. Treatment effects were estimated using a generalized linear model with a Gaussian distribution and log link. Treatment was included as the fixed effect and individual ID nested within isolation date as the random effect. Goodness of fit was assessed using QQ plots for all models. Significant differences among treatment groups were determined using Tukey's HSD post-hoc testing ($P < 0.05$). Plots show mean ± SE.

4.4 Results

4.4.1 Do female sex steroids independently modify EEC organization and gene expression?

EEC organization

Steroid treatment did not alter the density of gland-like structures (Fig. 3A, TRT: $F_{4,65} = 2.36$, $P = 0.06$), but it modified gland-like structure areas (Fig. 3B, TRT: $F_{4,2700} = 23.87$, $P < 0.0001$). Both estradiol treatments and 4 ng/ml progesterone treatment significantly decreased the average gland-like structure area (Fig. 3B, $P < 0.0001$ for all), whereas 40 ng/ml progesterone treatment resulted in average gland-like structure area that did not differ from vehicle-treated controls (Fig. 3B, P_4H vs. vehicle, z ratio = 1.63, $P = 0.47$; P_4H vs. all other treatments, $P < 0.0004$). Steroid treatment modified the density of tube-like structures (Fig. 3C, TRT: $F_{4,65} = 8.05$, $P = 0.00002$). Estradiol dose-dependently decreased the density of tube-like structures (Fig. 3C, Vehicle vs. E_2L, z ratio = 2.77, $P = 0.04$; Vehicle vs. E_2H, z ratio = 5.70, $P < 0.0001$), whereas progesterone had no effect on density of tube-like structures (Fig. 3C, $P > 0.36$ for all comparisons). Steroid treatment had no effect on average diameter of tube-like structure (Fig. 3D, TRT: $F_{4,540} = 1.69$, $P = 0.15$).

Relative gene expression

There was no difference in the expression of GLUT3 (Fig. 4A, $F_{3,46} = 0.86$, $P = 0.47$). All other genes were affected by steroid treatment (VEGF: Fig 4B, $F_{4,46} = 3.43$, $P = 0.02$; PGR: Fig. 4C, $F_{4,46} = 7.99$, $P = 0.0002$; IR: Fig 4D, $F_{4,46} = 4.13$, $P = 0.01$). VEGF expression was higher in the 4 ng/ml progesterone treatment compared to the 40 pg/ml estradiol treatment (z ratio = -3.06, $P = 0.01$), but not significantly higher that the 40 ng/ml progesterone treatment (z ratio = -2.43, $P = 0.07$). PGR expression was higher in the 40 pg/ml estradiol treatment compared to all other treatments ($P < 0.02$ for all comparisons), but did not differ among remaining treatments (E_2L, P_4L, P_4H; $P > 0.031$ for all comparisons). Finally, IR expression was higher in the 4 ng/ml progesterone group relative to the 40 pg/ml estradiol group (z ratio = -3.31, $P = 0.005$), and the 4 pg/ml estradiol and 40 ng/ml progesterone were intermediate ($P > 0.19$ for all other comparisons).

4.4.2 Do female sex steroids modify EEC organization and gene expression in physiologically-relevant combinations?

EEC organization
Gland-like structure density varied among *in vivo*-like steroid conditions (Fig. 5A, TRT: $F_{2,30} = 3.61$, $P = 0.04$). Relative to low steroid conditions in the interestrous-like (IE) group, high estradiol conditions (estrous-like, Es) decreased the gland-like structure density (Fig 5A, $z = -2.95$, $P = 0.009$), and high-progesterone conditions (pregnancy-like, Pr) resulted in an intermediate gland-like structure density (Fig 5A, $P > 0.12$ for all comparisons). Gland-like structure area also varied among combined treatments (Fig. 5B, TRT: $F_{2,944} = 7.51$, $P = 0.0005$). In this case, gland-like structure areas in the Pr treatment were larger than the IE treatment (z ratio = -3.65, $P = 0.0008$), while Es treatment gland-like structure areas were intermediate in size ($P > 0.08$ for all comparisons).

The density of tube-like structures varied among treatments (Fig. 5C, TRT: $F_{2,30} = 8.40$, $P = 0.001$). The density of tube-like structures was lowest in the Es treatment (Fig. 5C, vs. IE, z ratio = -4.45, $P < 0.0001$; vs. Pr, z ratio = -2.42, $P = 0.04$), and there was no difference in tube-like structure density between Pr and IE treatments (z ratio = 1.95, $P = 0.12$). Treatment had no effect on tube-like structure diameter (Fig. 5D, TRT: $F_{2,107} = 1.08$, $P = 0.34$).

Relative gene expression
There was a significant effect of treatment on expression of GLUT3 (Fig 6D, TRT: $F_{2,18} = 9.90$, $P = 0.001$) and expression of VEGF (Fig 6D, TRT: $F_{2,18} = 6.22$, $P = 0.008$). There was no effect of treatment on expression of PGR (Fig 6D, TRT: $F_{2,18} = 2.65$, $P = 0.09$) or IR (Fig 6D, TRT: $F_{2,18} = 1.56$, $P = 0.24$).

Expression of GLUT3 was lowest in the Pr treatment group (Fig. 6A, Pr vs. Es, z ratio = 3.32, $P = 0.002$; Pr vs. IE, z ratio – 4.47, $P < 0.0001$). The same pattern was evident in VEGF expression (Fig 6B, Pr vs. Es, z ratio = 2.89, $P = 0.01$; Pr vs. IE, z ratio = 3.49, $P = 0.001$).

4.5 Discussion

Sex steroids are associated with changes to function and organization of the endometrial epithelium cells (EEC) that are requisite for successful pregnancy. We explored the extent to which sex steroids modify the organization and function of primary feline EECs *in vitro*. Our experiments are the first to demonstrate that 3-dimensional (3D) structures formed by EECs in Matrigel are responsive to physiologically-relevant hormone regimes, and thus we offer novel evidence for the relevance of these structures to *in vivo* EEC organization. We also found that sex steroids enact different patterns of effects on EEC organization vs EEC function (gene expression), which suggest distinct regulatory systems within felid

decidualization that are not yet understood. Our experiments lay the groundwork for more comparative experimentation into the mechanisms controlling uterine preparedness in mammalian models while also proposing novel hypotheses about endocrine control of the endometrium in felids.

4.5.1 EEC organization reflects antagonistic interactions between sex steroids

EECs organize into similar 3D structures *in vitro* across species[197–200], and researchers often address their histological similarity in structure to the glands that form and grow in the endometrial epithelium during pregnancy preparation *in vivo*[197–200]. To our knowledge, no one has experimentally determined whether the abundance or size of these structures varies with sex steroid treatment as would be expected based on *in vivo* patterns (e.g., Fig 1A, [174,208]). Thus, even though the organization at the cellular level resembles endometrial glands, the extent to which their formation reflects biologically-relevant processes remains poorly understood.

Based on *in vivo* associations[174,201,205,208,209], we had predicted that estradiol would be associated with increased number of structures, (Fig 1, B,C). We instead found that estradiol and, to a lesser extent, progesterone exerted suppressive effects on structure number (relative to vehicle-controls, Fig. 3A,C; Fig 5A,C). Interestingly, these effects were more pronounced in tube-like structures, though the pattern is apparent for both gland- and tube-like structures. The functional implications of differences between tube- and gland-like structure sensitivity to sex steroids is not clear because all previous research has focused on gland-like structures only. An *in vivo* structure equivalent to these tube-like structures has not been determined based on histological comparisons of cell organization. However, we can note that their diameter appears largely fixed across sex steroid treatments, suggesting that it's organization and growth is distinct from the gland-like structures, and thus supporting it's separate structure class. Without more detailed information on form (cell polarity and structure within the tube), it is difficult to guess at their functional and biological relevance.

We had predicted that progesterone would increase structure size, but we found limited support for this hypothesis as well. Instead, both estradiol and progesterone exposure largely resulted in smaller gland-like structures relative to the vehicle control (Fig 3B). Interestingly, we find exceptions to these suppressive effects of sex steroids in the pregnancy-like treatment containing both sex steroids, where the combination of progesterone and estradiol appears to at least partially "rescue" tube- and gland-like structure density as well as gland-like structure size. That the absence of sex steroids *in vitro* results in the largest and most abundant 3D structures challenges the idea that sex steroids actively promote the increase in structure number and size to support early pregnancy. Instead, our results suggest that progesterone at high concentration is permissive to endometrial reorganization, likely by counteracting effects of estradiol. In this scenario, the differences in gland area in Fig 3B reflect absence of action of progesterone at high

concentration, rather than an active promotion of gland size. Thus, hormone treatments that modify the estradiol/progesterone ratio are likely to modify the preparatory organization of the uterine endometrium prior to pregnancy. By recapitulating some – but not all – predicated changes to EEC organization under physiologically-relevant sex steroid concentrations, our study suggests that *in vitro* models are an appropriate way to further explore the underlying processes as part of future studies.

4.5.2 GLUT3 regulation by progesterone is largely conserved in feline EECs

Overall, we found limited support for our hypothesis that sex steroids in isolation would exert similar effects on gene expression when administered in combination. In particular, despite evidence that progesterone suppressed GLUT3 expression in the endometrium of humans[182], mice[184], rats[183], and cats[201], we found no independent effect of either sex steroid on GLUT3 expression (Fig. 3A); instead, suppressive effects of progesterone on GLUT3 expression were only apparent when both sex steroids were combined, but progesterone was high (Fig. 5A). Taken together with the results on EEC organization described above, the ratio of estradiol to progesterone (within a biologically-relevant range) maybe a more informative metric for integrating *in vitro* and *in vivo* findings and generating mechanistic hypotheses to describe endometrial preparation.

4.5.3 Classic markers of decidualization in feline EECs do not respond to sex steroids

Surprisingly, we did not find any evidence for the up-regulation of classic markers of decidualization, VEGF and PGR, under any conditions. In fact, the combined steroid treatment we expected to be most likely to promote VEGF and PGR transcription (pregnancy-like, Pr) resulted in a significant suppression of VEGF expression, and a non-significant decrease in PGR expression (Fig. 6B,C). These genes are reliable markers for decidualization in canines and felines[203,205,210], and combined treatment with estradiol and progesterone is routinely effective for sustaining high expression of VEGF in human EECs *in vitro* [190,191]. Several possibilities could explain this striking difference. Most simply, failure to detect a decidualization response through these gene markers could reflect a relatively short window during which these genes are up-regulated under chronic treatment, and our sampling time point was simply past this critical window. In this case, quantifying gene expression after shorter-term treatment and/or protein abundance later in the culture period would be needed to determine whether feline EECs displayed a classic decidualization response *in vitro*. However, because these changes to gene expression reflect differentiation processes at the level of the cell and remain apparent over a week after chronic treatment in human EECs, we believe this is not the most likely explanation for our results.

Alternatively, these differences could reflect fundamentally unique aspects of felid biology. One such possibility is that decidualization-dependent changes to gene expression might be particularly dependent on prior differentiation processes that only occur under other steroid environments (i.e., Es-like conditions). In this case, measuring decidualization-dependent change to gene expression would require that treatment administration recapitulate natural transitions in the endocrine environment. Taking such a dynamic approach to the culture system would be an exciting way to test this hypothesis, and it would allow us to understand how dynamic changes occur more broadly in the uterine endometrium across the reproductive cycle.

The failure to detect a decidualization response by way of established markers could also suggest that the decidualization process and/or the signals that stimulate decidualization are unique within felids. Still, decidualization markers including VEGF are locally up-regulated in feline uteri *in vivo*[205], so up-regulating the expression of VEGF is probably a conserved part of the feline decidualization response. In contrast, primary canine EECs also failed to significantly up-regulate PGR as part of decidualization[203], though immortalized canine EECs were able to express a relatively subtle change in PGR expression during decidualization[204]. Defining a useful suite of decidualization markers for felines may therefore require a broader approach (e.g., microarray or -omics) to identify prominent markers.

4.5.4 Feline EEC organization and gene expression seem to reflect distinct integration pathways

When considered together, our gene expression results most clearly emphasize the importance of interactions between progesterone and estradiol signaling – gene expression under isolated steroids did not predict differential gene expression when steroids were combined for any of the gene transcripts we examined. These results stand in somewhat sharp contrast to our results regarding EEC organization, where the effects of estradiol and progesterone when isolated were informative for understanding the results from combined treatments. Understanding whether these differences are reflections of truly distinct organizational and functional regulatory processes or a product of observing variation at different levels of biological organization remains to be tested. The molecular mechanisms that modify EEC organization (e.g., production and localization of cell adhesion proteins) in felines remain largely unknown but identifying these markers and examining their response to sex steroids will be particular informative for integrating the organizational and functional results presented here.

Our experiments suggest that 3D structures formed by feline EECs *in vitro* and some gene markers are indeed responsive to sex steroids in predictable ways, but they also highlight remaining unknowns. Based on our reported interactive effects of progesterone and estradiol, varying the ratio of progesterone to estradiol as well as the concentration of each across a range will likely lead to important

insight into the relative role of each. The simpler approach of isolated sex steroid treatments appears to yield limited insight into felid EEC function. Mixed sex steroid manipulations will be especially important for understanding the mechanisms that might underlie reproductive failure. We can use known ratio and values of sex steroids along with the known reproductive outcomes from *in vivo* reproductive attempts to understand more specifically which organizational and functional changes to EECs might be most important for success.

Identifying additional endocrine or paracrine signals apart from the sex steroids examined here will also likely be necessary to make meaningful progress. A complex suite of factors may be necessary to coordinate the organizational and functional aspects of the decidualization response, perhaps through modulating sensitivity to sex steroids and thereby altering effects reported here. Because the set of signals that leads to decidualization in felines is poorly defined, exploring the contribution of other hormones to the organization and function of feline EECs will be an important line of future research.

4.5.5 Conclusions

By defining the role for sex steroids in preparing EECs for pregnancy, we have taken several steps toward not only understanding EEC regulation, but also advancing the broader application of these techniques to complex culture systems. For example, by combining these approaches with established *in vitro* fertilization technologies (e.g., [211,212]), we will be able to more directly interrogate implantation failure by combining known-quality blastocysts with EECs that are differentiated under various endocrine environments. Ultimately, these lines of research will allow us to exert targeted control over specific mechanisms and thus improve *in vivo* reproductive success rates. By approaching these goals from a comparative perspective, interested particularly in the conservation of function, we increase the likelihood that mechanisms emerging from this work will be both efficient and adaptable, ultimately leading to improvements to both animal and human health.

4.6 Acknowledgments

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4.7 Tables

Table 1 Treatments used in this study and sample sizes (individuals) for morphological and gene expression outcome measures

Treatment	Treatment ID	[Estradiol]	[Progesterone]	N, morphology	N, gene expression
Vehicle-only	VEH	-	-	19	N/A
E_2	E_2L	4 pg/ml	-	8	9
E_2	E_2H	40 pg/ml	-	18	16
P_4	P_4L	-	4.0 ng/ml	8	9
P_4	P_4H	-	40.0 ng/ml	17	16
Interestrous-like	IE	10 pg/ml	0.4 ng/ml	11	7
Estrous-like	Es	50 pg/ml	4.0 ng/ml	11	7
Pregnancy-like	Pr	10 pg/ml	30.0 ng/ml	11	7

Table 2 Media components

Component	Catalogue info	Transport media	Basal media	Matrigel media
Base media		MEM with Hank's Salts [11575-032, Gibco]	DMEM/F12 (phenol-red free) [21041-025, Gibco]	DMEM/F12 (phenol-red free) [21041-025, Gibco]
HEPES buffer	15630-080, Gibco	-	0.1%	0.1%
Penicillin	P7794, Sigma-Aldrich	100 IU/ml	100 IU/ml	50 IU/ml
Streptomycin	S1277, Sigma-Aldrich	100 µg/ml	100 µg/ml	50 µg/ml
Ascorbic acid	A61, Fisher Chemical	0.25 mM	-	-
Amphotericin B	15290-018, Thermofisher	-	0.1%	0.1%
ITS+	41400045, Thermofisher	-	1%	1%
Matrigel (phenol-red free)	356237, Corning	-	-	3%
EGF Recombinant human protein	PHG0315, Themofisher	-	-	10 ng/ml

Table 3 Primer sequences used for quantitative PCR analyses

Gene	NCBI Accession Number	Forward	Reverse	T_A(° C)	[µ M]	Product Length	Efficiency (%)
GAPDH	NM_001009307.1	AGTATGATTCCACCCACGGCA	GATCTCGCTCCTGGAAGATGGT	59	0.5	102	96.7
RPL2	NM_001128842.1	CACGAGGCCAGAGGTGATCT	CTCCACCATTGTAGCGTCGG	62	0.2	225	97.2
YWHAZ	XM_006943327.4	GAAGAGTCCTACAAAGACAGCACGC	AATTTTCCCCTCCTTCTCCTGC	62	0.5	115	100
PGR	XM_019811582.2	GTGGCAGATTCCACAGGAGT	TTTGCCTCAGACCAATTGC	58.4	0.5	179	98.5
GLUT3	XM_019834462.2	TTGGCTACAACACGGGAGTC	CCAACGGAGAAGGAGCCAAT	60	0.3	175	98.5
IR	DQ835565.1	CTGCCCTGCCACTGTCA TC	GCAGACGGTCGGACAAACT	60	0.5	85	100.2
VEGF	NM_001009854.1	TTTCTGCTCTCTTGGGTGCATTGG	TGCGCTGGTAGACATCCATGAACT	60	0.6	139	95.6

4.8 Figures

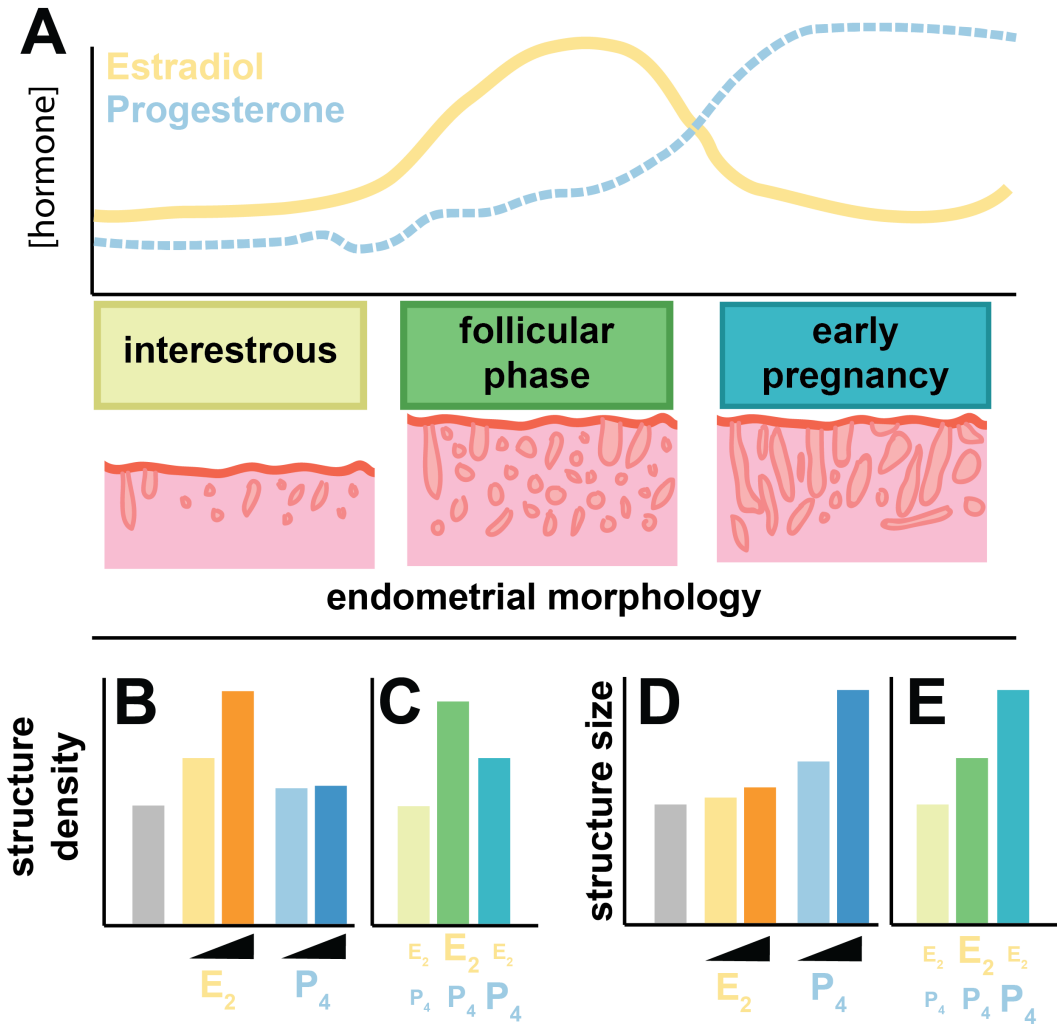

Figure 1 (A) Cyclic changes to the concentration of sex steroids in circulation and the organization of the domestic cat endometrium occur across the reproductive cycle *in vivo*. Hormone figure adapted from [206]; Cartoon representation of endometrium based on [174] (B-E) Predictions for endometrial epithelial cells organizational changes under different hormone treatments *in vitro* experiments were based on *in vivo* associations laid out in panel A.

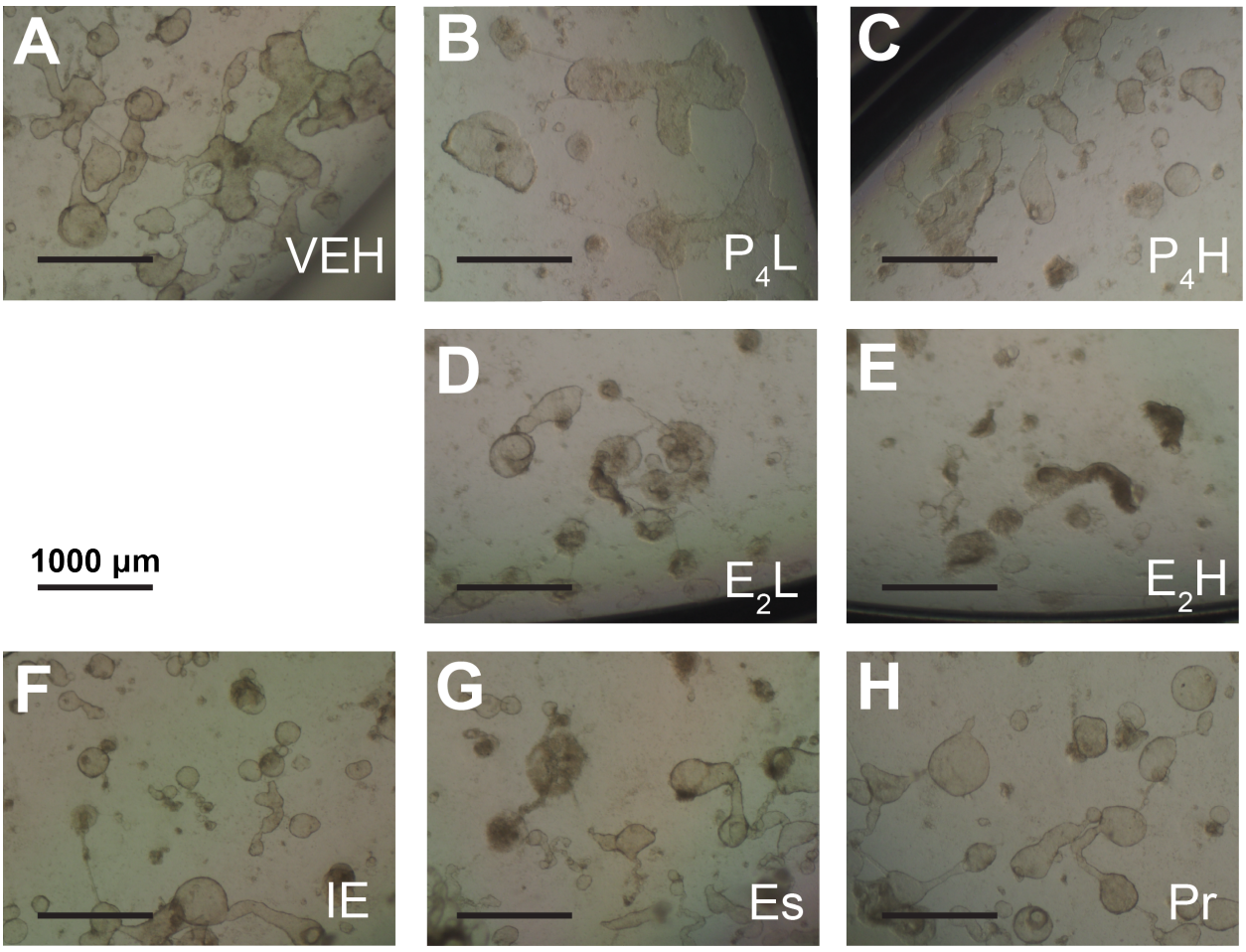

Figure 2 Representative brightfield images of endometrial epithelial cell (EEC) culture growth and 3-dimensional structures. Treatment IDs are indicated in the lower right of each image. Primary EECs organize into gland-like structures (arrow heads) and tube-like structures (arrows) when grown in Matrigel media. Images A-E show EEC organization under treatments from experiment 1, testing for effects of isolated steroids (progesterone, P_4, or estradiol, E_2) at a high (H) or low (L) physiologically-relevant concentration, where high and low are separated by an order of magnitude. Isolated sex steroid treatments were compared to a vehicle (VEH) control, containing no steroid hormones. Images F-H show organization under treatments from experiment 2, examining effects of combined steroid hormones at physiologically-relevant concentrations, determined by serum values considered normal for various stages of the reproductive cycle (IE, interestrous; Es, estrous; Pr, pregnancy). Bars on each image are equal to 1000 µm.

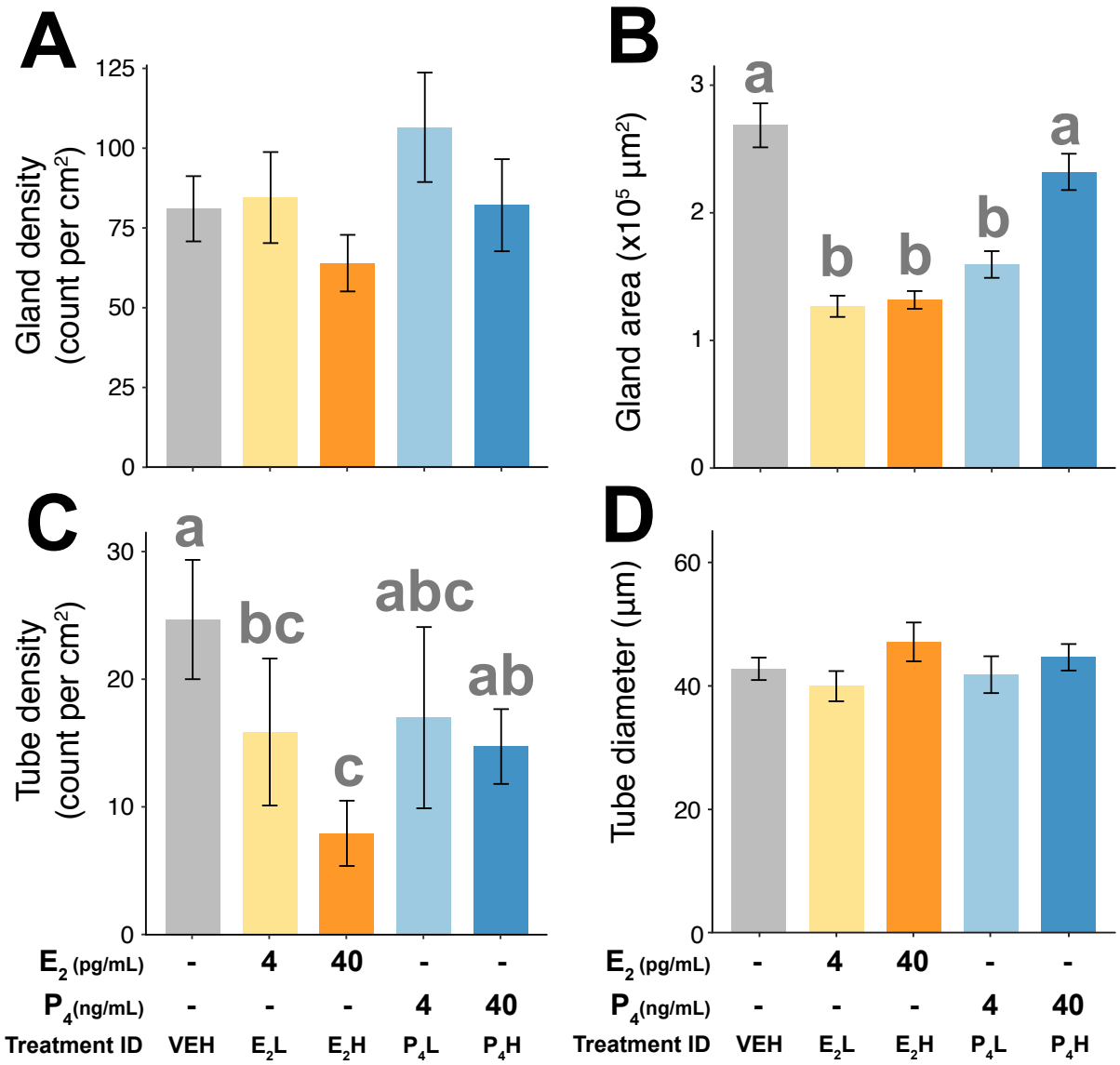

Figure 3 Modification of size and density of 3-dimensional structures treated with either progesterone or estradiol *in vitro*. Panels show gland-like structure density (A), gland-like structure area (B), tube-like structure density (C), and tube-like structure diameter (D). The concentrations of steroid hormones in each treatment are specified on the x-axis, and treatment IDs refer to these treatments containing isolated steroids (progesterone, P_4, or estradiol, E_2) at a high (H) or low (L) physiologically-relevant concentration, where high and low are separated by an order of magnitude. Vehicle control (VEH) contained no steroid hormones. Letters indicate significant differences among groups at $P < 0.05$.

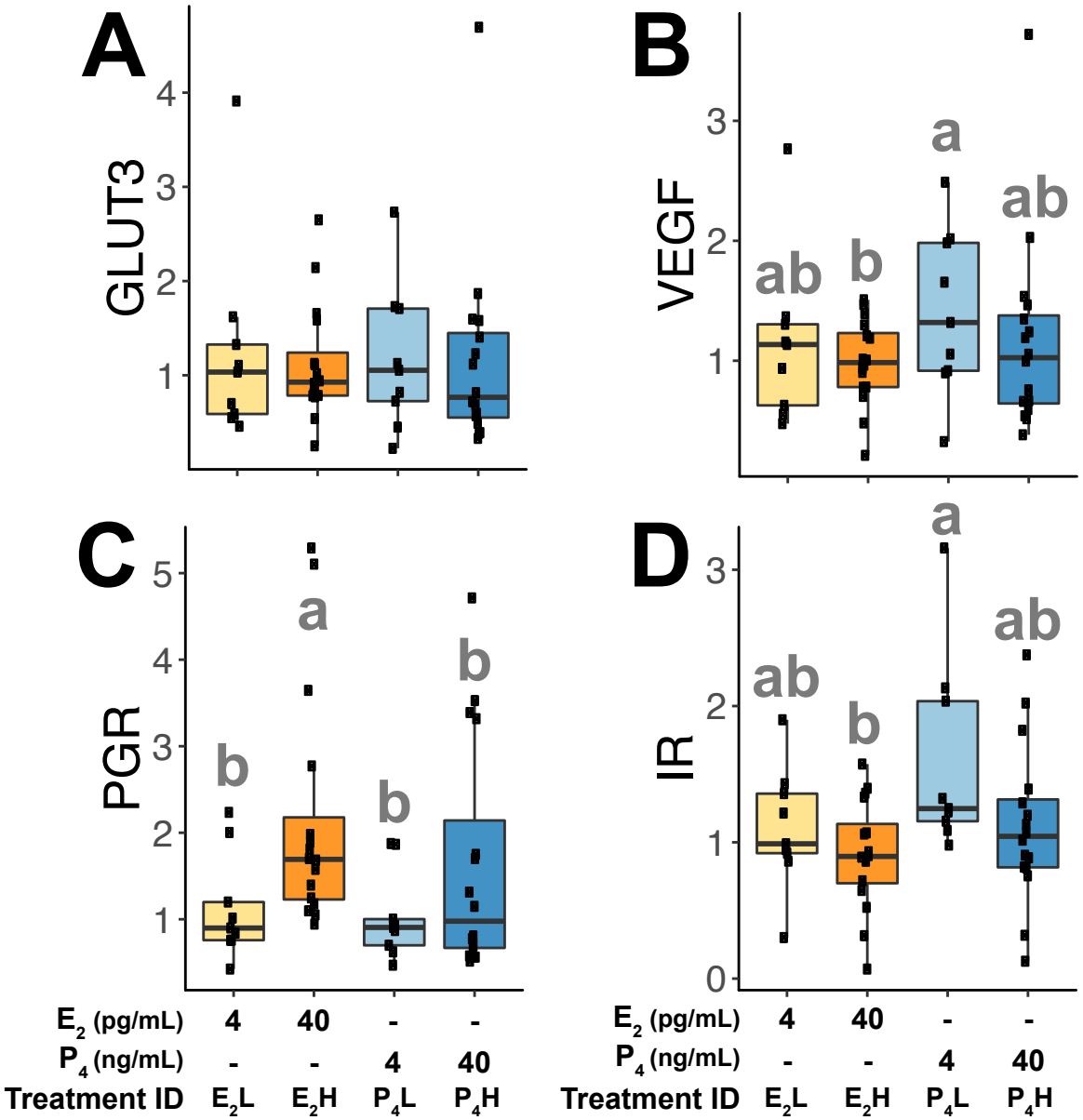

Figure 4 Differential expression of candidate genes in endometrial epithelial cells (EECs) treated with either progesterone or estradiol *in vitro*. We quantified expression of the candidate genes glucose transporter 3 (GLUT3; A), vascular endothelial growth factor (VEGF; B), progesterone receptor (PGR; C), and insulin receptor (IR, D). Gene expression shown as fold-change over the average expression in wells treated with 4 pg/ml E_2 (E_2L). The concentrations of steroid hormones in each treatment are specified on the x-axis, and treatment IDs refer to these treatments containing isolated steroids (progesterone, P_4, or estradiol, E_2) at a high (H) or low (L) physiologically-relevant concentration, where high and low are separated by an order of magnitude. Letters indicate significant differences among groups at $P < 0.05$.

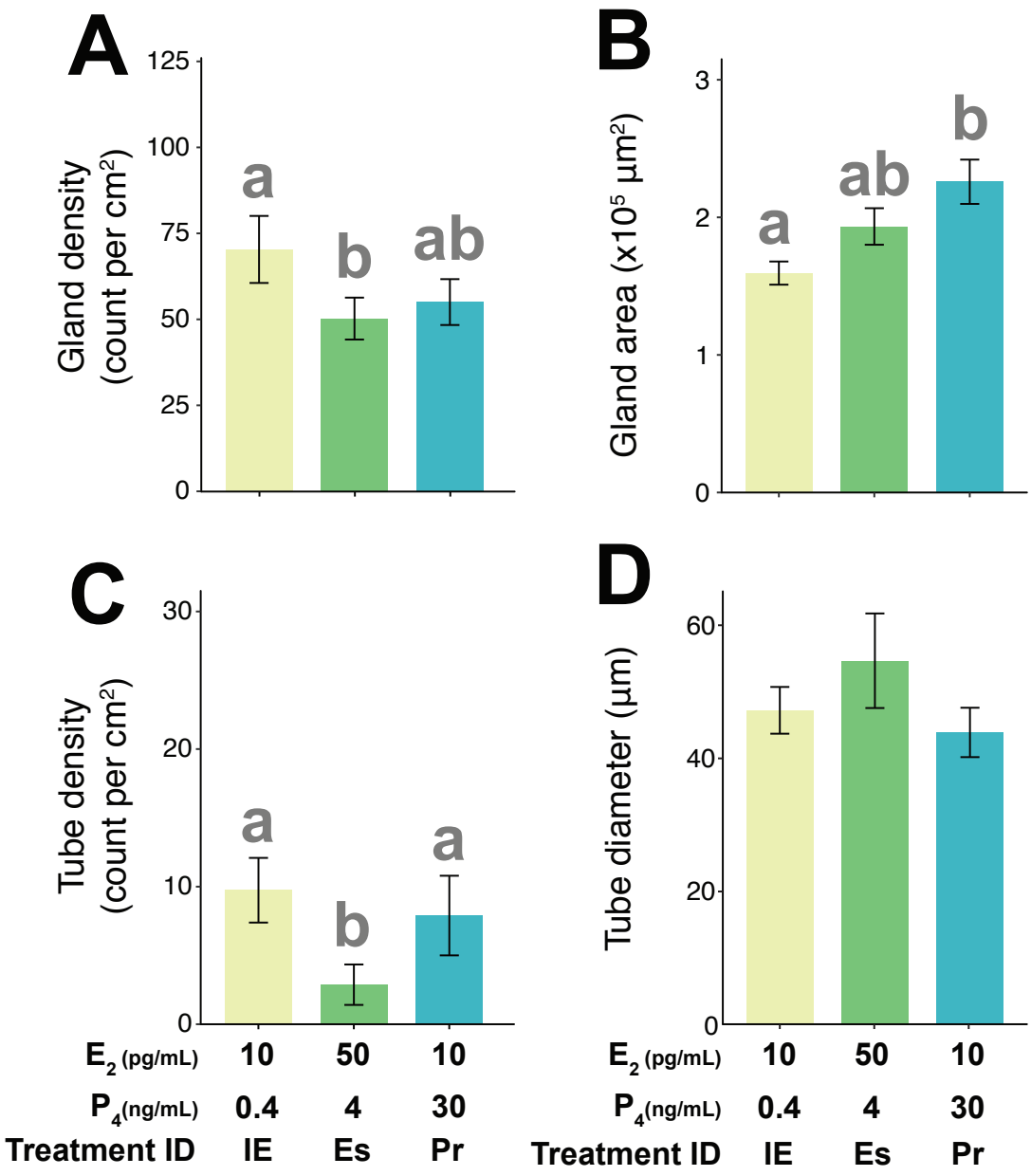

Figure 5 Modification of size and density of 3-dimensional structures endometrial epithelial cells (EECs) treated with estradiol and progesterone in combination at physiologically-relevant concentrations *in vitro*. Panels show gland-like structure density (A), gland-like structure area (B), tube-like structure density (C), and tube-like structure diameter (D). The concentrations of steroid hormones in each treatment are specified on the x-axis, and treatment IDs refer to treatments containing combined steroid hormones at physiologically-relevant concentrations, determined by serum values considered normal for various stages of the reproductive cycle (IE, interestrous; Es, estrous; Pr, pregnancy). Letters indicate significant differences among groups at $P < 0.05$.

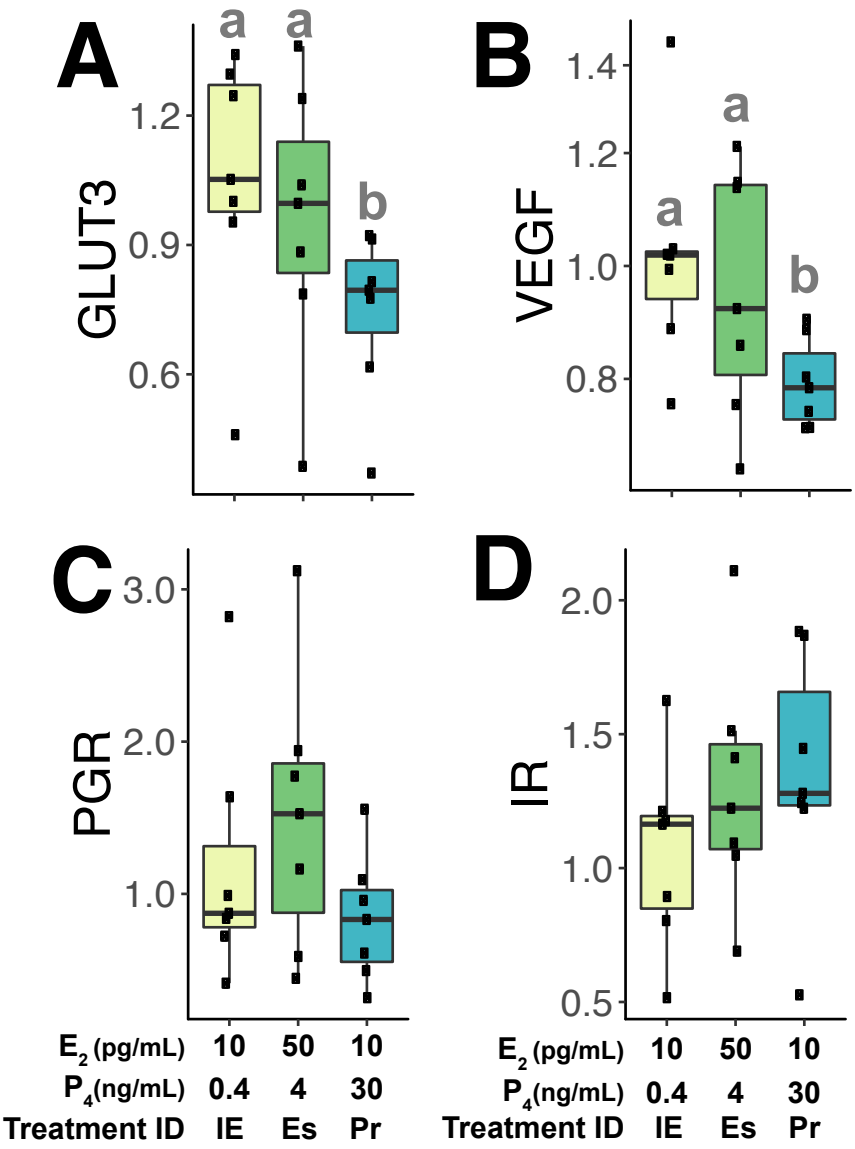

Figure 6 Differential expression of candidate genes in endometrial epithelial cells (EECs) treated with estradiol and progesterone in combination at physiologically-relevant concentrations *in vitro*. We quantified expression of the candidate genes glucose transporter 3 (GLUT3; A), vascular endothelial growth factor (VEGF; B), progesterone receptor (PGR; C), and insulin receptor (IR, D). Gene expression shown as fold-change over the average expression of EECs in the interestrous-like (IE) treatment. The concentrations of steroid hormones in each treatment specified on the x-axis, and treatment IDs refer to treatments containing combined steroid hormones at physiologically-relevant concentrations, determined by serum values considered normal for various stages of the reproductive cycle (IE, interestrous; Es, estrous; Pr, pregnancy). Letters indicate significant differences among groups at $P < 0.05$.

5. Pregnancy stage determines the effect of chronic stress on ovarian progesterone synthesis

5.1 Abstract

Stress-induced glucocorticoid release is thought to be a primary driver by which maternal stress negatively impacts pregnancy outcomes, but the neuroendocrine targets mediating these adverse outcomes are less well understood. We hypothesized that stress-induced glucocorticoid secretion influences pituitary hormone secretion, resulting in altered ovarian progesterone synthesis. Using a chronic restraint model of stress in mice, we quantified pituitary hormones, steroid hormone production, and expression of ovarian genes that support progesterone production at both early- (day 5) and mid-pregnancy (day 10). Females subjected to daily restraint had elevated baseline corticosterone during both early- and mid-pregnancy. However, lower circulating progesterone was observed only during early pregnancy. Lower progesterone production was associated with lower expression of steroidogenic enzymes in the ovary of restrained females during early pregnancy. There were no stress-related changes to luteinizing hormone (LH) or prolactin (PRL). By mid-pregnancy, circulating LH decreased regardless of treatment, and this was associated with down-regulation of ovarian steroidogenic gene expression. Our results are consistent with a role for LH in maintaining steroidogenic enzyme expression in the ovary, but neither circulating PRL nor LH were associated with the stress-induced inhibition of ovarian progesterone production during early pregnancy. We conclude that chronic stress impacts endocrine networks differently not only in pregnant and non-pregnant mammals, but also in different stages of pregnancy.

5.2 Introduction

Maternal stress increases the likelihood of adverse pregnancy outcomes in many mammals [213], including humans [214,215]. Adverse outcomes include total failure (miscarriage, or resorption) as well as a range of sub-lethal effects, including lower birth weight of offspring, slower growth rates, and altered social and anxiety behaviors [216–218]. One mechanism by which stress can produce these adverse outcomes is by increasing activity of the hypothalamic-pituitary-adrenal (HPA) axis: when animals experience stress, the HPA axis increases glucocorticoid release from the adrenal gland, and this release of glucocorticoids (above homeostatic levels) impacts pregnancy progression and fetal development [26,31]. Some of these effects result from the inhibition of the primary pregnancy maintenance hormone, progesterone. If progesterone is too low during early pregnancy, embryo implantation and/or the pregnancy will fail [219–221], and more broadly, low progesterone throughout pregnancy can adversely affect placental growth and development [222].

Circulating progesterone during early pregnancy in humans and other mammals is inversely correlated with circulating glucocorticoids [223–225], and

stress exposure during pregnancy is associated with lower circulating progesterone concentrations [225–227]. Despite clear evidence of these associations, the pathway by which glucocorticoids inhibit progesterone production during pregnancy is unknown [26,225].

In non-pregnant female mammals, glucocorticoids regulate ovarian function primarily through action on the hypothalamus and pituitary. For example, in non-pregnant females, glucocorticoids alter hypothalamic and pituitary hormone release (including luteinizing hormone [LH] and prolactin [PRL]), and these changes can result in lower sex steroid production (estrogens and progestogens) from the ovary [26]. The association between glucocorticoids and progesterone release during pregnancy could potentially reflect action through these same circuits. However, as described below, pregnancy requires substantial changes to regulatory networks and activity of endocrine axes (the reproductive axis being only one of many), and it is therefore possible that the association between glucocorticoid and progesterone production during pregnancy results from novel interactions among endocrine organs or from interactions that are less important in non-pregnant females. Furthermore, pregnancy is dynamic and the effects of chronic stress on endocrine outcome measures (including glucocorticoid and progesterone production) are likely to change across pregnancy progression.

In rodents, the amount of progesterone produced during pregnancy depends on steroidogenic activity in the corpora lutea (CL) in the ovary. Increased steroidogenic activity by the CL is a function of activity across two pathways [228,229]. First, inhibition of the enzyme *20aHSD*, which usually metabolizes progesterone; and second, increased expression of steroidogenic enzymes, especially P450 cholesterol side-chain cleavage enzyme (*P450SCC*). The pituitary hormones prolactin (PRL) and luteinizing hormone (LH) control these pathways, respectively [228–231]. Failure or decreased function of any of these signaling pathways within the ovary during early pregnancy can increase the likelihood of adverse pregnancy outcomes [230,232,233]. Though the placenta begins to contribute progesterone to circulation by mid-pregnancy [229,234], the ovary is thought to be required for pregnancy maintenance throughout gestation in mice [235].

We hypothesized that chronic stress affects ovarian steroidogenesis across the first half of pregnancy (early- to mid-pregnancy) by modulating the pituitary hormones (LH, PRL) that mediate these responses in non-pregnant animals. To test this possibility, we used chronic restraint to model chronic stress in mice, and we measured pituitary and ovarian hormone production, and gene expression in candidate ovarian steroidogenic pathways during early- and mid-pregnancy. We predicted that restrained females would have elevated circulating concentrations of glucocorticoids, specifically corticosterone, which would be associated with lower circulating progesterone. Furthermore, we predicted that the pituitary hormone signaling (circulating concentrations of PRL or LH, and receptor expression in the ovary) would be concomitantly lower in restrained females.

5.3 Methods

5.3.1 Animals

C57BL/6J mice were purchased from the Jackson Laboratory (Sacramento, CA) and housed in ventilated cages on a 14:10 light/dark cycle (lights on at 06:00, lights off at 20:00) with *ad libitum*. access to food and water. Experimental animals were pair-housed with the male throughout the experiment. All animals were allowed to acclimate for at least 1 week. Females used in these experiments were 8-10 weeks old. All protocols were approved by the UC Berkeley Office of Laboratory Animal Care and were consistent with NIH guidelines for the care and use of laboratory animals.

5.3.2 Experimental procedures

Successful mating was determined either through observation of at least two intromissions during timed mating trials or by the identification of a vaginal plug the morning following pairing. The morning after mating or on which a vaginal plug was found was considered Day 1 of pregnancy. Females were then pseudo-randomly assigned to restraint stress or control (unrestrained) groups such that assignment between groups was balanced across the length of the experiment (see Table 1 for total sample sizes). All females were weighed each morning prior to treatment. Animals assigned to the chronic restraint stress group were moved each morning, beginning on Day 1, to a separate room where they were restrained in a modified 50 mL plastic tube. Animals were also exposed to predator odor during restraint: each day, 15 uL of predator odor (undiluted fox urine, Minnesota Trapline, Inc; Pannock, MN) was freshly soaked into a new cotton ball and placed in the cage with each mouse during restraint. Daily restraint lasted four hours from 08:00 to 12:00 (relative to lights-on). Restraint was repeated daily until tissue collection. Unrestrained females remained in their home cages.

Females were euthanized on either day 5 (early-) or day 10 (mid-) pregnancy. All animals were euthanized via intraperitoneal injection of sodium pentobarbital (200 mg/kg) followed by rapid decapitation or perfusion. In animals euthanized via decapitation, trunk blood was collected into 1.5 mL Eppendorf tubes and the ovaries were rapidly dissected from the body, cleaned of fat, and flash frozen in isopentane on dry-ice. The number of developing fetuses for each side of the uterus was counted in females collected at mid-pregnancy, and fetal developmental abnormalities or resorption sites were recorded by an observer unaware of the individual's treatment. In animals euthanized via perfusion, blood was collected via the retro-orbital sinus immediately prior to perfusion; the uterus and ovaries were clamped and removed prior to perfusion, and these tissues were immediately dissected and frozen as previously described. Tissues were stored at -80°C until extraction and analysis. Blood was centrifuged at 1300 rcf for 10 min. and plasma removed. Plasma was centrifuged a second time for 1 min. and then aliquoted and stored at -80°C. Blood samples were collected an average of 2:41 ± 0:30 minutes from lifting the cage (Average ± St.Dev.), with a median time to collection of 2:37 (N = 46).

Samples collected more than 4 minutes after lifting the cage were excluded from analyses.

5.3.3 Hormone analyses

Progesterone was quantified using Cayman Chemical Progesterone ELISA (Item No. 582601, Ann Arbor, MI). Intra-and inter-assay variations for progesterone were 3.9% and 5.1%, respectively. Baseline corticosterone was quantified using Enzo corticosterone ELISA kit (ADI-900-097; Enzo Life Sciences, Inc., Farmingdale, NY) using the manufacturer's protocol for small sample volumes. Intra-and inter-assay variations were 4.8% and 7.9%, respectively. LH levels were quantified using an LH ELISA, modified from [154]. The protocol was kindly provided by Jens D Mikkelsen (Copenhagen University Hospital, Denmark). Briefly, 96-well microtiter plates were coated with 50µl of bovine LHß 518B7 monoclonal antibody (kindly provided by Lillian E Sibley, UC Davis) and incubated overnight at 4 C. Excess antibody was removed, and the plates were washed with 200µl/well of 10mM PBS with 0.1% Tween 20. The plates were blocked using 5% skim milk powder in PBS-T and incubated for 1 h at room temperature. Following washes, 50µl of sample or standards of mouse LH (mouse RIA kit, National Hormone and Pituitary program, University of California, Harbor Medical Center, Los Angeles, CA), diluted in assay buffer, were added per well in duplicates and incubated for 2 h at room temperature. The plates were washed and 50µl of Rabbit polyclonal LH antibody (AFP240580Rb, National Hormone and Pituitary program, University of California, Harbor Medical Center, Los Angeles, CA) were added into each well, then incubated at room temperature for 90 min. After washing, 50µL Polyclonal Goat Anti-Rabbit IgG conjugated to horseradish peroxidase (DAKO Cytomation, catalog # P0448) was added at 1:2000 dilution and incubated for 1 h at room temperature. After washing, 100µl of o-Phenylenediamine (OPD (Invitrogen, catalog # 00-2003)) in citrate buffer were added to all the wells. The color reaction was allowed to develop for 30 min in the dark. The enzyme was stopped by adding 50µl of 3M HCl per well and the OD of each well was immediately read at 490 nm with a reference of 655 nm.

Samples which did not reach the limit for detection for the LH assay were assigned the lowest measurable value (0.078 ng/mL; N = 7, all females from mid-pregnancy). Intra- and inter-assay variations were 5.9% and 3.59%, respectively.

Three samples (all in the mid-pregnancy group) gave values that were nearly 10 times greater than the average of all other samples (1.19, 1.71, and 2.12 ng/mL compared to the average of 0.18 ng/mL [range: 0.078-0.53 ng/mL]). Such values are comparable to LH values measured in ovariectomized mice that were run in the same assay as internal controls. However, we could not determine any reason to suspect that the values measured were inaccurate. Accordingly, we include these data points in the figure, and present analyses with and without these samples included.

Prolactin was assayed using the mouse prolactin ELISA kit from Abcam (ab100736, Cambridge, MA). Intra-assay variation for prolactin was 2.8%.

Some samples did not have sufficient plasma to quantify all hormones, thus sample sizes vary for different hormone measures.

5.3.4 Gene expression analysis

Total RNA was extracted from whole ovaries (ISOLATE II RNA Mini-kit, BIO-52073, Bioline USA Inc., Taunton, MA). The RNA quality of a random subset of samples (N = 10) were analyzed on an Agilent Technologies Bioanalyzer and yielded an average RNA integrity number (RIN) of 9.5 (Range: 8.8 to 10). We reverse transcribed 1.0 ug of RNA (iScript Advanced cDNA synthesis Kit for RT-qPCR, Bio-Rad Laboratories Inc., Hercules, CA). cDNA was diluted 1:25 in nuclease-free water immediately prior to performing quantitative PCR. Quantitative PCR was performed using duplicate 10 uL reactions with a 2-step amplification for 40 cycles followed by a melt curve. All primers used were validated prior to analyses by confirming single-peak melt curves, correct product length, and acceptable efficiency (all primer pairs between 85 and 101% efficiency). Primer sequences and annealing temp. are provided in Table 2. Any wells with aberrant melt-curves were excluded from expression analysis. C_T values were corrected for efficiency; relative expression was calculated using methods by Pfaffl and colleagues [73]. All data are expressed as fold-change over mid-pregnancy, restrained individuals.

5.3.5 Statistical analyses

All analyses were run in RStudio 0.98.1091 with the nlme and multcomp packages.

We evaluated the change in mass across pregnancy in the restrained and unrestrained groups by calculating percent change in mass per day (of initial body mass) from days 1-6 and days 6-9. We identified day 6 as the point at which chronically stressed females began to gain mass by visually inspecting mass across pregnancy (Fig. 1). Slope was statistically evaluated using a one-way ANOVA with post-hoc comparisons among means using a Holms-Sidak correction for multiple comparisons.

Progesterone, corticosterone, and LH were log-transformed for analyses to meet assumptions of residual normality. Blood collection method (retro-orbital vs. trunk blood) significantly affected progesterone measurements, and because samples collected from animals at day 5 were all collected via retro-orbital bleeds, we only included progesterone measurements from day 10 animals that were collected via the retro-orbital sinus in the analysis. We ran a one-way ANOVA with planned contrasts to test for differences among groups based on our *a priori* predictions. We tested for differences between restrained and unrestrained individuals during early- and mid-pregnancy (planned contrasts 1 & 2), and we tested for differences between early and mid pregnancy (planned contrast 3). The correlations between baseline

corticosterone and progesterone were assessed using Pearson's product-moment correlation. A difference in circulating prolactin between restrained and unrestrained animals during early pregnancy was analyzed using Welch's two sample t-test.

Gene expression analyses were carried out using a repeated-measures linear regression model including a random effect of individual to account for use of both ovaries. All genes were log-transformed to fulfill assumptions of normality of residuals. Again, we used planned contrasts to test for *a priori* differences. We used Pearson's product-moment correlation to examine correlations between expressed genes. All tests were considered statistically significant at $P < 0.05$. Because we used planned comparisons, we did not correct p-values for multiple comparisons. Figures show untransformed data and use mean ± S.E., except where noted.

5.4 Results

5.4.1 Maternal mass and litter size

Females exposed to chronic restraint stress lost body mass during early pregnancy, in contrast to unrestrained females, which gained mass (Fig 1A). Once chronically stressed females began gaining mass (after day 6), they gained mass at the same rate as unrestrained animals (One-way ANOVA: $F_{3,42} = 44.07$, $P < 5e^{-13}$; Fig. 1B). Regardless of treatment, pregnant mice gained mass between successful mating and mid-pregnancy (CON: 2.80±0.13 g; STR: 0.63±0.12 g), however unrestrained females gained more mass (Welch's T-test, $t_{17.5} = 12.03$, $P < 7e^{-10}$; Fig 2A).

There were no overall differences in total number of fetuses per female at mid-pregnancy (data not shown, $P > 0.3$). However, evidence of early resorption and underdeveloped fetuses was apparent in 2 of 15 females (13%) exposed to daily restraint stress, whereas 0 of 12 (0%) unrestrained females showed signs of resorption or underdeveloped fetuses.

5.4.2 Steroid hormones

Baseline corticosterone (CORT) increased as pregnancy progressed (Fig. 2A; AOV: $F_{3,28} = 10.65$, $P < 8e^{-5}$; Pregnancy: $t = 2.573$, $P < 0.015$). Chronic restraint stress elevated baseline CORT during both early and mid-pregnancy (Fig. 2A; AOV: $F_{3,28} = 10.65$, $P < 8e^{-5}$; planned contrasts: Early: $t = 2.554$, $P < 0.016$; Mid: $t = 4.11$, $P < 0.0003$). Chronic stress also resulted in lower circulating progesterone, but only during early pregnancy (Fig. 2B; AOV: $F_{3,26} = 16.26$, $P < 4e^{-6}$; planned contrasts: Early: $t = -5.776$, $P < 5e^{-6}$; Mid: $t = 0.492$, $P < 0.63$). Baseline CORT was correlated with circulating progesterone during early pregnancy (Fig. 2C; Pearson-R = -0.78, $t_7 = -3.32$, $P < 0.013$), but not during mid-pregnancy (Pearson-R = 0.16, $t_9 = 0.48$, $P > 0.60$).

5.4.3 Pituitary hormones

Circulating prolactin did not differ between unrestrained and chronically restrained females during early pregnancy (Fig. 3A; Welch's t-test $t_{7.984} = -1.27$, $P = 0.24$). When all LH measures are included the analyses, circulating LH did not vary across pregnancy or with treatment ($P > 0.15$ for all). However, these three points in the mid-pregnancy group (see Fig. 3B) are all at least two times greater than any other measured value and, when included, they are responsible for a 5-fold increase in standard deviation within the mid-pregnancy group. When these points are excluded, circulating LH was lower during mid-pregnancy relative to early pregnancy (AOV: $F_{3,26} = 4.711$, $P < 0.009$; planned contrasts: Pregnancy: $t = -3.72$, $P < 0.0009$), though it still did not differ between unrestrained and chronically restrained females (Fig. 3B; planned contrasts: Early: $t = 0.091$, $P < 0.93$; Mid: $t = 0.773$, $P < 0.45$).

5.4.4 Ovarian gene expression

During early pregnancy, the expression of two steroidogenic enzymes (Steroidogenic acute regulatory protein [*StAR*] and p450 cholesterol side-chain cleavage enzyme [*SCC*]) were lower in chronically-stressed animals compared to unrestrained females (*StAR*, Early: $t_{34,32} = -3.46$, $P < 0.0015$; *SCC*, Early: $t_{34,32} = -2.41$, $P < 0.0220$; Fig. 4). Expression of these genes in the ovary during mid-pregnancy was lower relative to early pregnancy, and there was no difference in expression between chronically stressed and unrestrained individuals during mid-pregnancy (*StAR*, Pregnancy: $t_{34,32} = -7.21$, $P < 0.0001$, Mid: $t_{34,32} = -0.043$, $P < 0.96$; *SCC*, Pregnancy: $t_{34,32} = -8.30$, $P < 0.0001$, Mid: $t_{34,32} = -0.414$, $P < 0.68$; Fig. 4).

Expression of the long prolactin receptor isoform (*PRLRL*) was lower in restrained females during early pregnancy (Early: $t = -2.28$, $P < 0.029$; Fig. 5A), but not during mid-pregnancy (Mid: $t = -0.084$, $P < 0.93$), and there was no overall difference in expression between early and mid-pregnancy (Pregnancy: $t = -0.772$, $P < 0.45$). Expression of the receptor for LH (*LH*R) and the enzyme *20αHSD* decreased during mid-pregnancy relative to early pregnancy (*LHR*, Pregnancy: $t = -4.62$, $P < 0.0001$; *20α*, Pregnancy $t = -5.22$, $P < 0.0001$), but there was no difference related to stress treatment (*LHR*: Early: $t = -0.59$, $P < 0.56$; Mid: $t = 0.43$, $P < 0.67$; *20α*: Early: $t = -0.077$, $P < 0.94$; Mid: $t = 0.62$, $P < 0.54$; Fig 5B,C).

We found a strong correlation between the expression of *PRLRL* and expression of the two steroidogenic enzymes (*StAR* and *SCC*; Fig. 6). The relationships between the steroidogenic enzymes and *PRLRL* were consistent between early pregnancy (Day 5; *SCC*: Pearson-R = 0.98, $t_{34} = 29.60$, $P < 2.2e^{-16}$; *StAR*: Pearson-R = 0.97, $t_{34} = 26.82$, $P < 2.2e^{-16}$), and mid-pregnancy (Day 10; *SCC*: Pearson-R = 0.83, $t_{32} = 8.37$, $P < 1.47e^{-9}$; *StAR*: Pearson-R = 0.89, $t_{32} = 11.25$, $P < 1.18e^{-12}$). The relationships between these gene transcripts shift in late pregnancy; the steroidogenic enzymes are down regulated, while there is no longer a difference between restrained and unrestrained females in *PRLRL* (see Fig 4A,B and Fig 5A). However the slope of the

line that explains the correlation between steroidogenic enzymes and PRLR-L appears to be similar (Fig. 8A,B).

5.5 Discussion

5.5.1 General conclusions

We found that ovarian progesterone production is sensitive to restraint stress. Our results suggest that glucocorticoids do not inhibit progesterone release during pregnancy via a top-down (hypothalamic-pituitary) mechanism within the HPG axis, because basal pituitary LH and PRL secretion were unaffected by stress. The down-regulated expression of steroidogenic enzymes in the ovary by mid-pregnancy suggests that the majority of circulating progesterone at this time point may no longer be from the ovary. In contrast to the ovary during early pregnancy, mid-pregnancy progesterone synthesis appears resilient to restraint stress and elevated baseline glucocorticoids. Though there have been concerted efforts to understand the extent to which chronic stress alters reproductive outcomes, our results make it clear that there is substantial work still needed to describe and test the basic interactions between the HPA and reproductive axes across different stages of pregnancy. Understanding the functional network between these and other endocrine axes during pregnancy will help to establish the mechanisms connecting maternal stress to reproductive failure.

5.5.2 Effects of stress-induced corticosterone (CORT) release on progesterone during early pregnancy

The inverse correlation we found between baseline CORT and progesterone production during early pregnancy is consistent with other rodent studies [224,236]. Direct action of CORT on ovarian progesterone synthesis is unlikely to explain the relationship between circulating CORT and progesterone (for more, see [26,30,228,237], and we found no evidence to support the hypothesis that chronic stress alters the basal release of pituitary hormones (LH and PRL) during early pregnancy. Instead, placental factors that regulate corticosterone metabolism (e.g., 11ß-HSD) and/or ovarian progesterone synthesis (e.g., placental lactogens) are promising areas for further study. Careful attention to placental endocrine activity and sensitivity *in vivo* during early pregnancy, especially related to glucocorticoid receptor isoform expression[238], may facilitate the identification of new functional mechanisms by which CORT impacts progesterone synthesis.

In addition, progesterone production and CORT secretion during pregnancy could be connected through other shared upstream regulators. Because restraint stress resulted in initial loss of body mass, suggesting that restrained females entered a negative energy balance, endocrine or metabolic signals associated with changes in energy balance could be responsible for changes to baseline CORT and progesterone production. For example, the adipose hormone leptin promotes ovarian progesterone production [239], is inhibited by chronic stress [240,241], and is inversely related to CORT during negative energy balance in mice [242]. Mapping the interactions between energy balance circuits and reproductive function

specifically in early pregnancy is likely to identify new connections between stress and adverse pregnancy outcomes.

5.5.3 Progesterone production during mid-pregnancy

In mid-pregnancy, *StAR* and *SCC* were dramatically down-regulated relative to early pregnancy, and there were no longer any differences in gene expression between restrained and unrestrained females. The decrease in expression of *StAR* and *SCC* suggests that ovarian steroidogenic activity is lower in mid-pregnancy. While these results counter the classic suggestion that the ovary is required for progesterone production throughout pregnancy in mice [235], they are consistent with the idea that decreasing pituitary LH release by mid-pregnancy causes a decline in ovarian progesterone production [229]. In further support of the latter idea, circulating LH concentrations were lower during mid-pregnancy relative to early-pregnancy in this study. We also found a novel correlation between *PRLRL* and *StAR* and *SCC*. The strong co-regulation between these genes and differential sensitivity to stress across pregnancy underscores the need to better understand the regulatory networks that control ovarian progesterone production.

Even though restrained females continued to exhibit elevated baseline CORT during mid-pregnancy, circulating progesterone no longer differed between restrained and unrestrained females. Moreover, even though progesterone remained elevated, steroidogenic genes in the ovary were considerably down-regulated, suggesting the ovary is much less steroidogenically active by mid-pregnancy. Circulating progesterone during mid-pregnancy may instead reflect placental steroidogenesis. Interestingly, circulating progesterone appears to be insensitive to chronic stress (elevated CORT) during mid-pregnancy. Further work to establish the source of mid-pregnancy progesterone is needed in order to determine how the apparent insensitivity to CORT develops across pregnancy.

5.5.4 Caveats

Though our results present a relatively clear picture of how chronic stress affects reproductive function during the first half of pregnancy, there are some important caveats. First, only ovarian mRNA, not protein, was measured, raising the possibility that protein expression and activity may differ meaningfully. Second, both prolactin and luteinizing hormones are released in a pulsatile fashion such that single-time point measurement may miss dynamic changes in the pulse rate or peak size for either hormone resulting from chronic stress exposure. The three LH samples showing exceptionally high values likely reflect the pulsatile nature of this hormone, whereas the majority (31/33) measures reflect basal levels as expected. Evaluating upstream changes in protein and gene expression within the pituitary and hypothalamus and/or serial blood samples would be required to conclusively determine whether temporal changes in pituitary hormone production and release could explain the relationship between glucocorticoids and progesterone during early pregnancy. However, most studies evaluating the effects of stress on pituitary hormone release (LH in particular) find differences using single time point

measures (e.g. [243,244]), and we were able detect a change in LH across pregnancy.

More broadly, it is worth considering that animals or people that experience chronic stress during pregnancy are likely to experience stress before pregnancy as well. Geraghty et al. [15] showed that chronic stress prior to pregnancy in rats was associated with lower reproductive success, but that these effects could be ameliorated by inhibiting production of a stress-induced neuropeptide, gonadotropin-inhibitory hormone (GnIH), in the hypothalamus leading up to pregnancy. Thus, their results demonstrate that effects of chronic stress on central (hypothalamic) processes can explain some stress-related reproductive failures that occur during pregnancy. Whether we can differentiate between mechanisms that come into play prior to pregnancy versus during pregnancy, and furthermore whether this difference is functionally meaningful, will be important moving forward.

5.5.5 Conclusions

Taken together, our results present a first step towards identifying the endocrine network that connects psychological stress to reproductive function during early pregnancy. Importantly, the effects of chronic stress on the reproductive axis during pregnancy do not seem to be acting through the well-known circuits that play a role in non-pregnant females. Combining hormone production measures and ovarian gene expression across pregnancy progression offers a new perspective for understanding the endocrine networks through which stress impacts pregnancy.

5.6 Acknowledgments

We would like to acknowledge Aimee Pepper, Pooja Srinivas, Amber Kirawala, Laura Reynolds, Damhee Hu, Emily Tang, Alvin Balmeo, and especially Veronica Kim, whose help made this project possible.

5.7 Tables

Table 1 Summary sample sizes used in experiment.

Treatment	Day of Pregnancy Collected		Total
	Day 5	Day 10	
Control	10	12	22
Restrained	9	15	24
		Total	46

Table 2 Primers used for quantitative PCR analyses of gene expression in C57BL/6J mice. Primers for *PRLRL* were taken from (5). T_A: Annealing Temperature, °C.

Target	Forward Primer	Reverse Primer	T_A
TBP	GGGAGAATCATGGACCAG	GGCTGTGGAGTAAGTCCTGT	55
PRLRL	ATAAAAGGATTTGATACTCATCTGCTAGAG	TGTCATCCACTTCCAAGAACTCC	60
StAR	CTTGGCTGCTCAGTATTGAC	TGGTGGACAGTCCTTAACAC	55
P450SCC	CGATACTCTTCTCATGCGAG	CTTTCTTCCAGGCATCTGAAC	55
LHR	CTCCAGAGTTGTCAGGGTCG	AGGTGAGAGATAGTCGGGCG	60
20αHSD	ATGAGCTTTTGCCTAAAGATGAG	GTTAGACACCCCGATGGAC	55

5.8 Figures

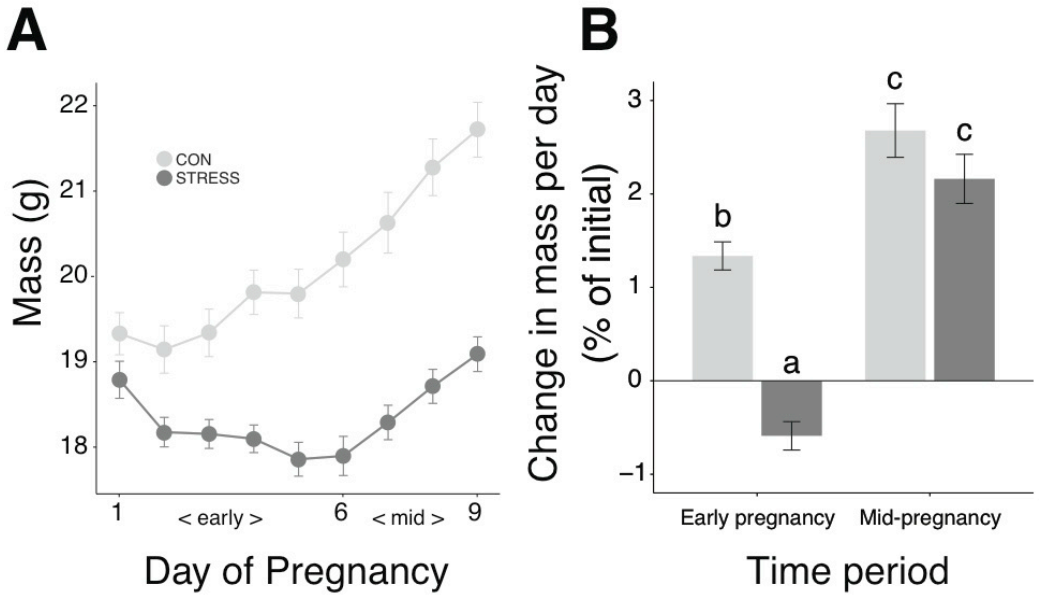

Figure 1 (A) Patterns of weight gain across pregnancy in unrestrained mice (CON, light line; N = 20) and mice exposed to chronic restraint stress (STRESS, dark line; N = 23). (B) When exposed to chronic restraint stress, pregnant female mice (dark bar) lost mass between days 1-6 relative to pregnant unrestrained mice, which gained weight (light bar). However, between day 6 and day 9, chronically stressed females gained weight at rates comparable to unrestrained females (though absolute mass is still less than unrestrained females; see 2A). Letters above bars indicate significantly different post-hoc comparison ($P < 0.03$ for all).

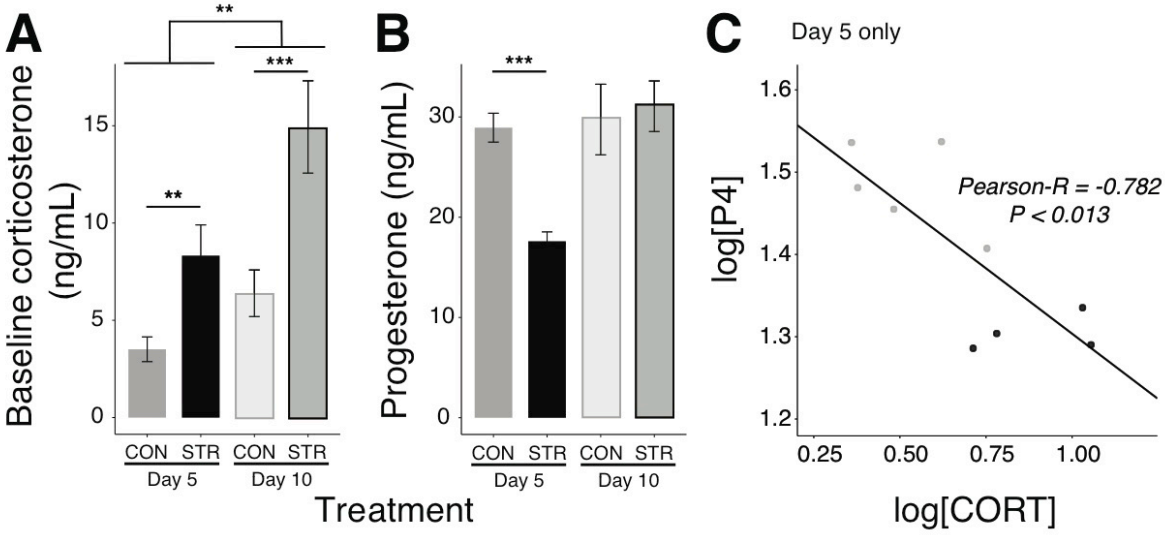

Figure 2 (A) Baseline corticosterone was elevated in chronically-stressed (STR), pregnant female mice relative to unrestrained (CON) females at both day 5 and day

10 of pregnancy. Baseline corticosterone increased from early to mid-pregnancy (day 5 to day 10), and was elevated in restrained females relative to unrestrained at both time points. Day 5: CON, N = 5; STR, N = 4; Day 10: CON, N = 11; STR, N = 12. (B) Baseline progesterone was lower in chronically-stressed (STR), pregnant female mice on day 5 relative to unrestrained (CON) females, but not on day 10. (C) On day 5, baseline corticosterone was inversely correlated with circulating progesterone. Day 5: CON, N = 10; STR, N = 9; Day 10: CON, N = 5; STR, N = 6. ** P < 0.02; *** P < 0.001, planned comparisons.

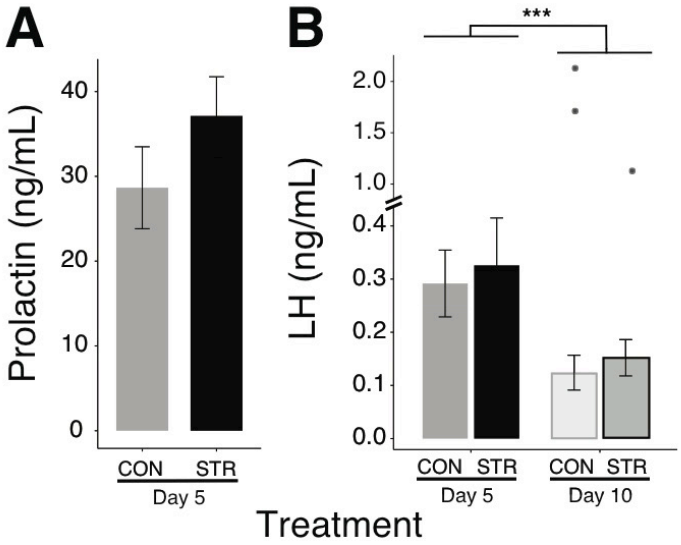

Figure 3 (A) Circulating concentration of prolactin did not differ between unrestrained (CON) and restraint-stressed (STR), pregnant mice on day 5 of pregnancy (P > 0.2) (B) Circulating concentration of LH varied between early and mid-pregnancy, but not with stress. PRL - Day 5: CON, N = 5; STR, N = 5. LH - Day 5: CON, N = 5; STR, N = 4; Day 10: CON, N = 9; STR, N = 12. *** P < 0.001, planned comparisons.

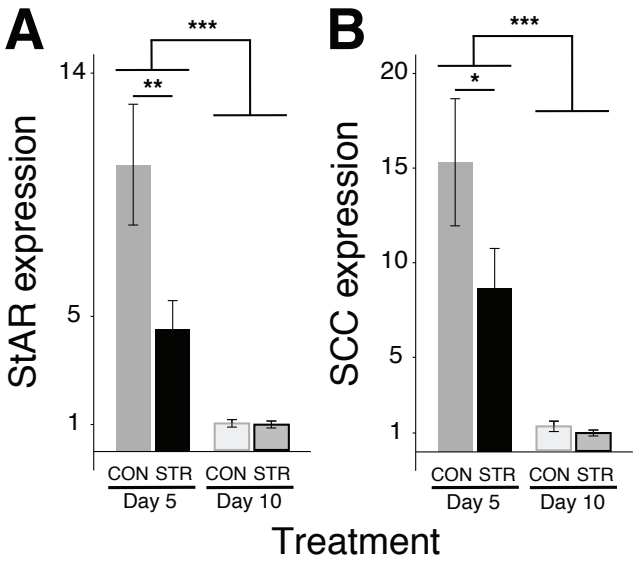

Figure 4 Expression of two steroidogenic enzymes in the ovary is modulated by stress and pregnancy progression. (A) Fold-change expression of steroidogenic acute regulatory protein (*StAR*) was decreased in restraint-stressed (STR) animals during early pregnancy related to unrestrained females (CON), but down-regulated in both groups during mid-pregnancy (B) Fold-change expression of cholesterol side chain cleavage enzyme (*SCC*) was also lower in restraint-stressed animals during early pregnancy, but down-regulated in both stressed and unrestrained animals during mid-pregnancy. Plot shows untransformed data. Day 5: CON, N = 20; STR, N = 16; Day 10: CON, N = 18; STR, N = 17. Samples sizes report number of ovaries. Both ovaries were used from most individuals within a repeated measures analysis (see section 3.3.4). * P < 0.05; ** P < 0.02; *** P < 0.001, planned comparisons.

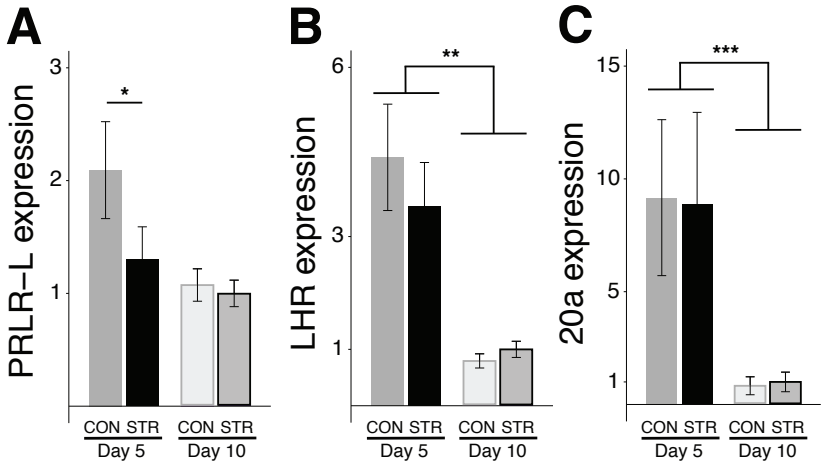

Figure 5 Expression of other candidate genes know to be important for ovarian progesterone production during early pregnancy are unaffected by restraint stress. (A) The long-form prolactin receptor (*PRLRL*) was not inhibited by chronic restraint during early pregnancy, but not during mid-pregnancy. (B) Luteinizing hormone

receptor (*LHR*) was not affected by restraint stress during early pregnancy, but showed substantial down-regulation by mid-pregnancy in both groups. (C) *20αHSD* (*20α*) was also not affected by restraint stress during early pregnancy, but was down regulated in the ovary by mid-pregnancy in both groups. Plot shows untransformed data. Day 5: CON, N = 20; STR, N = 16; Day 10: CON, N = 18; STR, N = 17. Samples sizes report number of ovaries. Both ovaries were used from most individuals within a repeated measures analysis (see section 3.3.4). * P < 0.05; ** P < 0.02; *** P < 0.001, planned comparisons.

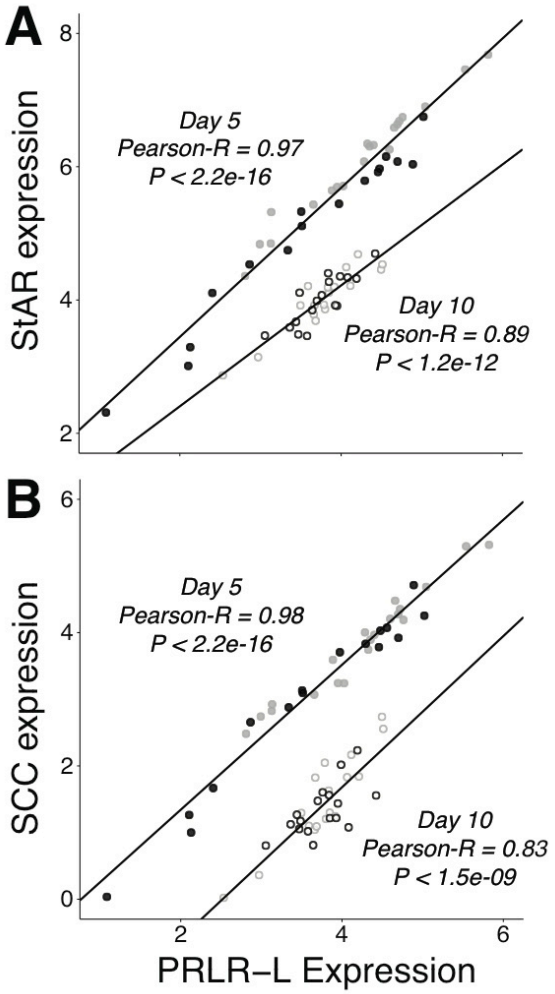

Figure 6 Expression of the long-form of the prolactin receptor (*PRLRL*) was correlated with expression of steroidogenic enzymes in the ovaries (Light orange: Day 5, Unrestrained; Dark orange: Day 5, restraint stress; Dark purple: Day 10, Unrestrained; Light purple: Day 10, restrained). (A) *PRLRL* covaries with expression of steroidogenic acute regulatory protein (*StAR*) across pregnancy. (B) *PRLRL* covaries with expression of *SCC* across pregnancy. Plot shows log-transformed data.

6. Conclusions

Flexible control of reproductive investment is crucial for females to maximize their reproductive fitness. The hypothalamo-pituitary-ovary (HPO) axis has historically been considered the core system for reproductive modulation. The past 50 years have allowed us to integrate the HPO axis with complex neural networks that deliver environmental and endogenous information to gonadotropin releasing hormone (GnRH) neurons in the hypothalamus. Changes to GnRH secretion can then cascade through the HPO axis to modify reproductive function. Mechanisms that modulate reproductive function but lie outside of the canonical HPO axis are a growing area of interest.

This dissertation has generated insight into four novel mechanisms by which cue integration can modulate female reproductive function, and it has done so across multiple levels of biological organization. Chapters 2 and 3 introduce new pathways by which ovarian function is regulated in the domestic cat and the mouse, respectively, by focusing on the isolated ovary (Chapter 2) and the isolated ovarian follicle (Chapter 3) as functional units. These studies build on a growing foundation of evidence suggesting that local signal integration can meaningfully alter ovarian function and thus drive variation in reproductive outcomes. Chapter 2 also highlights the possibility that such signals may also contribute to regulating other homeostatic processes. Though we recognize that endocrine signals such as sex steroids influence diverse physiological processes, actually integrating and understanding the adaptive value of this multi-system coordination remains intellectually challenging. Physiological studies such as those outlined here will lay the groundwork for such efforts into the future.

Experiments from Chapter 4 demonstrate the efficacy and adaptability of an *in vitro* approach for understanding uterine preparedness. Furthermore, these studies highlight the potential utility of the *in vitro* approach for comparative reproductive physiologists. Given that our understanding of implantation failure remains limited even in humans, these approaches offer exciting opportunities for progress in broad comparative biology of uterine function. The efficacy of this *in vitro* system may also allow us to more easily determine the importance of sympathetic innervation for uterine function, because it allows us to easily control the uterine environment.

Finally, Chapter 5 supports the hypothesis that HPO axis function is reorganized throughout pregnancy, and our current knowledge of feedback systems is insufficient to explain how this reorganization occurs. As with Chapters 2-4, experimental studies that make use of advances in gene editing, agonist/antagonist development, and *in vitro* reproductive technologies are going to be critical for advancing the field when our working model of reproductive function fails. The primary advantage of these newer tools is the ability to make tissue and even cell-specific modification to receptor function. Translating *in vitro* findings to *in vivo* function will depend heavily on these kinds of targeted approaches.

Despite the opportunities for stepwise advancement, there remain big challenges towards incorporating cue integration across the axes. In particular, traits relevant

to reproductive outcomes occur at many levels of biological organization within the axis. Steroidogenesis must occur sufficiently and appropriately at the level of the granulosa and thecal cells of particular ovarian follicle types, however it is the sum of those hormones that are available in circulation and which feedback to influence how the brain regulates behavior and axis function. Although chapters 2-4 in this dissertation illustrate functional changes at multiple levels of biological organization, these studies do not on their own allow us to scale function up to organismal outcomes. Evidence for function does not necessarily translate into biological relevance! For this reason in particular, scaling molecular function to organismal trait or outcomes is an essential goal for reproductive physiologists to keep in mind as we make advances in identifying cues or integration points.

For some researchers focused on the ultimate patterns of life history variation and evolution, deconstructing the underlying mechanisms may seem "in the weeds". Are the nuanced mechanisms important if we are ultimately interested in explaining broad-scale variation? To the contrary, pursuing questions in the absence of physiologically-sound theories directly undermines our ability to appropriately answer ultimate questions of interest. For example, if we know that glucocorticoids indeed alter sex steroid secretion by the ovary, but only through action on granulosa cells in mature follicles, we can generate hypotheses about how the system should behave at different stages in the reproductive cycle as the follicular make-up of the ovary changes. By making progress on the mechanisms, we avoid generating theories about life history evolution that are disconnected from the biological function, which could ultimate make them impossible for evolution to act on.

Though the big questions about life history evolution will require much more research into scaling mechanisms to organ-level function and organism-level traits, useful progress on ultimate questions is dependent on us first pursuing the low hanging fruit. Comparative physiologists can make major contributions to understanding hormone evolution and conserved or convergent function simply by filling in gaps of functional data related to paracrine neuroendocrine function in the ovaries and uterus. Additional attention to the unique endocrine components of discrete stages of reproduction, especially pregnancy and lactation, will help clarify what comprises a functional and flexible reproductive system at each stage. Last, careful *in vitro* and *in vivo* manipulations are necessary to directly evaluate speculative mechanisms currently driving many hypotheses and predictions about flexible reproductive physiology in ecologically-relevant contexts. This breadth of opportunity combined with the depth of molecular access that current tools offer us portends an exciting future for reproductive physiologists. Progress will require careful attention to applying hypothesis-driven approaches explicitly designed to test reproductive system function, and approaches most useful for this will likely be adapted from technologies developed for use in human and agricultural animal health.

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