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Biomarkers of reproductive health in wildlife and techniques for their assessment

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ABSTRACT

Historically, reproductive health in wildlife species has been evaluated primarily via immunoassay detection of fecal and urinary steroid hormone metabolites. This combination of sample type, biomarker category, and assay has been preferred for decades due to the ease of assessing reproductive health through the evaluation of stable compounds in easily collected biological samples using a cost-effective method. Increasingly, beginning with high performance liquid chromatography (HPLC) and more recently with convergence chromatography and ultra HPLC coupled with mass spectrometry (MS), wildlife studies are incorporating more sensitive and specific high-throughput technologies for the assessment of not only steroid hormone metabolites but proteins as well. Of note, a comprehensive health evaluation requires the measurement of biological readouts that modulate reproduction such as: glucocorticoids, leptin, insulin, thyroid hormones, melatonin, the microbiome, and markers of inflammation. Emerging modulatory biomarkers of reproductive health include acute phase proteins, microRNAs, and reactive oxygen species. Several of these biomarkers require application of newer technologies such as LC-MS/MS and sequencing, which demonstrates the need for the field of wildlife reproductive biology to diversify from its reliance on immunoassays. Importantly, endocrine disrupting chemicals adversely affect many aspects of reproductive function and evaluation of these compounds requires high throughput technology such as LC-MS/MS. The application of sequencing, particularly Next Generation Sequencing of bulk RNA (RNA-Seq) and single cell RNA-Seq, is uncommon in studies of wildlife reproductive health. However, as the cost of these methods decreases and consortiums of wildlife researchers band together to raise funds in support of studies using these technologies, their use will become more routine. Future research should focus on integration of known biomarkers of related systems into comprehensive reproductive assessments and the development of new biomarkers which are sensitive, precise, and employ non-invasive methodologies for the assessment of reproductive health of wildlife species.

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1. Introduction

Over the last few decades, endocrine techniques have been applied using non- or minimally-invasive sample types to generate knowledge of the reproductive status, health, and well-being of diverse taxa and to promote wildlife conservation. Endocrine analyses are key to understanding basic reproductive physiology (such as seasonality [1–3], rut [4–7], cyclicity [8,9] and pregnancy [10,11]), investigating abnormal reproductive function [12,13], and for applied reproductive management [14–16]. Steroid hormone metabolites are commonly used to establish reproductive mechanisms in wildlife due to their relative ease of measurement across sample matrices. However, these biomarkers may provide only a portion of the full reproductive health status of an animal.

In the last 5–10 years, the wildlife reproduction field has shifted its focus to include measurement of non-hormone biomarkers which provide information about both normal reproductive physiology and pathophysiology. Through adaptation of innovative methodologies, exploration of mechanisms, and application of commonly used biomarkers in humans and domestic animals, wildlife reproductive biologists can improve their ability to address critical challenges affecting wildlife reproductive health. This review describes the current status as well as the future of the wildlife reproduction field. This review discusses commonly measured hormones and techniques as well as emerging biomarkers and precise and sensitive technologies commonly used in other fields and in studies of humans and domestic animals.

The radioimmunoassay (RIA) was developed in the late 1950's and allows for precise measurement of parts per trillion of material per milliliter of blood [17]. By 1975, John Wingfield modified RIA methods to analyze circulating concentrations of androgens, estrogens, and corticosteroids in small blood samples taken from wild birds [18,19]. His work revolutionized the study of endocrinology in wildlife species and gave ecologists a tool to study hormone modulation of behavior, ontogeny, life history stages, and immunology [20]. Development of protocols for measurement of steroid hormones in urine and feces in the 1980's and 1990's permitted the non-invasive monitoring of daily hormonal fluctuations and cyclic patterns as well as the definition of normal concentrations of steroid hormones in lab, zoo, and free-living species (reviewed by Fusani, [20]; Graham, [21]; Monfort, [22]). The design of enzyme immunoassays (EIAs) to measure fecal metabolites of steroid hormones [23] has resulted in an increase in the number of studies on reproductive endocrinology in a diverse range of taxa.

Measurement of biomarkers of reproductive health has directly benefited the conservation of species *in situ* and *ex situ,* improved captive breeding outcomes, and supported the development and application of assisted reproductive techniques. The field of wildlife endocrinology has expanded our knowledge of basic wildlife physiology through the application of immunoassays to the measurement of biomarkers in non- or minimally-invasive collected specimens. Over the last 30 years, wildlife conservationists have developed immunoassay techniques to measure biomarkers of reproduction and health in diverse biomaterials such as saliva [24–28], claws [29,30], feathers [31,32], hair [33,34], skin sheds [35] and secretions [36], and whale blubber [37], blow [38,39], and baleen [40] and have also used biochemical, molecular, and genomic technologies to advance knowledge on the importance of novel endocrine molecules [41].

Compared to the depth and breadth of knowledge on reproductive physiology in domestic species, knowledge of wildlife reproductive endocrinology is less well-developed, particularly with respect to molecular mechanisms [42]. However, as research labs identify new biomarkers of reproductive status [43–46], it is expected that the wildlife biology field will catch up. For application to the study of wildlife reproductive health, useful biomarkers must: 1) have a characterized relationship with the process of interest (i.e. biologically meaningful), 2) be measurable in circulation or in at least one non- or minimally-invasive sample matrix, 3) have a good signal to noise ratio, and 4) have a relatively rapid response to changes in condition. It is essential to employ a uniform methodology for both sample collection and development of new lab techniques and research protocols. Standardization supports the generation of repeatable data, eliminates bias, minimizes error, and permits the ability to compare results within and among species [47,48].

2. Biomarkers of reproductive function

2.1. Sex steroid hormones

Due to external and/or internal stimuli, the hypothalamus releases the gonadotrophin-releasing hormone (GnRH), which in turn results in the release of gonadotrophins – follicle stimulating hormone (FSH) and luteinizing hormones (LH) (see the Protein-hormone section) [49]. After sexual maturity, gonadotrophins activate the gonads in both sexes resulting in the production of sex steroid hormones: progesterone, estrogens, and androgens [50,51]. All steroid hormones are synthesized from cholesterol, which is first converted to pregnenolone [52]. After steroid hormones are released into the bloodstream for transportation to their respective target tissue(s) to exert their effects [48,53], circulating steroids are subsequently metabolized by the liver and excreted often as conjugates, via the kidneys into the urine or via bile into the gastrointestinal tract [54,55].

Progesterone is produced from pregnenolone by two biosynthetic reactions in the gonads and adrenal glands [56]. This sex steroid acts a precursor for androgens and, subsequently, estrogens and plays an important role in maintenance of pregnancy through progesterone receptors in the endometrial stroma, which releases growth factors that support the conceptus during implantation and promote fetal growth [57–59]. Estrogens are biosynthesized from progesterone by a three-step pathway in the gonads [60] and in other tissues such as adipose tissue [61]. Estrogens drive the development of female primary and secondary characteristics, such as the development of genital and mammary tissue [62]. Increases in circulating estrogen concentrations suppress progesterone production, increase LH concentrations, and stimulate growth and development of ovarian follicles [63]. After ovulation, ovarian follicles form the corpus luteum (CL), which produces progesterone to decrease estrogen production in many species through a negative feedback loop [64]. High circulating progesterone levels are maintained after successful fertilization. However, in the absence of fertilization the CL disintegrates which leads to a drop in progesterone and a gradual increase in estrogen. Assessment of progesterone and estrogen metabolites has established species-specific estrous cycles and gestation periods in mammalian wildlife species housed in captivity such as Roan antelope (*Hippotragus equinus*) [65], Steller sea lions (*Eumetopias jubatus*) [66], and bottlenose dolphins (*Tursiops truncatus*) [67].

Dehydroepiandrosterone (DHEA) is a weak androgen produced from pregnenolone primarily in the adrenal cortex and gonads and to a lesser extent in other tissues [68]. DHEA regulates immune function and the production of cytokines, as well as serving as a glucocorticoid antagonist [69,70] (see Section 3.1.3). Additionally, DHEA is also a precursor for more potent androgens and for estrogens [52,68]. Testosterone and 5-alpha-dihydrotestosterone are potent androgens responsible for the development of male primary and secondary sexual characteristics. High intratesticular concentrations of testosterone are required for normal spermatogenesis [71]. Testosterone also is critical for normal male sexual behaviors (i.e. courtship and mating) and reproductive seasonality. For example, African bush babies (*Galago moholi*) show grooming and mating behavior only when urinary androgen metabolites are relatively high [72]. Similarly, wild caught southern African spiny mice (*Acomys spinosissimus*) have increased androgen levels only during long photoperiods [73].

Monitoring sex steroids and/or their metabolites in relation to reproductive function has been an effective tool in a wide range of wildlife research of males and females [74]. Females also produce

androgens from the theca interna and the adrenal cortex and demonstrate a species-specific pattern with respect to the dominant androgen (i.e. testosterone versus androstenedione). For instance, in addition to monitoring serum progesterone and LH to time breeding and artificial insemination [75], monitoring serum testosterone in pregnant Asian elephants (*Elephas maximus*) can predict the sex of calves [76]. Sex steroids also are useful for understanding the link between reproductive hormones and social rank in cooperative breeding species. For example, in the cooperatively breeding Meerkat (*Suricatta suricatta*) [77], dominant females have higher androgens and estrogens compared to males.

2.2. Protein hormones

2.2.1. Anti-Müllerian hormone

Anti-Müllerian hormone (AMH) is a member of the transforming growth factor-β (TGF-β) family. During embryonic development, AMH secreted by Sertoli cells in the developing testis causes regression of the Müllerian or paramesonephric ducts and inhibits the formation of the female duct system [78,79]. In the female, AMH is produced by granulosa cells in follicles, with expression highest in the preantral and small antral follicles [80]. AMH inhibits follicle recruitment by FSH [79,81]. AMH is a marker of fertility in women [82], cattle [83], goats [84], pigs [85], and mice [86], with circulating concentrations positively correlated to the number of morphologically healthy oocytes in the follicular reserve [87,88]. In the human, serum AMH is used to determine the onset of menopause, a state of reproductive cycle cessation due to ovarian follicle depletion [89–91]. Similarly, for water buffalo [92], macaques [93], and rabbits [94], the concentration of AMH in follicular fluid has a negative correlation with size of follicle diameter. These data suggest that in these species AMH may play a direct role in promoting pre-antral follicular growth and selection of dominant follicles. AMH is widely used as a tool in the selection of individual dairy cows for superovulation programs and as a predictor for the number of transferable bovine embryos [95,96]. Studies investigating AMH in female elephant and rhino species, cheetah, and beluga whales have shown that the highest AMH concentration occurs in prepubertal females, with variation in AMH concentrations between individuals of the same age and a decline in AMH with age [97–100]. In addition, treatment of cheetah with deslorelin, a long-acting GnRH agonist, significantly lowers serum AMH concentrations compared to AMH concentrations in untreated females of the same age and reproductive status [100]. For male equid [101–103], elephant [97], and rhino species [98], cattle [104], and beluga whales [105], serum AMH concentration is higher in post pubertal, reproductively active males when compared to females of the same age. In horses, serum AMH is higher in stallions compared to geldings; therefore, serum AMH concentration can be used to determine cryptorchidism in young, pre-pubertal colts [102].

2.2.2. Follicle stimulating hormone

FSH is a glycoprotein produced by gonadotrophic cells in the anterior pituitary in response to pulsatile secretion of hypothalamic GnRH [106] and granulosa cell derived activin [107]. FSH receptors are found on granulosa cells within developing preantral follicles and stimulation of these receptors promotes folliculogenesis [108,109]. FSH specifically acts on granulosa cells to convert testosterone, produced by neighboring theca interna cells, into estradiol (E2) by increasing the activity of aromatase [110]. In several mammalian species, E2 is transported by sex-hormone binding globulin via the vascular circulation to target estrogen receptor alpha (ERα) in the arcuate nucleus of the hypothalamus [50,111–113]. Binding of ligand to the receptor allows for the negative feedback regulation of tonic GnRH secretion and subsequent luteinizing hormone (LH) production by gonadotrophic cells in the anterior pituitary [114]. As GnRH neurons do not express ERα, E2 is believed to work indirectly through other neurotransmitters to inhibit GnRH neurons, thereby regulating FSH synthesis and secretion [115,116]. For males, FSH is shown to promote spermatogenesis and Sertoli cell development during the fetal and neonatal period in the primate or during puberty in the rat [117,118].

Similar to its effects in ruminants and rodents, FSH recruits follicles in canids and felids, and initiates successive follicular waves in elephants [119–122]. Bioactive levels of urinary FSH have been shown to demonstrate two peaks during the follicular phase preceding a midcycle surge of FSH in the lowland gorilla [123]. By contrast, in the bottlenose dolphin, urinary FSH concentration peaks during the follicular phase and decreases immediately before ovulation occurs [99]. Understanding the physiological response to FSH and LH has been instrumental in the development of contraception for wildlife populations. Male and female cheetah (*Acinonyx jubatus*), African leopard, and African lion implanted with deslorelin have decreased plasma progesterone and testosterone concentrations and a subsequent drop in fertility [124].

2.2.3. Inhibin

Inhibin, a heterodimeric glycoprotein and member of the TGF-β family, is produced by the granulosa and theca cells of developing ovarian follicles and by Sertoli cells in the testis [125,126]. It exerts its suppression of FSH secretion by binding to serine-threonine receptors on gonadotropic cells in the anterior pituitary [127]. In humans and sheep, inhibin from granulosa cells in estrogenic follicles has a paracrine effect on theca interna cells resulting in an increase in androgen production which helps to drive aromatization of androgens to estrogens [128,129]. By contrast, serum inhibin concentrations in elephants increase after luteinization of the follicle following the first LH peak [130–133]. Therefore, the primary source of inhibin in elephants is cells in luteinized follicles and accessory corpora lutea [21].

One of inhibin's main effects is the antagonism of activins [134,135]. Activins, also TGF-β family members, are both stimulatory to and stimulated by FSH [136]. Although inhibin receptors have been identified in Sertoli, Leydig, and germ cells, the mechanisms and effects of inhibin within the testis are unknown [137,138]. In both male and female prairie dogs, which are seasonal breeders, immunochemical staining in the gonad during seasonal regression supports the theory that inhibin plays a role in the negative feedback of androgens in the male and progesterone in the female [139]. In wild male Japanese black bears (*Ursus thibetanus japonicus*), serum inhibin is higher during the breeding season and positively correlated with testosterone and LH [140], suggesting that this biomarker could be a useful indicator of testicular function in this species. By contrast, a negative correlation between testosterone and inhibin has been found for male beluga whales. Furthermore, although no seasonal changes have been observed in inhibin in beluga whales, inhibin concentrations do decrease with age in this species [141]. In male Italian wall lizards (*Podarcis sicula*), testicular-derived inhibin acts at the hypothalamus to suppress gonadotropin release and at the testis to regulate germ cell proliferation and decrease local steroidogenesis [142]. Immunohistochemistry of both African [143] and Asian [144] elephant testicular tissue has revealed inhibin and/or activin presence in the Sertoli and Leydig cells of elephants but circulating concentrations of these two protein hormones have not been assessed in these species. Although inhibin is commonly used to assess follicular growth and development in domestic species, additional work is needed to ascertain the full potential of this biomarker in wildlife species [145].

2.2.4. Luteinizing hormone

Similar to FSH, LH is regulated by hypothalamic GnRH and synthesized and secreted by gonadotrophic cells in the anterior pituitary [114]. Acting on membrane-bound receptors on theca interna cells in developing follicles, LH is critical to the growth and maturation of large antral follicles and triggers ovulation [146–148]. Exogenous GnRH administration to marsupial species such as the tammar wallaby [149] and fat-tailed dunnart [150] results in increased circulating FSH and LH, follicular development, ovulation, and subsequent mating. The binding of LH to theca interna initiates the multistep conversion of cholesterol to

testosterone [151,152]. Immediately preceding ovulation, granulosa cells of large antral follicles also develop LH receptors. Binding of LH to these receptors on the granulosa cells triggers ovulation in response to the LH surge [153]. As such, LH is the primary biomarker used to determine the timing of ovulation. In the giant panda [154,155], bottlenose dolphin (*Tursiops truncatus*) [156], and killer whale (*Orcinus orca*) [157] detection of urinary LH is used as a proxy for timing of estrus. Unlike other mammalian species, African and Asian elephants do not exhibit one but two LH peaks which occur three weeks apart with the second LH peak triggering ovulation [158,159]. Unlike other species, urine is not a useful sampling matrix for detection of the LH surge in female elephants [160]. As the timing of the ovulatory LH surge is predictable and species-specific, the LH surge has been instrumental to scheduling natural mating attempts and artificial insemination (AI) in managed wildlife [75,121,161,162].

For induced ovulators such as camelids [163], Sumatran rhino [164], lion [165], black bear [166], common hare [163], mink [163], Koala [167,168], and brush-tailed bettong [169], the LH surge is triggered by cervical stimulation. Interestingly, in Koala [168], alpaca [170] and camel species [171], simply the presence of semen in the female reproductive tract can trigger ovulation. Thus, there is a semen-induced effect on LH secretion. For these species, a rise in serum LH can provide proof of mating and ovulation even when mating may not have been observed. Following ovulation, LH triggers luteinization of theca interna and granulosa cells to form the CL and stimulates subsequent synthesis and secretion of progesterone from this structure [172]. In the male, LH is responsible for testosterone production by Leydig cells in the testes [173] and is increased during musth in the elephant [174] and during the breeding season in male Pallas' cats where it also correlates with morphological measures of sperm quality [175].

2.2.5. Oxytocin

Synthesized in magnocellular neurons in the supraoptic nuclei of the hypothalamus, oxytocin, Greek for "rapid birth", is one of two proteohormones secreted from the posterior pituitary [176]. Acting on receptors in uterine myometrium, oxytocin initiates smooth muscle contractions during parturition to facilitate expulsion of fetus and extraembryonic membranes. Postpartum, oxytocin stimulates milk ejection from the mammary during lactation. In addition, it also stimulates the synthesis and secretion of prostaglandin F2a (PGF2a), which regulates luteolysis, from the endometrium and decidua [177,178]. Oxytocin receptors are also found on lactotroph cells in the anterior pituitary. After release from hypothalamic oxytocinergic neurons, oxytocin is transported to the anterior pituitary via the portal vessels where it causes a release of prolactin stores [179,180]. In the male, along with nitric oxide, dopamine, and glutamate, oxytocin helps regulate erectile function [181]. In humans oxytocin receptors are found in the epididymis and prostate and regulate seminiferous tubule contraction and modulation of steroidogenesis [182,183].

Interestingly, oxytocin is also a strong neuromodulator. In macaques, oxytocin facilitates prosocial behavior and affiliative behavior in infants toward caregivers [184]. Similarly in chimpanzees and the naked mole-rat, urinary oxytocin concentrations are highest after grooming events with socially bonded partners [185,186]. By contrast, increases in salivary oxytocin concentration were not observed in managed male gorillas following positive reinforcement training with primary human caregivers [187]. On the other hand, salivary oxytocin concentrations in dogs [188], pigs, and cattle [189] increase upon interaction with humans. These data support the use of oxytocin as a potential biomarker of stress in wildlife. Surprisingly, the administration of oxytocin to semi-wild African lions causes an increase in time spent near in-group lions and a decrease in aggression toward out-group individuals [190]. Urinary oxytocin has also been linked to reproductive abnormalities in African elephants as it is elevated in hyperprolactinemic females compared to those with normal physiological prolactin levels [191].

2.2.6. Prolactin

Synthesized and secreted by lactotrophic cells in the anterior pituitary gland, prolactin, a single-chain peptide hormone, is involved in a variety of biological processes, most notably mammary gland development and lactation [192–195]. Prolactin release is under constant inhibitory control by dopamine from hypothalamic tubero-infundibular dopamine neurons in the arcuate nucleus [196]. Several species- and cycle stage-dependent mechanisms of action for prolactin are of importance for management of wildlife species [197]. Although the exact role of prolactin in male reproductive biology is unclear, in humans and rodents, prolactin is involved in the meiotic division of germ cells [198] and regulates testicular function via modulation of gonadotropin receptors on Sertoli and Leydig cells [199,200].

In the rodent, prolactin is the main luteotropic hormone which maintains progesterone secretion from the CL via a decrease the progesterone metabolizing enzyme 20α-hydroxysteroid dehydrogenase [201–203]. For felids, blood prolactin and relaxin concentrations increase after mid-gestation, which permits diagnostic use of these hormones for pregnancy determination in these species [204,205]. Prolactin concentrations also increase during pregnancy in African and Asian elephants [206]. Additionally, prolactin has anti-atretic properties in preovulatory follicles of cattle [207]. In seasonal wildlife species, such as the Spanish ibex (*Capra pyrenaica hispanica*) [208] and pangolin [209], prolactin concentrations are elevated in females during the breeding season. In the African, but not the Asian, elephant prolactin increases during the follicular phase of the estrous cycle, suggesting it plays a role in follicular recruitment and development [121,210]. However, hyperprolactinemia, a common cause of infertility in women [211], also is associated with abnormal ovarian activity in African elephants [191]. Similarly, in males, high prolactin concentrations inhibit reproductive function by suppression of GnRH and subsequently LH which causes loss of libido and impotence [212]. Prolactin is also a powerful modulator of reproductive behaviors in birds [213]. High prolactin concentrations are positively associated with brood rearing, including nest building and protection, incubation, and good parental care of hatchlings [214–218].

2.2.7. Relaxin

Relaxin, a two-chain non-glycosylated polypeptide consisting of an alpha and beta chain linked by two disulfide crosslinks, is an important hormone produced during pregnancy [219,220]. Relaxin, which is primarily synthesized by the CL in the pig, rat, and human, and by the placenta in the cat, dog, rabbit, and horse, loosens the ligaments in the pubic symphysis during parturition [221–223]. Granules containing relaxin have been isolated from the CL of pregnant sharks and marsupials [224]. Interestingly, the amino acid composition of prolactin from sharks is more similar to porcine insulin than mammalian prolactin [224,225]. During the second half of pregnancy relaxin softens the cervix by reorganizing its collagen fiber distribution and organization which increases its size [226,227].

Relaxin is a useful tool for pregnancy recognition which is used to develop breeding management strategies for wildlife species. Serum prolactin and relaxin can be used to diagnose pregnancy in the second half of gestation in felids [204,205,228–230]. For one study in domestic cats, increases in urine relaxin were detected 28 days post-copulation [230]. In elephants, blood relaxin increases by gestational week 20 and reaches its maximal concentration in the second trimester with a second peak eight weeks before parturition [231,232]. Similarly with Sumatran rhinoceros, there is an increase in relaxin by 24 weeks gestation, with concentrations continuing to rise before peaking two weeks prior to parturition [233]. Relaxin in the pregnant killer whale can only be differentiated from luteal phase prolactin at 32 weeks of gestation [234]. As with the Sumatran rhino, relaxin in the killer whale continues to increase until it peaks in the final month of pregnancy [234].

With respect to male reproductive physiology, prolactin is

synthesized in the testes [235], seminal vesicles, [236], and prostate gland [237,238] of mammals and in the reproductive organs of birds [239,240] and elasmobranchs [241,242]. Although it has been hypothesized that relaxin plays an important role in modulating semen quality, this theory has yet to be proven.

2.2.8. Prostaglandins

Prostaglandins (PG) are a family of lipid compounds first discovered in human seminal fluid [243,244] which are now known to be ubiquitous in mammalian tissues [245,246]. All PGs are synthesized from the essential fatty acid arachidonic acid and have a similar base structure of 20 carbon atoms with a cyclopentane ring [245]. The first primary PGs isolated were PGE1 and PGF1α. Additional primary PGs were isolated and named according to added double bonds – PGE2, PGE3, PGF2α, and PGF3α [245,247]. Although the chemical structure of PGs may be similar, the hormone-like effects of different PGs are often dissimilar and/or opposing [245,247]. PGEs and PGFs perform essential roles in the reproduction such as regulation of CL-life cycle [248,249], maternal recognition of pregnancy [250–252], and parturition [253–255]. These fatty-acid derived compounds are produced locally in or near the tissues of action [245,246] and are metabolized quickly either in the tissue or in the lungs by enzymatic breakdown into PG metabolites excreted in urine and feces [256].

PGF2α metabolites (PGFM) isolated from the urine of Iberian lynx (*Lynx pardinus)* [257], domestic cats [258], and dogs [249,259] increase during pregnancy. Over the last decade pregnancy in wild felids [260,261] and the giant panda [262] has been diagnosed using fecal and urinary metabolites of this biomarker. PGFM also have been routinely measured in the holding water of several fish [263] and amphibian [264] species under both field and laboratory conditions [265] to assess sexual receptivity of the females and for determining the quality of eggs. Moreover, in fish taxa, PGF2α is released as a hormonal pheromone which attracts males of the target species, and its measurements have contributed toward prediction of the timing of courtship and spawning [263].

3. Endogenous modulatory biomarkers of reproductive function

3.1. Metabolism

3.1.1. Thyroid hormones

Triiodothyronine (T3) is the biologically active thyroid hormone created by deiodination of thyroxine (T4), the primary hormone secreted by the thyroid gland. T3 and T4 influence growth, maturation, metabolism, and nutritional physiology [266–268]. Decreases in T3 and T4 depress metabolic rate, conserve energy, and decrease lipolytic activity (break down of fats) in energy stores which increases the chance of survival when food is scarce [266,269,270].

Thyroid hormones also regulate reproductive homeostasis in seasonal breeders [269,271,272] via activation of thyroid hormone in the medio-basal hypothalamus which regulates seasonal GnRH secretion [271,272]. Additionally, thyroid hormones contribute to reproductive tract development [267,269]. The relationship between changes in energetic needs and reproductive function has been demonstrated in several wildlife species. For example, fecal T3 metabolite levels increase during pregnancy in mantled howler monkeys (*Alouatta palliata*) [273] and serum free and total T4 decrease during musth, a reproductive state associated with mate seeking and decreased food intake, in male elephants [274].

3.1.2. Leptin and insulin

Leptin is a polypeptide hormone made by adipocytes and, as such, the amount of circulating leptin correlates with an animal's energy reserves [275]. Leptin plays a key role in the regulation of energy homeostasis, including energy expenditure and food intake [275]. Leptin deficiency or resistance contributes to infertility via leptin signaling in the hypothalamus [275,276]. Leptin increases during pregnancy in humans and primates with contributions from both the placental trophoblast and the maternal adipose tissue [277,278]. Pregnancy induces physiological hyperleptinemia and leptin resistance, which results in the uncoupling of eating behavior and metabolic activity to regulate nutrient partitioning during the high energy state of pregnancy [275]. Increased leptin-like immunoreactivity has been observed during egg-laying in wild female European starlings (*Sturnus vulgaris*) which corresponds with a concurrent decrease in body and fat mass [279]. In the Japanese black bear (*Ursus thibetanus japonicus*) serum leptin concentrations increase gradually from September to mid-November in association with increasing body weight in preparation for hibernation. Serum leptin in this species peaks in late November at the beginning of hibernation and presumed embryo implantation and declines to baseline during hibernation approximately one month before parturition [280].

Leptin's role in regulation of reproduction spans the entirety of the reproductive lifespan of an animal from puberty to reproductive senescence. When food availability is low, puberty may be delayed, reproductive periods may be shortened, less offspring may be born or reproduction may be forfeited all together [276,281]. Alternatively, when food resources are abundant, puberty may occur at a younger age, more offspring may be produced, and reproductive periods can become longer [276,281]. Therefore, leptin provides critical signals to the hypothalamus to modulate GnRH neuronal activity which forms a bridge between energy reserves and the capacity for successful reproduction [275,281].

The relationship between circulating insulin, leptin, and adipose tissue in mammals connects the neuroendocrine system to the energy status of the animal [281]. Increased plasma glucose and insulin production during lactation and fasting in Northern elephant seals (*Mirounga angustirostris*) suggests that glucose recycling in this species helps to balance the energetic demands of milk production and the maternal metabolic needs for survival during fasting [282]. Insulin's primary role is control of glucose homeostasis via regulation of glucose uptake, oxidation, and storage. However, evidence from a number of studies suggests that insulin also has a permissive role in the pulsatile and surge secretion of GnRH and LH [283]. For example, in a study of managed African elephants, acyclic females with the highest body scores, had elevated leptin and insulin levels and a lower glucose to insulin ratio than cycling females with the same body score [284]. Such studies demonstrate the critical link between circulating insulin and leptin levels and normal reproductive function in females.

3.1.3. Glucocorticoids

Physiological concentrations of glucocorticoids (GCs), principally cortisol and corticosterone, are a vital component of normal reproductive function [285–287]. However, many studies of the relationship between GCs and reproduction have conflicting findings. As discussed by Fanson and Parrott [288], a possible explanation for the conflicting results (as reviewed in [289]) is that GCs are intrinsically linked to reproductive function yet are modulated across different reproductive stages. As such, disruptions to hypothalamic-pituitary-adrenal (HPA) activity via effects on patterns of secretion and on GC concentration can impair reproduction. Among seasonally breeding species, increased baseline GCs during the breeding season are adaptive, allowing individuals to meet the high energetic needs of reproduction [290]. One example of this is in the European starling (*Sturnus vulgaris*). Starlings housed outdoors have increases in baseline and stress-induced GCs and display appropriate breeding behaviors. However, starlings in an indoor setting have no change in baseline or stress-induced GCs and few signs of breeding activity, which suggests a role of GCs in facilitating the transition from the non-breeding to breeding season in this species [291]. However, there can be species or context differences in effects of GCs on reproduction. For example, in spotted salamanders (*Ambystoma maculatum*) GCs stimulate [292] but in rough-skinned newts (*Taricha granulosa*) GCs suppress [293] male reproductive activity. For species where

opportunities to mate during the breeding season are limited, stress-induced suppression of reproduction might be maladaptive, especially when stressors are acute. Experimental manipulation of oviparous females can provide insights into the mechanisms by which HPA dysregulation impacts reproductive success. In fact, parental investment in oviparous females may be either decreased or increased by potential stressors. In female house wrens (*Troglodytes aedon*), increased corticosterone results in increased pre- and postnatal investment in offspring [294,295]. In contrast, in Adélie penguins (*Pygoscelis adeliae*) [296] parental investment is reduced in corticosterone-treated females. GC-treated female garter snakes (*Thamnophis elegans*) produce more stillborn offspring and exhibit higher reproductive failure than control females [297].

Research across a variety of taxa has demonstrated that GCs modulate female reproduction from gametogenesis through pregnancy and lactation [285]. GCs play an important role in follicle maturation, ovulation, and luteinization [298,299], regulate production of and sensitivity to gonadotropins [286], and increase in circulation prior to ovulation in several species [300–303]. Administration of GCs during proestrus in the musk shrew (*Suncus murinus*) has been shown to promote receptive behavior [288]. Similarly, short-term administration of GCs stimulates testosterone production in bulls [304]. In contrast, disruption of GCs can lead to delayed puberty [305,306], impaired follicle development [307], and irregular estrous cycles [286,308–310]. GCs also play an important role in pregnancy, facilitating implantation [265], regulating immune processes [311], and mobilizing maternal energy stores [312]. The activity of the HPA axis increases as pregnancy progresses, and the vital role of fetal-derived GCs in the initiation of parturition for some species has been well characterized [313,314]. GCs also contribute to mammary development [315,316] and modulate important lactation genes [317]. The presence of GC receptors within the ovary [318,319], uterus [320], placenta [321,322], and testes [323–326] indicates that GCs can directly regulate reproductive tissues.

Although studies to characterize the exact patterns of GC production and potential mechanisms of action can be difficult in wildlife, several studies have revealed consistent cyclic changes in GCs across the estrous cycle in Asian elephants [327], black rhinoceros [328], and giant pandas [300]. Additionally, in mated female Tasmanian devils that did not successfully produce live offspring, the ovulatory peak in fecal GCs is delayed by four days which corresponds with higher GCs during the sperm storage phase [329]. These studies suggest that an understanding of normal GC concentrations and patterns in individual species can be helpful to ascertain the causes of reproductive failure.

GCs are important to the suppression of reproductive function. From a practical perspective, measuring GCs as a proxy for HPA axis activity is useful tool for wildlife, especially due to the wide variety of potential sample matrices [285]. However, it is important to put observed changes into context, as some physiological patterns of GC secretion are beneficial to normal reproduction. In such cases, deviation from normal GC levels can lead to disruption of normal reproduction and fertility. When examining the role of GCs in normal as well as impaired reproduction, it is essential to consider species differences in both underlying physiology and the metabolism and excretion of GCs (both identity of metabolites and routes of elimination).

3.2. Melatonin

Melatonin, an indole hormone produced enzymatically from the amino acid tryptophan by the pineal gland, is produced at night in both diurnal and nocturnal species and is commonly associated with the regulation of circadian and seasonal rhythms [271,330]. Melatonin and its receptors control the hypothalamic secretion of GnRH in photoperiodic, seasonally breeding animals [330]. Melatonin also contributes to ovarian follicle health in females [330]. The initiation of seasonal reproduction is controlled by the photoperiod which times reproduction for the optimal offspring survival [271,331–333].

Studies have demonstrated that the short, highly synchronized breeding season of the nocturnal brown antechinus (*Antechinus stuartii)* is controlled by endogenous circannual rhythm, day length, and the rate of change of photoperiod (related to altitude and latitude) and that administration of exogenous melatonin can disrupt the timing and duration of reproductive events [331,332]. In the Tasmanian devil (*Sarcophilus harrisii*), another nocturnal marsupial species, alterations in photoperiod and rate of change of day length changed the timing and length of the breeding periods [333]. Therefore, it is not surprising that the potential consequential effects of human-induced light pollution on photoperiod affects melatonin production and the control of seasonal reproduction in wildlife species. For example, light pollution has been shown to mask seasonal changes to ambient light cues in tammar wallabies (*Notamacropus eugenii*) which suppresses melatonin levels and results in delayed, poorly synchronized births [334]. The breeding season of the Pallas cat (*Otocolobus manul*), a species with pronounced reproductive seasonality [335], is triggered by a transition from short-day to long-day photoperiod. Extension of photoperiod due to a zoo's "Festival of Lights" event during winter (effectively adding 4–5 hrs onto the 10 hr light period) stimulated ovarian activity outside the normal breeding period for this species [336]. Concentrations of salivary melatonin in the aye aye (*Daubentonia madagascariensis*) are decreased when these species are kept under red as opposed to blue lights [337]. Although the effects of this light change on melatonin in the aye aye have not been explored with respect to reproduction in this species, it is possible that such a husbandry change could be used to modulate reproduction in managed aye ayes.

3.3. Markers of oxidative stress

Oxidative stress is caused by an imbalance between antioxidants and the pro-oxidants, reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS are created during a wide range of cellular processes involving oxygen metabolism and are kept in delicate balance by antioxidants. As is the case with GCs and the neuroendocrine stress response, ROS are important mediators in a range of normal physiological processes, such as sperm capacitation [338] and ovulation [339]. However, if the correct balance of ROS is disrupted, subfertility including impaired gametogenesis, complications during pregnancy [340,341] and reproductive diseases such as endometriosis [342] and polycystic ovary syndrome [343] (see [344–347] for reviews) can result. Biochemical oxidative stress might not equal biological oxidative stress if adaptive mechanisms designed to protect the organism are activated appropriately [348].

In the ovary, there is a complex relationship between ROS and antioxidants during the development of a follicle cohort. ROS in the preovulatory follicle play a key role in ovulation [349] and RNS are involved in oocyte maturation [350,351]. ROS are also produced in the CL and are key to luteal regression in the non-pregnant female [352] and development of a subsequent wave of follicles. In the pregnant female, production of ROS is a physiological process as increased placental mitochondrial activity supports fetal growth [353]. However, excessive ROS can negatively affect embryo implantation and lead to pregnancy loss.

In the male, oxidative stress is also important to the function of spermatozoa. The generation of ROS drives sperm capacitation, the acrosome reaction, and sperm-oocyte fusion [345,354–357]. However, ROS production which exceeds the antioxidant capacity of seminal plasma disrupts cell membrane characteristics critical for fertilization which leads to sperm DNA damage, fertilization failure, abnormal embryonic development, and premature pregnancy loss [358]. Excessive ROS production has also been associated with measures of sperm viability including sperm motility, concentration. and morphology [345, 359]. However, this may vary within and between species, depending on the type and concentration of ROS [360–362]. Species differences in polyunsaturated fatty acids in the sperm cell membrane are related to

this variable sensitivity to oxidative stress [363–365].

Several risk factors for development of systemic oxidated stress include obesity [366], heat stress [367], and exposure to environmental contaminants [368,369]. In the long-lived wandering albatross (*Diomedea exulans*), oxidative damage may be a physiological cost of reproduction as individuals with higher levels of organic or non-organic contaminants have higher oxidative damage [370]. In male lizards (*Eremias argus*), exposure to pollutants is associated with increased oxidative stress markers and reduced testes size [371]. Red deer (*Cervus elaphus*) living in polluted areas have increased DNA fragmentation, associated with decreased sperm motility, viability, and acrosome integrity [372,373]. Similar to GCs, the relative cost of reproduction as measured by biomarkers of oxidative stress does not always provide consistent results [374]. This may be due to the fact that intrinsic and extrinsic factors can influence oxidative state [347] and ultimately impact the reproductive success of a given animal [375].

A variety of biomarkers can be used to quantify oxidative stress, including oxidized DNA, lipids, proteins, and total antioxidant capacity [376]. However, in conservation and ecological studies, there are some limitations to the samples that can be collected and methodological issues to consider. Distinguishing between controlled (adaptive) and uncontrolled (pathological) processes and combining biomarkers to account for the complexity of oxidative balance systems [377] are essential to the appropriate application of such biomarkers. Minimally invasive samples including urine [378,379], saliva [376,380], exhaled breath condensate [381,382] and semen/seminal plasma [359] may be used to measure ROS in wildlife species. Gametes are also extremely sensitive to damage by ROS. As ROS compounds can be generated during cryopreservation, antioxidant supplementation may increase the success of assisted reproductive technologies (ARTs) [344,345]. The potential use of antioxidant supplementation (including Vitamins C and E, and natural plant antioxidants) in extenders for cryopreservation to counteract oxidative stress has been explored in domestic animals [383], but more research is needed to evaluate this approach for wildlife species.

3.4. MicroRNAs

MicroRNA (miRNAs) are small non-coding RNA molecules that regulate post-transcriptional gene expression involved in a wide range of biological processes, including reproduction. Research from humans (reviewed in [384,385]) and domestic animals (reviewed in [386]) demonstrates that miRNAs regulate processes involved in sexual development, gametogenesis, CL function, fertilization, embryo development and pregnancy, as well as reproductive pathologies [387,388] and pregnancy-related diseases [389,390]. Although much of the work on the role of miRNAs in reproductive mechanisms has characterized miRNAs in tissues, the potential for measurement of miRNAs in various biological fluids such as plasma, serum, urine, saliva, semen, and milk, and the stability of miRNAs despite extended storage and freeze-thaws [386] render miRNAs exciting biomarkers for application to wildlife species.

Across many different species, miRNAs associated with normal physiological processes or differences in reproductive output have been identified. Studies in sheep have found differences in ovarian miRNAs associated with follicular development and ovulation between breeds with differing fecundity [391]. Additionally, miRNAs related to endocrine signaling and follicular development differ between ewes in estrus during the breeding season as opposed to the non-breeding season [392]. Salivary miRNAs have been used as a non-invasive tool to improve estrus detection methods in buffalo [393]. In this species miRNAs associated with follicular development, steroidogenesis and ovulation were differentially expressed during estrus as compared to diestrus. These biomarkers are also useful to understand reproductive pathology. For example, in luteal tissue [394] several miRNAs are preferentially expressed in non-regressed versus regressed CLs. Abnormal miRNA function can also contribute to reproductive disease. In mares, some miRNAs are elevated in individuals with endometritis [395], which is a significant cause of infertility in horses [396].

Circulating miRNAs may also be used as specific biomarkers for pregnancy detection. In the horse, several signaling- and endocrine pathway-associated miRNAs have are differentially expressed between pregnant and non-pregnant mares during late gestation [389]. Specifically, miRNAs implicated in embryonic, fetal, and placental development are expressed at 25–45 days of gestation [397]. Guelfi and colleagues explored miRNAs involved in progesterone-mediated oocyte maturation and pregnancy in Italian Mediterranean buffalo (*Bubalus bubalis*) and found differential expression of three miRNAs at day 25 and of four miRNAs at day 40 of gestation compared to non-pregnant and non-inseminated control [398]. In pigs, changes related to embryo-maternal communication during pregnancy can be detected in maternal serum as early as day 16 of gestation [399], which is around maternal recognition of pregnancy in this species. miRNAs can also be used to detect early pregnancy loss. Extracellular vesicle-derived miRNAs can differentiate cows that are pregnant (day 30 gestation), non-pregnant, or had pregnancy loss at (days 17–30 gestation) [400].

Characterization of miRNAs may also provide insight into male reproduction. Comparison of immature and mature testes in the boar [401,402] and the bull [403] have found differential expression of numerous miRNAs with roles in development and spermatogenesis. Indeed, characterization of miRNAs from sperm [404] and seminal plasma [405] of high and low fertility individuals could identify potential mechanisms for improvement of sperm production and fertility [406].

The application of miRNAs as tools to assess reproductive dysfunction in wildlife is in its infancy. Studies investigating the effects of perfluoroalkyl acids (PFAAs) on peregrine falcons (*Falco peregrinus*) [407], immune function in the giant panda (*Ailuropoda melanoleuca*) [408], physiological adaptations to hibernation in brown bears (*Ursus arctos*) [409]), and miRNAs as potential biomarkers of iron overload in Sumatran (*Dicerorhinus sumatrensis*) and black (*Diceros bicornis*) rhinoceros [410] have been published relatively recently. Research in giant pandas has explored the measurement of miRNAs in milk as potential regulators of cub development [411] and in seminal plasma as a proxy for sperm tolerance to cryopreservation [412]. Thus far, studies have been limited to the measurement of miRNAs in serum, sperm, and seminal plasma in males; however, the potential to measure miRNAs in urine and saliva [393,413,414] could expand options for use of miRNAs as biomarkers of reproductive function in wildlife [415].

3.5. Microbiome

Host-associated microbial communities referred to as microbiomes have an important role in a wide range of biological functions including immunity, metabolism [416], and reproduction [417,418]. The microbiota, which consists of bacteria, fungi, archaea, viruses, and protozoa [419], inhabit various ecological niches in the body. The most widely studied microbiome in humans includes the gut, oral cavity, skin, and female reproductive tract [420]. The importance of the microbiome to wildlife species and the implications for conservation have gained increasing attention [421–423], with a focus on anthropogenic disturbance [424], *in situ* vs. *ex situ* comparisons [425,426], and disease [427,428]. Fewer studies have directly addressed questions related to wildlife reproductive health (see [429,430] for recent reviews). The reproductive tract microbiome may directly influence reproduction, but microbiome communities in other areas of the body also can contribute to successful reproduction. Microbiomes in distant locations may impact internal reproductive mechanisms, signal reproductive status and mate quality (i.e. olfactory cues), reproductive behavior, and/or offspring survival [430]. With respect to managed breeding programs, it may be necessary to consider the compatibility of female and male genital tract microbiome [431] to maximize reproductive success.

Increasing evidence from humans and model organisms suggests a bidirectional relationship between the microbiome and hormone production [432]; gut microbiota can produce biologically active molecules, change host gene expression, and influence the metabolism of hormones [433], and, in return, the hormone milieu can influence the structure (richness and diversity) and function of the host microbiome [434]. Systemic estrogen concentrations may be increased by gut microbes [435,436], GCs can be converted to androgens [437] and the microbiome of the female reproductive tract is strongly influenced by ovarian cycle phase, with certain taxa correlated with progesterone concentrations [438]. This bi-directional communication is also true of the HPA axis; microbiota regulate the development and function of the HPA axis [434], and GCs modulate the microbiome, affecting both composition and diversity [439]. In free-ranging southern white rhinoceros (*Ceratotherium simum simum*), stressful events such as chemical immobilization result in shifts in the microbiome characterized primarily by a decrease in the *Spirochaetia* class, the third most common microbial class in free-ranging southern white rhinoceros [440].

There are several examples in wildlife species of the importance of the microbiome during transitions in reproductive state or of the microbiome being associated with reproductive success. The vaginal microbiome differs substantially by reproductive state in wild baboons, with the vastly different microbial communities during pregnancy, postpartum amenorrhea, and ovarian cycles, especially around ovulation [441]. In Tibetan antelope (*Pantholops hodgsonii*) [442] and Tibetan macaques (*Macaca thibetana*) [443], fecal microbial composition differs between late pregnancy and the postpartum period [442], with several genera associated with metabolic and adrenal hormones. Such data suggest a shift of the microbiome to accompany energy requirements and metabolism during pregnancy and lactation. Data from cynomolgus macaques (*Macaca fascicularis*) [438] and Phayre's leaf monkey (*Trachypithecus phayrei crepusculus*) [444] demonstrate that progestogens contribute to gut microbial shifts during the ovarian cycle and pregnancy. In *ex situ* black rhinoceros (*Diceros bicornis*), gut microbiota composition differs according to reproductive state, with different gastrointestinal bacterial communities found in bred and unbred females and during pregnancy and after parturition as compared to follicular and luteal phases of the estrous cycle [432]. Around a third of bacterial genera show more than a 10% correlation with either progestogen and/or GC concentration. In the white rhinoceros (*Ceratotherium simum simum*), reproductive success of *ex situ* females is negatively impacted by a sensitivity to dietary phytoestrogens [445]. Recent comparison of gut microbiota in managed white rhinoceros suggests that the estrogenic activity of several abundant bacterial taxa and microbially-derived phytoestrogen metabolites could be contributing to reduced reproductive output in some females [446].

Due to the bidirectional nature of the microbiome-endocrine relationship, the microbiome has the potential to be critical to the assessment and management of wildlife reproductive health. Mueller and Sachs [447] outline a process by which microbiome research can be used to alter host performance. In the first instance, characterizing the microbiome and correlating microbial taxa with measures of host performance provides useful insight when different wildlife populations are compared (i.e. in situ vs. *ex situ*, breeding vs. non-breeding, and pregnant vs. non-pregnant). The next step is to infer functional properties of microbial taxa that differ between these populations and to determine whether the microbial composition can be manipulated via dietary changes or transfer of microbes between hosts [448–451]. Although more work is needed to determine the efficacy and feasibility of this approach, a more complete understanding of a healthy microbiome would be beneficial to many wildlife conservation efforts.

3.6. Immune function

Biomarkers of immune function have been growing in popularity for evaluation of both general health and reproductive fitness in wildlife species [43,452–456]. Research in humans and domestic animal species has investigated the role of the immune system in normal reproductive processes [457,458], reproductive pathologies [459,460], and reproductive failure [461].

Cytokines are protein mediators of the immune response that recruit and control the activity of various immune cells [462]. Cytokines also interact bidirectionally with hypothalamic and pituitary hormones of the HPA and HPG axes [463] and are regulated by steroid hormones such as progesterone [464]. In humans, pregnancy is characterized by a T helper 2 (Th2) dominated anti-inflammatory cytokine pattern [464], whereas women who experience recurrent miscarriage have elevated numbers of T helper 1 (Th1) cells which produce proinflammatory cytokines [465]. Cytokines also are important regulators of ovarian function, especially with respect to folliculogenesis, oocyte maturation [466], and luteolysis [467,468]. In the male, cytokines contribute to testes development and function [460] and are produced by both somatic and spermatogenic cells. Although examples of the use of cytokines to assess reproduction in wildlife are limited, a study in the gilthead seabream (*Sparus aurata*) [469] describes gene expression of several cytokines in the gonads. This study found that the cytokine levels differed according to reproductive stage which suggests these biomarkers could play a role in gametogenesis.

Another group of immune proteins that could be useful as indicators of wildlife reproductive health are acute phase proteins (APPs) which are part of the innate acute phase reaction [470,471]. Acute phase proteins such as serum amyloid A (SAA), C-reactive protein, and haptoglobin can be useful indicators of inflammation and have been used for the diagnosis and monitoring of reproductive disorders in dogs, cats, ruminants, and horses (reviewed in [472]). Other studies have begun to investigate SAA concentrations in reproductive processes, such as parturition [473] and artificial insemination [474]. However, before biomarkers such as APPs can be applied more widely, it is important to establish species-specific reference intervals [462]. Although most wildlife studies of APPs have focused on their potential application to the understanding of health and disease [475], one exception to this is the study of ceruloplasmin. Ceruloplasmin binds free copper in circulation and protects against free iron and free radical oxidation during inflammation [470]. In the giant panda, urinary ceruloplasmin is elevated from the first week of pregnancy until 20–24 days prior to parturition [476], making it a potentially useful biomarker for the diagnosis of pregnancy in this species. Additionally, recent research has found that APPs are quantifiable in saliva [477] which is useful matrix to be applied to wildlife studies.

4. Assessment of compounds related to reproductive dysfunction

It is impossible to understand and quantify threats to reproductive health without a comprehensive understanding of species-specific reproductive endocrinology and biology. Hormone analysis techniques can be used to describe normal reproductive endocrinology as well as to confirm the potential effects of threats to the reproductive health of wildlife.

4.1. Glucocorticoids (GCs) to monitor reproductive dysfunction

The measurement of GCs, principally cortisol and corticosterone, has been used widely as an indicator of HPA activity in most species [478] or of the hypothalamic-pituitary-renal axis in fish [479]. For many years, studies in humans, model species, and domestic livestock have examined the negative effects that chronic stress (and therefore increased amounts of GCs) has on reproduction. These studies indicate that stress can disrupt reproductive function at several levels [480,481] and both the timing and nature (including chronic vs. acute) of the stress response is important [482]. Physical [482], behavioral [483], social [484], nutritional [485], and environmental [486] stressors all lead to impaired

reproduction through activation of the HPA response.

In wildlife studies, GCs have been implicated in the disruption of reproduction across a range of taxa. Elevated fecal GC excretion due to social changes is associated with estrous cycle irregularity in female douc langurs (*Pygathrix nemaeus*) [287], and increased variability in GC concentrations have been reported in irregularly cycling black (*Diceros bicornis*) [328] and non-cycling white (*Ceratotherium simum*) rhinoceros [487]. In the pygmy rabbit (*Brachylagus idahoensis*), higher GCs during the breeding season were associated with lower reproductive success, both in terms of conception and litter survival [488]. Stress-related GC production also has a role in the suppression of subordinate reproduction in cooperatively breeding species, such as the meerkat (*Suricata suricatta*). Chronic elevation of GCs following eviction from the group by the dominant female is associated with reduced pituitary sensitivity to GnRH and increased abortion rates in this species [489]. Among avian species, egg quality (zebra finches (*Taeniopygia guttata*) [490], hatching date (yellow-legged gulls (*Larus michahellis*) [491], parental care (American kestrels (*Falco sparverius*) [492], and fledgling survival (yellow-eyed penguin (*Megadyptes antipodes*) [493] are all negatively associated with GC concentrations.

4.2. Other biomarkers of reproductive dysfunction

In addition to monitoring GCs, several other hormones have been measured to evaluate threats to the reproductive health of wildlife species, particularly in the context of exposure to endocrine disrupting chemicals (EDCs). EDCs are defined as ‘‘exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)- populations’’ [494]. EDCs are present in the environment due to human production, use, and disposal and are typically produced in agriculture (pesticides, herbicides), industrial manufacturing (waste, chemicals such as bisphenol A), and in the pharmaceutical industry (synthetic or bioidentical hormones such as 17α-ethinylestradiol). Most EDCs interfere with organisms’ metabolic pathways either by disrupting the normal pathways or due to the similarity between their chemical structure and that of endogenous hormones. For example, exposure to imidacloprid (a neonicotinoid insecticide) causes decreased reproductive hormone levels and testicular regression in a red munia (*Amandava amandava*). These effects in the red munia are due to the pesticide’s interference with thyroid function during a critical phase of reproductive development [495]. A recent study in adult zebrafish (*Danio rerio*) demonstrated that the herbicide metachlor interferes with the expression of HPG axis-related genes resulting in reduced levels of testosterone in females and reduced estradiol and testosterone in males [496]. Hayes et al. [497] confirmed that atrazine, the most common herbicide found in ground, surface, and drinking water, causes feminization of male gonads and decreased synthesis and secretion of androgens in several vertebrate classes. Rats exposed to industrial effluents of the leather industry for 20 days have decreased levels of gonadotropic hormones (LH and FSH) as well as developed hyperplasia of the seminiferous tubules [498].

17α-ethinylestradiol (EE2), a synthetic estrogen commonly used in oral contraceptives for women, mimics endogenous estradiol and, thus, produces feminization of male aquatic organisms and leads toward skewed sex ratios and disrupted population growth in fish and amphibian species [499]. For example, in a study by Mackenzie et al. [499], Northern leopard frog and wood frog tadpoles exposed to microgram concentrations of EE2 during larval development had intersex gonads, altered gonadal morphology, and an abnormal vitellogenic state at metamorphosis. These examples clearly demonstrate the potential of hormone analysis techniques to monitor cause-effect relationships of EDCs which negatively impact reproductive health. Scientists need a better understanding of the effects of both low dose and multi-EDC exposures both of which are challenging to determine because of critical knowledge gaps in the reproductive endocrinology of a wide range of wildlife species [500].

5. Techniques to measure biomarkers of reproductive function and health

5.1. Sample matrix extractions

Steroid hormones can be measured in a variety of matrices. However, proper sample preparation is essential to the acquisition of reliable and repeatable data. In blood samples as well as other matrices that contain proteins and lipids, steroids need to be isolated from matrix components that can interfere with antibody-antigen binding in immunoassays. Separation of steroid hormones is achieved through either liquid-liquid extraction (i.e. dichloromethane, diethyl ether) or solid phase extraction [501]. In some species with high serum fatty acid and lipid content a double extraction may have to be performed with a diethyl ether extraction followed by a hexane-methanol extraction to remove a maximum amount of lipid from the serum [502]. The most widely used method of steroid isolation from fecal samples involves mixing a specific amount of feces in an alcohol-water solution to create a steroid suspension [503]. A similar extraction approach is used with hair, feathers, and scales, but the sample must be washed and finely minced prior to extraction [504,505]. It is critical to conduct biochemical validation of the immunoassay with the extraction to ensure that the extraction approach is optimal for the sample type and species.

5.2. Immunoassays

Competitive binding immunoassays are the most widely used measurement technique in wildlife endocrinology. This technique was first developed in the 1950’s but did not become widely used until the 1980’s [17]. In this technique, both a fixed concentration of labeled ligand (tracer) and an unknown concentration of unlabeled ligand (sample) are incubated with an antibody and compete for binding sites. The amount of tracer bound to the antibody is inversely related to the amount of hormone in the sample and provides a quantitative measurement of hormone concentration. Radioimmunoassays (RIAs) were the first type of immunoassay developed and use radioactive molecules to label the tracer molecule [17]. More recently, enzyme-immunoassays (EIAs) were developed, using enzyme tags to catalyze a color change that can be easily quantified via absorbance (optical density). As the performance and sensitivity of EIAs has improved, there has been a shift away from the use of RIAs due to the hazards of working with radioactivity. Immunoassays are cost-effective, and the high demand has resulted in a plentiful supply of easy-to-use kits. However, separate assays need to be conducted for each target hormone of interest. Therefore, for studies that evaluate more than one hormone, other techniques such as mass spectrometry, may be more appropriate. Furthermore, immunoassays do not allow accurate identification of specific molecules in the sample.

5.3. (U)HPLC for enzyme immunoassay validation

In terms of planning assisted reproduction, endocrine monitoring is often urgent. Therefore, non-invasive endocrine monitoring is generally based on the previously described, cost-effective EIAs [41,506]. However, one of the biggest challenges of non-invasive monitoring with EIAs is that both the metabolism and route of excretion of hormones are species- and hormone-specific [21]. For example, Wasser et al. [507] found that estradiol metabolites are almost exclusively excreted in urine, while progesterone metabolites are excreted in both urine and feces, in the African elephant (*Loxodonta africana*). In the white rhinoceros (*Ceratotherium simum*), however, most progesterone is excreted via urine [508], while in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) progesterone is almost exclusively excreted via feces [509]. The same level of variation has been reported in metabolism as Pribbenow et al. [510] detected certain fecal GC metabolites in the Iberian lynx, but

not in the Eurasian lynx. Furthermore, different types of GC metabolites were found between male and female Iberian lynx indicating possible sex-specific variation. The latter point clearly illustrates that any EIA used for endocrine monitoring needs to be fit-for-purpose and validated across every single (sub)species and matrix [41,48].

(Ultra) high performance liquid chromatography ((U)HPLC) has been used as a tool for EIA validation and is particularly powerful in combination with radiometabolism studies. In the past, species specific-metabolism and -excretion of steroids (and prostaglandins) was confirmed by radio-metabolism studies in which radio-labeled isotopes (^{14}C and or ^{3}H) were injected followed by regular sampling of blood, urine and feces [511–513]. Subsequently, high performance liquid chromatography (HPLC) was applied to collected fractions based on the elution (retention time) of steroid and prostaglandin standards (including their (conjugated) metabolites). These fractions were then subjected to EIAs with relevant cross-reactivities for identification. The latter technique is currently still applied for EIA validation (specificity) but suffers from a lack of accurate compound identification as this is based on merely retention time with no orthogonal confirmation (no detection source directly coupled to the HPLC). Different potentially immunologically cross-reacting steroid metabolites or other cross-reacting molecules may elute within the same fraction and hamper correct molecular identification.

5.4. Mass spectrometry: validated identification and quantification of metabolites

Correct identification of metabolites, particularly those with similar structures eluting in the same HPLC fraction, can be significantly improved if a mass spectrometer (MS) is hyphenated as a detector. Similar compounds can then be discriminated based on their accurate mass (high resolution, HR) and/or fragmentation patterns (MS/MS). Mass spectrometer devices can be coupled to both gas chromatography and liquid chromatography (LC). Gas chromatography is applied primarily in the context of pheromone research [260]. Steroids also can be examined via gas chromatography but due to the need for elaborate derivatization steps, (U)HPLC has rapidly gained popularity for the analysis of non-volatile small molecules, including steroids [514].

Depending on the set-up, (U)HPLC-MS can be used for analysis of small metabolites (metabolomics) or larger peptides/proteins (proteomics) [515,516]. While investigation of small metabolites in non-invasive matrices such as urine and feces has high potential to unravel new biomarkers for reproductive monitoring and health at the species level, non-invasive matrices (particularly feces) hold more challenges for proteomics research, due to their enzymatic capacities, including bacterial degradation [517]. (U)HPLC-MS applications can be used for targeted and untargeted screening, with each of those approaches benefiting from specific MS equipment [515,518]. In most cases, targeted screening will be more sensitive (optimized for the detection and quantification of a limited number of known compounds) whilst untargeted screening will allow a broader screening of a variety of unknown compounds that can be identified, but not quantified in the same run. Untargeted analysis is often referred to as ‘metabolomics’. Metabolomics is a promising research tool that has demonstrated great potential for the discovery of biomarkers and the unraveling of pathways from (patho)physiological alterations. Indeed, identification of the metabolome of a species characterizes all small molecules present in cells, tissues, and body fluids and offers the most accurate reflection of an organism’s (patho)physiological status as opposed to other ‘omics’ techniques. When the focus of an untargeted approach is restricted to steroid hormones, steroid metabolites, and downstream molecules, the analytical approach is termed ‘steroidomics’ [519,520]. While studies employing targeted analysis, including the optimization of multi-compound methods, are becoming more common in wildlife reproduction [521,522], untargeted metabolomics is still relatively unexplored in this field.

Most MS-based studies relate to research in marine mammals, mainly in the context of environmental contaminant (i.e. endocrine disruptors) screening [523,524]. Recently, the number of MS-based studies including other wildlife species has been steadily increasing. Santamaria et al. [525] conducted untargeted metabolic profiling of fecal cortisol metabolites, which facilitated proper EIA selection to support future studies on non-invasive assessment of stress in Koalas. A more targeted approach was used by Kothmann et al. [440] to identify glucocorticoid, progesterone, and androgen metabolites in feces of the southern white rhinoceros and the information was used to cross-validate several EIAs for non-invasive endocrine monitoring. Similarly, Azevedo et al. [526] succeeded to cross-validate targeted LC-MS/MS with EIA for the identification and quantification of hair sex steroid and glucocorticoid metabolites in Iberian lynxes.

Applying a (U)HPLC- steroidomics - workflow for compound identification per species and per matrix, prior to the selection of immunoassays, is indeed a powerful tool to exclude erroneous EIA validation by disclosing information on the identity and (changing) abundance of compounds present in the respective and contrasting research samples. MS-based gold standard identification techniques are unfortunately largely inaccessible for wildlife endocrinology laboratories and assay validation is therefore still a well-known pitfall in noninvasive wildlife endocrine monitoring. The absence of accessible MS-technologies in wildlife endocrinology is indeed an important shortcoming which highlights that: 1) even when (U)HPLC fractionation is used to study the immune response of assays, it is never indisputably confirmed what signal is being picked up by the assay; 2) immunoassay selection is often based on trial-and-error due to scant knowledge of compounds per species and matrix; 3) analytical and biological validation does not always confirm the validity of an immunoassay; 4) cross-reactivity for immunoassays is only described for those compounds that have been included in the validation; and 5) pathway elucidation based on immunoassays is challenging and may even lead to erroneous conclusions [527]. Yet, replacing EIA by MS analysis is not an option because most MS-based methods are not feasible in the field, and MS methods are more expensive than immunoassays. These factors support the choice for EIA as the primary tool for evaluation of hormones in wildlife endocrinology. The optimal EIA is chosen based on the research question and after accurate cross validation of the relevant metabolites present in a species and/or matrix. Advanced analytical validation can be achieved in the future by subjecting the carefully separated immunoreactive fractions obtained by (U)HPLC to an MS-based steroidomics HR-MS/MS method, thereby guaranteeing accurate identification of the immunoreactive compounds in a specific species and matrix. Installation of an MS platform in non-invasive wildlife endocrinology would offer a necessary tool for cross-validation of antibody and EIA specificity in the field of wildlife monitoring.

5.5. Reverse transcription quantitative PCR and microarrays

Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a highly sensitive molecular technique that amplifies trace amounts of specific DNA fragments in a sample pool of DNA [528]. Most RT-qPCR is conducted using reverse transcribed cDNA from RNA extracted from the sample of interest. As RNA is translated into proteins, measurement of RNA provides a more functional assessment of the gene activity in a sample than extracted DNA. DNA primers with defined sequences complementary to a specific gene of interest are utilized as a template upon which a DNA product is built by a DNA polymerase enzyme [529] using free nucleotides. The amount of product made within a set period of amplification time is correlated directly to the amount of the gene of interest in the sample such that samples with ample amounts of the gene of interest will produce product more quickly to reach threshold than samples with scant amounts of the gene of interest. RT-qPCR utilizes fluorescence to detect the size of the DNA product in each well of a plate. The most common fluorescent systems

utilized in RT-qPCR are SYBR Green I, a cyanine dye with (CYN) with an excitation of 497 nm and an emission of 552 nm, and TaqMan®, a 6-carboxyfluorescien (FAM) with an excitation of 493 nm and an emission of 517 nm [530]. TaqMan differs from SYBR Green I in two main ways: 1) the fluorophore is attached to the 5' end of the specific probe-primer for the gene of interest; 2) a quencher is attached to the 3' end of the specific probe-primer for the gene of interest. Thus, the primers upon which gene products are built in the TaqMan® system are inherently part of the fluorophore. SYBR Green I [531], on the other hand, requires the design of primers specific not only to the gene of interest but also the species of interest. While the TaqMan® system is more sensitive and specific, it is not applicable to a wide range of species because probe-primers are only routinely available for predetermined, primarily domestic species [531].

Quantification of gene expression levels in a sample can be conducted via two main approaches: 1) absolute quantification with a standard curve [532] or 2) relative quantification to one or more endogenous housekeeping gene(s). While absolute quantification is ideal as it provides a more accurate assessment of copy number for the gene of interest, it is labor intensive, and results are only as good as the standards utilized in the assay. Standards can consist of PCR-amplified target sequences from DNA pool from the same sample type [533], plasmids with the target sequence [534], or commercially prepared DNA [535]. The cycle values for each sample are related to the cycle values of the standard curve to determine actual copy numbers for target genes [536]. The standard curve is generated by a correlation coefficient (R^2) of plotted points. For the reasons mentioned, relative quantification is the more common approach to quantification of gene expression levels. The validity of results generated via relative quantification is highly dependent on the selection of appropriate housekeeping gene(s) [537]. An ideal housekeeping gene has similar copy numbers irrespective of treatment condition within a given sample type. Some commonly employed housekeeping genes include beta actin (ACTB), glyceraldeyde-3-phosphate dehydrogenase (GAPDH), ribosome small subunit (18S) ribsosomal RNA (rRNA), Ubiquitin C (UBC), hypoxanthine guanine phosphoribosyl transferase (HPRT), succinate dehydrogenase complex, subunit A (SDHA) and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) [538, 539]. As many of these common housekeeping (control) genes can vary in expression under certain circumstances, one should always verify that a potential housekeeping gene is stable for a particular RT-qPCR experiment by testing a pool of cDNA from the given sample type [507]. Of particular note, RNA levels of GAPDH are androgen-dependent [540] which is of importance to studies of reproductive biology. Relative quantification is calculated by the 2-ΔΔCt method wherein the difference between the number of cycles of the target gene and the housekeeping gene for a treated sample (ΔCt) is compared to the difference of the target gene and the housekeeping gene for a control sample (ΔCt) [541]. Results are expressed as the fold difference of the gene of interest relative to the housekeeping gene. While relative quantification is an easier approach to RT-qPCR analysis than absolute quantification, drawbacks include that common reference genes may not be consistently expressed in all samples and that the technique assumes a PCR efficiency of 100% which is rare [542]. One way to improve the potential bias of this technique is to utilize more than one housekeeping gene and average the gene fold differences relative to each housekeeping gene.

RT-qPCR be performed in a 96 or a 384 well plate. Use of a 384 well plate decreases the cost of reagents and uses less samples as the total reaction volume is lower than that required for a 96 well plate. However, for each sample on the plate the gene of interest and the housekeeping gene must be run in triplicate. Therefore, there is relatively little information generated for reagent, time, and sample utilized. One solution to this problem is the implementation of microarrays. Microarrays are 96 well plates with stabilized species-specific gene primers in each well for genes of interest related to a particular disease or organ system (i.e. diabetes, cancer) as well as several housekeeping genes. The main advantage of microarrays is the large number of genes analyzed per sample. However, the disadvantages are that they are expensive and each 96 well plate only generates data for a single sample. With respect to wildlife, microarrays are not applicable unless they are custom made which is costly. However, microarrays that measure genes of interest specific to infectious diseases could be useful as the genes would be specific to the disease and not the animal species.

5.6. Sequencing

5.6.1. Next Generation Sequencing

With the advent of annotated species-specific genome assemblies, Next Generation Sequencing (NGS), which is commonly done with RNA but also can be conducted with DNA, took off at the turn of the 21st century. As compared to microarrays, RNA-sequencing (RNAseq) permits full sequencing of the whole transcriptome which allows the researcher to examine splice variants as well as non-coding transcripts such as miRNAs and long non-coding RNA (lncRNA) [543]. Such data not only gives a more complete picture of a specific individual animal's particular gene expression profile but also can provide mechanistic insights into disease status, genetic lineage, sensitivity to certain therapeutics, and mutations [544]. RNAseq characterizes the transcriptome for a biological sample during a set time for a particular physiologic state. Advantages of RNAseq include: the ability to quantify the transcript expression level of all genes simultaneously, the low amount of RNA required, high throughput, an extremely high resolution down to single base pairs, and the ability to distinguish different gene isoforms [544]. The main disadvantages to RNAseq are: the cost per sample, the need for specialized bioinformatic analytical skills for analysis and interpretation, and, with specific reference to wildlife species, the reliance on genomic sequence. As of July 2023, 30,131 species or 10% of known species have had their genome sequenced [545] and made available in the National Center for Biotechnology Gene Database [546, 547]. Most species in this database are vertebrate species (769) which is 32 times more gene assemblies than there are in the database for arthropods [545]. Although genome assemblies exist for over 3000 species, only 32.4% of these genomes are annotated which limits their utility for RNAseq data set analysis. It is imperative that wildlife research continues to push for the generation of genome assemblies not only for more species but for underrepresented taxa. However, to support such endeavors both the total amount of money and the money per an award must increase in support of wildlife research projects. Many scientists have banded together in support of such efforts and have resorted to crowd-funding approaches. One such example is Genome10K [548] out of which Vertebrate Genomes Project (https://vertebrategenomesproject.org/) [549] developed. The Vertebrate Genomes Project began in 2017 with the goal of creating genome assemblies for all 71, 670 vertebrate species and is primarily funded by donations from the public. However, invertebrates lack such platforms in support of genome assembly development. Government and foundation sources of funding must work to support genome research for invertebrate species.

One interesting application of sequencing technology to the wildlife conservation sphere is the measurement of environmental DNA (eDNA) (reviewed in [550]). Historically, monitoring of biomarkers in wildlife has been conducted on a single animal- or species-specific basis. However, such monitoring approaches make it difficult to assess biodiversity in an ecosystem or to conserve the entire ecosystem. Obtaining information from whole communities of organisms through retrieval of eDNA may hold the key to solving many challenges with biodiversity assessment [551]. eDNA can originate from skin [552], feather [553] and scale sloughs [554,555], urine [556] or decomposing organisms [557]. Once collected, samples of soil and water are extracted, genetic material is amplified by polymerase chain reaction, and DNA sequencing is performed. Sequencing of genetic material across taxonomic groups from soil or water samples enables identification of which organisms are (or were) in the environment. However, one caveat to this approach is that

organisms' genetic material must have been previously sequenced and identified. Additionally, without concurrent carbon dating of the organic material in samples it may be difficult to ascertain through eDNA alone whether the species identified are current as opposed to historical residents in an ecosystem [558]. Nevertheless, eDNA has been instrumental to answering research questions across a wide range of fields from ecology to paleontology.

5.6.2. Single cell sequencing

Single cell sequencing applies NGS techniques to the analysis of RNA or DNA expression levels in individual cells within a sample type to provide knowledge about each cell's function in the context of the microenvironment from which it is derived [559]. Single cell sequencing is especially useful for analysis of heterogenous samples, phenotypes conferring mosaicism, and microbes that cannot be cultured. Single cell RNA sequencing (scRNAseq) is preferred over bulk NGS RNAseq, because it enables examination of the cell-to-cell interactome and it delineates differences between individual cells within mixed cell populations [560]. As of 2022, scRNAseq is the gold standard for describing cell phenotypes [561]. scRNAseq requires isolation and capture of single cells, cell lysis, reverse transcription of the RNA to cDNA, cDNA amplification and library preparation followed by application of NGS. Due to the amount of RNA material in each cell, it is impossible to obtain information on every RNA in each cell. However, one can uncover gene expression patterns through analytical tools for gene clustering analysis [562]. It is also possible to perform single nucleus RNA sequencing (snRNAseq) which is especially useful for cell types without a lot of cytoplasm such as adipocytes. snRNAseq also can be applied to frozen samples and minimizes artificial transcriptional stress responses which are common in scRNAseq [563].

With respect to reproductive biology, heterogeneous tissues of multiple different cell types such as the endometrium, ovary, and testis are ideal candidates for scRNAseq or snRNAseq. Additionally, studies of spermatogenesis in mice and humans have benefited from delineation of the transcriptome changes that occur throughout this process [564]. One drawback to the application of scRNAseq in wildlife species is lack of genome annotation and existence of single cell atlases. A single cell atlas is a comprehensive profile of all genes in all cell types from a given organism [565]. With the increasing number of zoonotic infectious diseases, such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 or COVID19), whose origins can be traced to wildlife species, there has been an increased push to develop single cell atlases for potential wildlife reservoirs [566]. However, to date most of the scRNAseq performed in non-domestic animals has been limited to invertebrate species and plants [567–569]. There is great potential for the application of scRNA or scDNA sequencing to wildlife species, especially with respect to both infectious and non-infectious diseases.

5.6.3. 16 s rRNA sequencing

16 s ribosomal RNA sequencing is a culture-free way to compare bacteria and characterize the microbiome in a sample. 16 s rRNA sequencing characterizes both the phylogeny and taxonomy of microbes in a variety of samples including but not limited to feces, saliva, milk, vaginal secretions, and semen [570]. 16 s sequences are clustered based on operational taxonomic units (OTUs) and OTU sequences are then compared with reference databases to identify bacterial taxa [571]. Bioinformatics analysis with sophisticated computational packages is required to process the OTUs and determine the taxonomic diversity as well as species richness [572]. Despite the requirement for specialized approaches to data analysis and unlike some of the other sequencing types described above, there has been widespread application of 16 s rRNA sequencing to wildlife species including a wide variety of invertebrate species, white and black rhinoceros, giant panda, non-human primates, lemurs, Tibetan antelope, peccaries, amphibians, lizards, birds, and rodents [429].

6. Future directions: a multifaceted approach to wildlife reproductive evaluation through adaptation of biomarkers and techniques used in humans and domestic animals

Although measurement of sex steroid and protein hormones is commonly used for pregnancy diagnosis in domestic species, there are limitations to these approaches in wildlife species. This is particularly true of species where pregnant and non-pregnant luteal phases are indistinguishable for part or all of gestation (i.e. pseudopregnancy) or differentiation requires serial/longitudinal or invasive sampling. Additional reproduction-specific biomarkers that yield earlier or clearer diagnosis of pregnancy would be beneficial to management of wildlife species and would provide additional insight into reproductive processes. In ruminants, pregnancy specific protein B, a glycoprotein produced by cells within the trophectoderm of the embryo, can be measured in blood from day 24–28 of gestation in cattle [573,574] and day 21 in ewes [575]. Several studies in wildlife have taken a discovery approach to identify pregnancy-specific biomarkers, including the polar bear (*Ursus maritimus*) [576] and American mink (*Neovision vision*) [577]. In cheetah, the immunoglobulin J-chain is upregulated in pregnant cheetah within 4 weeks of mating [578,579]. In a variety of felid species fecal prostaglandin metabolites are used to distinguish pregnancy and pseudopregnancy [580].

Another area of wildlife reproductive health that would benefit from additional biomarkers is contraception. Although many products are used to manage reproduction and maintain sustainable population numbers while minimizing inbreeding, contraceptive options available to zoos are not specifically designed for use in wildlife species. Therefore, it is important to understand efficacy, latency to and duration of effect, and time to reversal (i.e. resumption of cyclicity, ovulation and pregnancy) [581]. In several species, the reversal of contraceptives can be highly variable, so techniques to measure active compounds, such as deslorelin [582] or synthetic progestins, could be beneficial to management of captive wildlife populations.

As our understanding of reproductive function in wildlife species expands through continued research efforts, future efforts to evaluate reproductive health will require an integrated approach which includes measurement of biomarkers of metabolism, host microbiome, and immune function. Foundational studies have identified the physiological significance of these biomarkers in the context of reproduction. However, there are a paucity of comprehensive studies of reproductive health in wildlife that incorporate these biomarkers in the context of reproductive parameters. To support integration of markers of metabolic and immune function into reproductive health assessments of wildlife species, scientists in the field must establish species-specific references for each of these biomarkers and analyze these alongside relevant traditional reproductive biomarkers. Due to the synergistic nature of the reproductive system and physiologic processes associated with metabolism, immune function and microbiome health, future studies should consider measurement of these biomarkers as important as measurement of gonadotropins and sex steroid hormones.

Specifically, in the case of metabolic biomarkers like leptin, insulin, glucocorticoids, and thyroid hormones, results in wildlife species demonstrate that energetic and reproductive biological processes are intricately linked. The intimate connection between reproduction and metabolism is supported by the fact that one system exerts effects on the other. Reproduction requires available metabolic fuel to proceed and reproductive hormones are significant contributors in how energy is made, stored, and used during numerous reproductive events [485]. Therefore, metabolic biomarkers as indicators of metabolic function should be of substantial interest to wildlife scientists evaluating the reproductive health of wildlife populations. Because reproductive dysfunction could be either a cause or symptom of the body trying to acquire, store, or use metabolic fuels, establishing species-specific reference ranges of these metabolic biomarkers is a high priority for future wildlife theriogenology studies.

Table 1
Biomarkers of reproductive health in wildlife.

Biomarker	Sample types	Information derived from biomarker	Measurement techniques	Limitations	Citations
Steroid Hormones					
Androgen metabolites	serum, plasma, urine, saliva, feces, hair, nails/horn, feathers, baleen	1) reproductive status - males 2) adrenal function	1) radioimmunoassay 2) enzyme immunoassay 3) high performance liquid chromotography 4) liquid chromatography tandem mass spectrometry 5) ultraconvergence chromatography tandem mass spectrometry	1) most useful if samples of serum/plasma, urine, feces, and saliva assessing steroids are collected 3–5 times per week from focal individuals which can be difficult to do in wildlife 2) steroid concentrations from hair/nails/horn/feathers/baleen can be difficult to interpret in the context of time without knowing how long the given sample was growing prior to collection	[18];[22];[42];[48–80];[214];[239–249];[473–494]
Estrogen metabolites	serum, plasma, urine, saliva, feces, hair, nails/horn, feathers, baleen	evidence for follicular maturation			
Glucocorticoids	serum, plasma, urine, saliva, feces, hair, nails/horn, feathers, baleen	adrenal response to stress			
Progesterone metabolites	serum, plasma, urine, saliva, feces, hair, nails/horn, feathers, baleen	1) evidence for ovulation and corpus luteum formation 2) estrous cycle length and characteristics 3) pregnancy diagnosis: felids, giraffe species			
Protein Hormones					
Anti-Mullerian Hormone	serum, tissue samples	1) follicular reserve 2) testicular maturation	1) radioimmunoassay 2) enzyme immunoassay 3) liquid chromotography tandem mass spectrometry	can only be measured in serum, urine (LH/FSH), or tissue samples which limits utility in free ranging wildlife as these types of samples are hard to collect in such populations	[18];[22];[42];[81–194];[214];[229–238];[473–494]
Follicle Stimulating Hormone	serum, urine	1) role of pituitary in follicular growth 2) role of pituitary in Sertoli cell development and spermatogenesis			
Inhibin	serum, tissue samples	1) function of ovarian granulosa cells or FSH suppression required for normal follicular cycling in females 2) function of Sertoli cells and subsequent spermatogenesis in males			
Insulin	serum	elevations in insulin are correlated with infertility in captive housed wildlife			
Leptin	serum	1) increases during pregnancy in many species 2) directly correlated to body fat quantity as it is an adipokine			
Luteinizing hormone	serum, urine	1) surge in this hormone is correlated with evidence for ovulation 2) role of pituitary in Leydig cell secretion of testosterone			
Oxytocin	serum, urine, saliva	1) maternal and paternal behavior & bonding with offspring 2) negatively correlated with aggression in animal species that live in social groups 3) milk letdown			
Prolactin	serum	Species specific roles in reproduction: 1) rodents - luteotropic 2) felids/canids/elephants - luteotropic & pregnancy maintenance 3) African elephant - follicular development, but also linked to acyclicity (hyper or hypoprolactinemia 4) kangaroo - embryonic diapause 5) all species - milk production			
Relaxin	serum, urine	1) corpus luteum function: swine, rats 2) placental function: felids, canids, lagomorphs, equids 3) pregnancy diagnosis: felids, elephants			
Miscellaneous Hormones					
Melatonin	serum, saliva	1) correlated with seasonal reproduction 2) light pollution drives down serum	1) radioimmunoassay 2) enzyme immunoassay	1) can only be measured in serum, feces (T3/T4, prostaglandin metabolites), saliva (melatonin)	[18];[22];[42];[196–228];

(*continued on next page*)

Table 1 (*continued*)

Biomarker	Sample types	Information derived from biomarker	Measurement techniques	Limitations	Citations
		melatonin levels and interferes with reproduction	3) liquid chromotography tandem mass spectrometry	which limits utility in free ranging wildlife as these types of samples are hard to collect in such populations	[285–291]; [473–494]
Prostaglandins	serum	1) corpora lutea establishment 2) maintenance of pregnancy/ placental function 3) changes in concentrations related to onset of parturition 4) onset of spawning and quality of eggs			
	Triiodothyronine (T3) / thyroxine (T4)	serum, feces			
	1) basal metabolic rate 2) seasonal regulation of GnRH secretion				
Emerging Biomarkers					
Acute Phase Proteins	serum, plasma	non-specific markers of inflammation including: -serum amyloid A (SAA) -C reactive protein -haptoglobin -ceruloplasmin	1) radioimmunoassay 2) enzyme immunoassay 3) fluorescent immunoassay 4) liquid chromotography tandem mass spectrometry	1) primarily measured in serum or plasma which limits utility in free ranging wildlife (although ceruloplasmin in urine) and saliva in domestic species (i.e. pigs) so potential for expanding options for wildlife	[18];[22];[42]; [292–433]; [473–530]; [550–558]
Cytokines	serum, plasma, tissue	1) pregnancy specific patterns 2) regulate folliculogenesis 3) control oocyte maturation 4) contribute to luteolysis 5) contribute to testis function & development	1) radioimmunoassay 2) enzyme immunoassay 3) fluorescent immunoassay 4) liquid chromotography tandem mass spectrometry 5) immunohistochemistry	1) primarily measured in serum, plasma or tissue which limits utility in free ranging wildlife. Urinary measures are possible, but would require validation 2) some methods of detection are costly	
Microbiome	feces, milk, semen, vaginal secretions, tissue	1) immune status 2) relative stress an animal is under at the microbial composition is correlated with glucocorticoid levels 3) for GI microbiome both related to estrogen in circulation and dietary estrogens	16 s sequencing	1) costly to perform sequencing 2) need to know how to perform bioinformatics analysis or have a collaborator that has this expertise	
microRNAs	serum, plasma, milk, urine, semen, tissue	1) related to fecundity 2) correlated with stage of estrous cycle 3) inflammation (i.e. endometritis) 4) pregnancy detection 5) correlated with spermatogenesis	1) qPCR 2) microarrays 3) RNA sequencing	1) costly to perform sequencing 2) need to know how to perform bioinformatics analysis or have a collaborator that has this expertise if running sequencing 3) for qPCR need to design primers which could be challenging because of differences in gene homology between domestic species and wildlife	
eDNA	soil, water	which organisms comprise the biodiversity of a specific ecosystem	qPCR coupled with DNA sequencing	1) costly to perform sequencing 2) to identify organisms in the ecosystem their genome has to have been previously sequenced and identified 3) cannot differentiate between current or historical ecosystem residents unless carbon dating techniques are coupled with the DNA sequencing	
Reactive oxygen species	serum, plasma, saliva, exhaled breath condensate, semen, IVF/IVM media, tissue	1) correlated to normal ovulation 2) made by a healthy corpus luteum 3) correlated with normal sperm capacitation and acrosome reaction 4) elevated levels in males correlated with sperm DNA damage	1) chemiluminescence or fluorescence 2) chromatography 3) fluorescent proteins 4) electron spin resonance. 5) spectrophotometry 6) flow cytometry or confocal microscopy with fluorescent dye DAF-FM	1) costly methods of detection 2) specialized equipment needed for detection 3) unique expertise needed for some of detection methods	

Similarly, studies suggest that host microbiomes, particularly those in the gastrointestinal tract, and reproductive processes work in a bi-directional relationship. Understanding of species-specific differences and the relationship between the host microbiome and reproductive state is in its infancy in the wildlife reproduction field. Knowledge of the functional role of existing microbial taxa is paramount to understanding associations between microbiota and reproductive biology. Of particular interest is the difference in gastrointestinal microbiomes between managed and free-ranging animals which is likely due to significant dietary variation between these two types of populations. Such

differences in the microbiome may have a critical role in the infertility in many managed species of conservation interest [430]. Future investigations should not only examine microbial communities alongside traditional biomarkers of reproductive health but also work to understand if manipulation of specific microbial species could promote reproductive success.

As a biomarker of immune function, cytokines have a similar bidirectional relationship to reproduction like metabolic and microbial biomarkers. Cytokines can modulate gonadal function and are themselves regulated by hormones during different reproductive events [460, 463]. A reproductive evaluation that does not incorporate cytokines could miss information potentially vital to characterizing an animal's health and reproductive potential. However, both cytokines and acute phase proteins require more information on species-specific reference intervals before their utility in reproductive assessments can be fully realized.

Increasingly, the health of the free-ranging populations of wildlife species is severely impacted by land transformation, urbanization, climate change, and other anthropogenic disturbances. Thus, conservation efforts for species will depend heavily on accurate assessment of health, including measurements of reproductive rate, population growth, occurrence and spread of diseases, and measures to control reproduction. Future research endeavors should focus on development of new biomarkers and sensitive, precise, and non-invasive methodologies for the assessment of reproductive health of species in natural and free-ranging conditions. Integration of biomarkers of metabolic and immune health into comprehensive reproductive assessments is also critical to the advancement of the wildlife theriogenology field and to the conservation of wildlife species (Table 1).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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