Using an on-site laboratory for fecal steroid analysis in wild white-faced capuchins

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ABSTRACT

Hormone laboratories located "on-site" where field studies are being conducted have a number of advantages. On-site laboratories allow hormone analyses to proceed in near-real-time, minimize logistics of sample permits/shipping, contribute to in-country capacity-building, and (our focus here) facilitate cross-site collaboration through shared methods and a shared laboratory. Here we provide proof-of-concept that an on-site hormone laboratory (the Taboga Field Laboratory, located in the Taboga Forest Reserve, Costa Rica) can successfully run

endocrine analyses in a remote location. Using fecal samples from wild white-faced capuchins (*Cebus imitator*) from three Costa Rican forests, we validate the extraction and analysis of four steroid hormones (glucocorticoids, testosterone, estradiol, progesterone) across six assays (DetectX® and ISWE, all from Arbor Assays). Additionally, as the first collaboration across three long-term, wild capuchin field sites (Lomas Barbudal, Santa Rosa, Taboga) involving local Costa Rican collaborators, this laboratory can serve as a future hub for collaborative exchange.

KEY WORDS

androgens, cortisol, estradiol, estrogens, field laboratory, glucocorticoids, progesterone, progestogens, testosterone, validation

INTRODUCTION

Our ability to extract steroid hormones from the fecal samples of wild animals has revolutionized the kinds of questions we can answer in the field of wildlife behavioral endocrinology. For example, fecal hormones provide a relatively easy and non-invasive approach for monitoring hormone trajectories in wild subjects where darting or capture is not possible or ethical. Fecal steroids are not without limitations, and researchers should consider these carefully when adopting this method (Palme, 2005). For example, the fecal steroid concentrations obtained directly from immunoassay kits are not absolute measures of circulating steroids. Rather, they are relative measures that are specific to that species and substrate (i.e., feces), the hormone extraction method, storage time, and the hormone assay. Even for the same species, hormone values obtained from different extraction methods and different immunoassays cannot be compared across studies. Because different field sites have adopted different methods

(based on site limiations or a history of using particular method), it is difficult to make cross-site comparisons within a species. One obvious solution is for studies on the same species to follow the same protocol for extraction and analysis (and, ideally, within the same laboratory). With this manuscript we move closer to this goal by validating six different steroid hormone assays for use in a single taxon, the white-faced capuchin (*Cebus imitator*), located at three different research sites in Costa Rica. Although we carry out various steps of these validations at two different laboratories (one in the U.S. and one in Costa Rica), the primary objective is to eventually have all hormone analyses take place at the laboratory in Costa Rica for these projects. An in-country laboratory (i.e., located in the same country as the species under study) can facilitate cross-site hormone and behavior comparisons due to shared equipment, protocols, and analyses.

Moreover, there are several additional benefits to shifting analyses to an in-country laboratory. First, this eliminates the need for export/import permits and minimizes the logistics and expenses of sample shipment and preservation in-transit (since samples typically must remain frozen). Second, for in-country laboratories located directly "on-site" (i.e., located at the same site where samples are being collected), researchers can harness daily measures of hormones (and other biomarkers) to guide same-day or next-day behavioral observations on individuals with known or suspected physiological states. Most field endocrine studies are unable to obtain measures for fecal hormones until months, sometimes years, later. Finally, an on-site laboratory can facilitate capacity building, since local research assistants can be trained on-site in hormone extraction, measurement, analyses, and troubleshooting. This has the potential to move the entirety of the scientific process, from sample collection to write-up, to the country where the work is being conducted. With this manuscript, we focus primarily on the

86 cross-site collaboration made possible when all researchers collect and analyze samples using
87 similar methods.
88 Here, we introduce an on-site laboratory, the Taboga Field Laboratory, located in the
89 Taboga Forest Reserve, Costa Rica. This laboratory abuts the Taboga Forest (outside of Cañas,
90 Costa Rica), largely characterized by seasonally dry tropical forest, featuring a closed canopy
91 and seasonal deciduousness (Janzen, 1988; Miles et al., 2006). The Taboga Forest is home to two
92 non-human primates, white-faced capuchins (*Cebus imitator*) and mantled howler monkeys
93 (*Alouatta palliata*), often found in the trees surrounding the laboratory itself. The Taboga Forest
94 is the home of the Capuchins at Taboga research project – investigating the cognition,
95 endocrinology, and behavior of wild white-faced capuchins. Facilitating collaborative efforts, the
96 Taboga Field Laboratory is located less than 100 km from two other long-term field sites
97 studying white-faced capuchins, the Lomas Barbudal Monkey Project and the Santa Rosa
98 Primate Project. This publication represents the first collaborative effort across all three sites. At
99 present, our knowledge of steroid hormone production and steroid metabolism in white-faced
100 capuchins is fairly limited, and most of what we know derives from wild studies on fecal
101 hormones.
102 Our primary objective was to validate six commercial steroid hormone assays in white-
103 faced capuchins (all assays are available from Arbor Assays), including assays from two
104 different glucocorticoids assays (DetectX®, ISWE), two different androgens assays (DetectX®,
105 ISWE), one estrogens assay (DetectX®), and one progestogens assay (DetectX®). All three long-
106 term capuchin sites have a history of collecting fecal samples for the extraction and measurement
107 of steroid hormones; and all three sites use similar extraction protocols (extraction and storage in
108 ethanol, EtOH), which will facilitate comparative studies in behavioral endocrinology for this

taxon. We specifically selected Arbor Assays as our supplier for three reasons. First, they have been extremely flexible in working with us to ship reagents internationally, including helping us make our own stop solution in-country, since this component is designated as "corrosive" and not easily shipped internationally (this is why we added the ISWE version to our validation for GCs and T). Second, they often troubleshoot their assays under less-than-perfect "field" conditions, which helps ensure their assay kits work under low-budget conditions. And, third, we have found Arbor Assays to provide generous technical help when helping us troubleshoot our assay results or shipping the reagents to the site.

Behavioral endocrinologists working with fecal hormones from wild populations routinely measure four broad classes of steroid hormones: glucocorticoids, androgens, estrogens, and progestogens. Fortunately, steroid hormones show up in the fecal samples of most vertebrates in smoothed proportions relative to recent episodic fluctuations in the bloodstream (Palme, 2005). These are some of the most useful hormones to accompany behavioral monitoring in wild animals. Glucocorticoids track metabolic, social, and other environmental challenges that demand energy (Beehner et al., 2005; Creel et al., 2013); they are often called "stress hormones" because secretion increases in response to stressful stimuli (although this term has been criticized (MacDougall-Shackleton et al., 2019)). Androgens track male development, reproduction, and challenges to reproductive success (Hau, 2007; Pappano and Beehner, 2014; Schoof et al., 2014); most studies focus on testicular secretion in males (Muller, 2017) although females secrete androgens as well (Hammes and Levin, 2019). Estrogens and progestogens track female development and reproduction (Beehner et al., 2006; Deschner et al., 2004; Möhle et al., 2005).

However, because steroids excreted in fecal matter are highly metabolized downstream products (and because different taxa metabolize hormones differently (von der Ohe and Servheen, 2002)), it is critical that all methods for extracting and measuring hormones be validated using fecal samples for each species of interest (Touma and Palme, 2005; Ziegler and Wittwer, 2005). The manuscript includes three parts: (a) a deconjugation analysis to ascertain whether excreted hormones are conjugated or not; (b) an analytical validation to ascertain whether the assay measurements are accurate and precise; and (c) a biological validation to ascertain that the assay is producing biologically meaningful results. Here we validate six Arbor Assays antibodies for use in wild white-faced capuchins. We then draw attention to the differences in glucocorticoid concentrations from the wet to dry seasons across the three study sites (Lomas, Santa Rosa, and Taboga) and to the normative life history trajectories for androgen concentrations in maturing males for Lomas and Taboga.

METHODS

Sites and study subjects

For all three study sites, routine censuses are conducted on the white-faced capuchin groups living in their natural habitats in Costa Rica. Each project conducts observational sampling on multiple habituated groups, noting presence/absence of each group member, additions of any group members (via birth or immigration), overall health (e.g., the presence of wounds), and reproductive state (e.g., pregnancy is assigned based on abdominal protrusions and confirmed after the birth of an infant; lactation is assigned based on the presence of a nursing infant). Individual animals are identified by distinct markings on their faces, heads, and bodies. All research for this study was non-invasive and carried out with IACUC permission from our

154 respective U.S. universities as well as permission from the Costa Rican government (UTN,
155 MINAE, SINAC, CONAGEBIO).
156
157 *Lomas Barbudal.* The Lomas Barbudal Monkey Project (hereafter, "Lomas") monitors the
158 capuchins in the Lomas Barbudal Biological Reserve and the surrounding private farm and ranch
159 lands adjacent to the Rio Cabuyo, Rio Salto, and Rio Pijije. The Lomas project was started by
160 Dr. Susan Perry in 1990 (Perry et al., 2012), with behavioral and demographic data collected
161 near-continuously since then. The Lomas Barbudal site is a tropical dry forest that includes
162 riverine and oak forest and includes a lot of steep terrain. This area also experiences a dry season
163 that usually begins in mid-Nov and ends in mid-May each year. There are 12 groups under
164 intensive study, although the monitoring of groups changes from year to year. During the period
165 when hormone samples were routinely collected (2006-2018), the project monitored an average
166 of 9 groups per year (range 6-11 groups), with each group censused about once a week. The
167 number of individuals monitored across this time ranged from 154-245 (average of 206/yr).
168
169 *Santa Rosa.* The Santa Rosa Primate Project (hereafter, "Santa Rosa") monitors the capuchins in
170 the Santa Rosa Sector (SSR) of the Área de Conservación Guanacaste. The Santa Rosa Project
171 was initiated by Dr. Linda Fedigan in 1983, with behavioral and demographic data collected
172 nearly continuously since that time (Fedigan and Jack, 2012; Melin et al., 2020). The project is
173 currently co-directed by Dr. Fedigan (University of Calgary), Dr. Katharine Jack (Tulane
174 University), and Dr. Amanda Melin (University of Calgary). The ~10,000 ha sector comprises
175 mainly tropical dry forest in various stages of regeneration, with several groves of old forest
176 growth and riparian forest edges along seasonally flowing streams. The area experiences a

distinct dry season from mid-December to mid-May; nearly all of the annual rain arrives during the wet season (Fedigan and Jack, 2012). There are currently ~48 groups of capuchins residing in the SSR, with 5 of these groups under intensive study comprising a study population of 119 individuals (40 adult females, 19 adult/subadult males). The Santa Rosa primate project conducts twice-monthly censuses on each of these 5 capuchin study groups, with additional intensive behavioral and ecological studies often occurring simultaneously.

Taboga. The Capuchins at Taboga Project (hereafter, "Taboga") monitors the capuchins in the Taboga Forest Reserve and the Finca Experimental (owned by the Universidad Técnica Nacional, UTN). The Taboga Project (established in June 2017) is the newest of the white-faced capuchin projects and is co-directed by Drs. Jacinta Beehner, Thore Bergman (University of Michigan), and Marcela Benítez (Emory University). The Taboga Forest is a tropical dry forest located in the foothills of the Guanacaste Mountains and part of the Tempisque River Basin, near Abangares and Cañas. The full forest is 789 ha (of which 516 ha are protected and the focus of this study) with extensive edge habitat and significant fragmentation. Although the area has a high degree of seasonality (Tinsley Johnson et al., 2020), the water availability is tempered by a series of year-round, artificial water sources (irrigation canals, including a "river" which derives from the irrigation canals) used to water the nearby rice and sugarcane fields. The irrigation provides a water source to the fauna species in the Taboga Forest year round, but it also appears to irrigate the flora in direct contact with the canals, especially during the dry season. There are also numerous fruit trees surrounding the forest including mango and banana trees that the capuchins use for foraging. The Taboga project monitors four habituated capuchin groups on a near-daily basis, with group sizes ranging from 16-33 individuals (Tinsley Johnson et al., 2020).

Fecal sample collection and extraction

The first stage of hormone analysis from wild subjects is to collect samples from known individuals within a few minutes of defecation, to preserve those samples immediately, and to extract the hormones from the fecal matrix. Santa Rosa and Taboga share an identical extraction method, while the Lomas method of extraction is slightly different. However, the Lomas extraction method overlaps the other two in the following ways: (a) samples were collected in their entirety and placed on ice for several hours prior to freezing, (b) hormones were extracted using 80% EtOH, and (c) extracts were stored in 80% EtOH (not buffer).

Santa Rosa and *Taboga.* The Santa Rosa and Taboga projects have adopted an identical collection and extraction method. Fecal samples are identified opportunistically from known individuals. Immediately following defecation, observers wearing gloves collect the entirety of the fecal sample using a wooden stick and place it into a polypropylene vial labeled with the individual ID, date, and time. Seeds and detritus are avoided, and samples contaminated in any form (e.g., with urine or standing water) are rejected. Labeled tubes are placed into cooler bags with ice packs until they are transferred to a freezer kept at -20˚C, where they remain until extraction.

For hormone extractions, researchers allow all samples to come to room temperature (~ 1 h). Then, each sample is thoroughly mixed with a metal spatula (1 min), 0.2 g (wet weight) of fecal material (taking care to avoid seeds) is weighed, and this aliquot is added to a labeled tube (15 ml Falcon polypropylene tube). Two ml of 80% EtOH are then added to each tube and vortexed for 10 min using a multi-tube vortexer. Following vortexing, all samples are centrifuged for 10 min (3000 rpm), then 1.5 ml of supernatant is gently transferred to a labeled cryovial with an O-ring cap. Extracts are then stored in the freezer until the time of assay. Lastly,

223 the Falcon tubes containing the wet fecal sample are left uncovered allowing them to air dry.
224 Once samples have dried completely, the dry sample is weighed again (to the nearest 0.001 g).
225 For all analyses, fecal hormone measurements are calibrated according to this dry weight (not the
226 0.2 g wet weight from earlier). Although not used in the final concentration calculation, the wet
227 weight helps approximate the similar amounts of fecal material for each extraction.
228
229 *Lomas.* The Lomas project collects fecal samples opportunistically from known individuals using
230 a very similar collection protocol with only one difference: once the field team returns to camp,
231 the samples are placed in a -20˚C freezer until the time of oven-drying (within a month of
232 collection). While we do not suspect this will dramatically alter hormone concentrations across
233 sites, we will specifically test this in the future prior to making direct comparisons.
234 For oven-drying, samples are brought to room temperature, thoroughly mixed, and placed
235 in an oven for 2-3 hours at 80-115˚C. After drying, undigested plant and insect material is
236 removed from the samples before grinding them into a fecal powder. The samples are then stored
237 in WhirlPak bags at room temperature until they are shipped to the laboratory where they are
238 then stored in a freezer (-20˚C) until extraction. To extract samples, samples are brought to room
239 temperature. Then, 0.15 g of dry fecal powder is weighted and extraced using the same
240 procedure as above.
241
242 **Deconjugation analysis**
243 Although for most catarrhine primate species steroid hormones are excreted into feces
244 primarily in the "free" unconjugated form (Heistermann, 2010), this is less often the case for the
245 platyrrhine primates of the Americas (Eastman et al., 1984; Ziegler and Wittwer, 2005). Because

246 the assay kits we use here were primarily designed to measure the free forms for these hormones,
247 the hormone signal may be improved by first deconjugating the steroids (typically by hydrolysis
248 or solvolysis). A previous study on white-faced capuchin fecal hormones identified that, while
249 hydrolysis was not necessary, solvolysis released a substantial amount of conjugated androgens
250 and a smaller amount of conjugated glucocorticoids (Weltring et al., 2012). Here, we aim to
251 establish that the non-conjugated portion of the steroid metabolites in feces are strongly
252 correlated with the conjugated proportion, allowing us to bypass this step in our hormone
253 extraction protocol for this species. Therefore, we conducted solvolysis on a subset of samples
254 and examined whether the immunoassay results with and without solvolysis were sufficiently
255 correlated. Although Weltring and colleagues (2012) demonstrated that androgens had the
256 highest levels of sulfate conjugation, we nevertheless compared all four categories of steroid
257 hormones.

258 To establish whether solvolysis was needed for white-faced capuchin steroid hormones,
259 we used a range of 52 samples from the Lomas Barbudal project (extracted in 80% EtOH). This
260 analysis was conducted in the Beehner endocrine laboratory at the University of Michigan (we
261 conducted this part of the validation before the field laboratory was in operation). These samples
262 derived primarily from those used in the biological validation (see below) to ensure we were able
263 to test solvolysis across the full range of hormone values. In brief, we added a strong acid
264 (sulfuric acid) and ethyl acetate to our samples (pH of ~1.0), incubated for one hour (at 55˚C),
265 then separated the organic layer (containing de-conjugated hormones) from the aqueous one. We
266 transferred the organic layer to a new tube, returned the sample to neutrality (~6.6, using
267 potassium hydroxide), dried it down, and reconstituted it in our extraction solvent (in this case,

80% EtOH). We then ran assays on all samples – solvolysized and non-solvolysized – for the four DetectX® hormones.

Hormone assays

Because we are measuring downstream metabolized products of hormone secretion (fecal hormone metabolites), most immunoassays measure several different hormone metabolites, not just the specific steroid hormone. Therefore, to distinguish among the different assay kits, we refer to the specific steroid (cortisol: CORT; testosterone:T; estradiol:E2; progesterone:P4), but when referring to our measurements, we refer to the class of hormones that the assay antibodies are cross-reacting with (glucocorticoids, androgens, estrogens, and progestogens). We also recognize that metabolites from the degradation of secreted hormones may cross over from one class to another, which is why a biological validation of fecal steroid hormones is so important (Touma and Palme, 2005). We validated measurements of these four hormone classes from fecal extractions in white-faced capuchins via competitive enzyme-linked immunosorbent assay (ELISA) using the DetectX® Immunoassay kits (cortisol, testosterone, estradiol, progesterone) and the ISWE mini-kit assays (cortisol, testosterone), all from Arbor Assays (Ann Arbor, MI, USA). We initially selected the DetectX® assays because we already had a working relationship with Arbor Assays. We then added the ISWE CORT and T assay because the kits are easier to ship internationally (the stop solution contains hydrochloric acid and requires special permits to ship internationally, the ISWE kits do not include this solution facilitating shiping). All validation procedures, the assays and hormones validated, the fieldsite contributing the samples, and the laboratory where the work was completed are listed in **Table 1.** The cross reactivity with other steroid metabolites are listed in **Table 2** for each assay.

Table 1. Summary table of validation steps, hormones validated, assay kit used, fieldsite the samples derived from, the test employed, and the outcome.

INSERT TABLE 1 HERE

Table 2. Cross reactivity (%) with other steroid metabolites for each Arbor Assays hormone kit as indicated by the kit literature; all cross reactivities not listed are less than 0.1%.

INSERT TABLE 2 HERE

Glucocorticoids. To measure glucocorticoid metabolites, we validated two cortisol enzyme immunoassay kits; the DetectX® CORT Enzyme Immunoassay kit (Arbor Assays, K003) and the ISWE CORT Mini-Kit (Arbor Assays, ISWE002). The DetectX® kit uses a mouse monoclonal antibody, a cortisol-peroxidase conjugate, with a plate coated with goat anti-mouse IgG. The ISWE kit uses a rabbit polyclonal antibody, a cortisol-peroxidase conjugate, with a plate coated with goat anti-rabbit IgG. The rest of the protocol for both kits is the same. Standards (7 standards, ranging from 50-3200 pg/ml) and samples (diluted in assay buffer from 1:16 to 1:128) were added to each plate in duplicate (50 µl/well), followed by the addition of 25 µl of the cortisol conjugate and 25 µl of the cortisol antibody (note that this protocol is halved from the kit literature for the ISWE kit). Plates were placed on the plate shaker and incubated at room temperature for 1 h (DetectX® CORT assay) or 2 h (ISWE CORT assay). Plates were then washed 4x with 300 µl wash buffer per well, followed by the addition of 100 µl of TMB substrate, and another 30 min incubation without shaking. The reaction was terminated with 50 µl of stop solution (1M HCl). All plates were read using a microtiter plate reader (BioRad

iMark®) at a wavelength of 450 nm. Concentrations in pg/mL based on optical density values and accounting for sample dilution were calculated using MyAssays® software. Final concentrations were calculated in pg/g based on the dry weight of the fecal sample. The sensitivity for each assay (i.e., the lowest hormone value where the assay can statistically differentiate from background) is as follows: DetectX® CORT assay = 27.6 pg/mL; the ISWE CORT assay = 11.2 pg/mL.

Androgens. To measure androgen metabolites, we validated two testosterone enzyme immunoassay kits; the DetectX® Testosterone Enzyme Immunoassay kit (Arbor Assays, K032) and the ISWE Testosterone Mini-Kit (Arbor Assays, ISWE001). Both kits use a rabbit polyclonal antibody, a testosterone-peroxidase conjugate, and a plate coated with goat anti-rabbit IgG. Both kits follow an identical protocol. Standards (7 standards, ranging from 40.96-10,000 pg/ml) and samples (diluted in assay buffer from 1:8-16 for non-adult males, 1:250-1000 for adult males) a were added to each plate in duplicate (50 µl/well). The rest of the protocol follows that for the two glucocorticoid assays. The DetectX® T assay has a sensitivity of 9.92 pg/mL; the ISWE T assay has a sensitivity of 5.03 pg/mL.

Estrogens. To measure estrogens, we used the DetectX® Estradiol Enzyme Immunoassay kit (Arbor Assays, K030). Standards (5 standards, ranging from 39.06-100,000 pg/ml) and samples (diluted in assay buffer from 1:16-32 for lactating females, 1:128-256 for pregnant females) were added to each plate in duplicate (50 µl/well). The rest of the protocol follows that for the other assays. The DetectX® E2 assay has a sensitivity of 39.6 pg/mL.

Progestogens. To measure progestogens, we used the DetectX® Progesterone Enzyme Immunoassay Kit (Arbor Assays, K025). Standards (7 standards, ranging from 50-3200 pg/ml) and samples (diluted in assay buffer from 1:20-80 for lactating females, 1:5000-10,000 for pregnant females) were added to each plate in duplicate (50 µl/well). The rest of the protocol follows that for the other assays. The DetectX® P4 assay has a sensitivity of 47.9 pg/mL.

Analytical validation

Analytical validation establishes that the assay (mainly the antibody) is operating as expected after accounting for any matrix interference and the full range of the dose-response curve (typically 20-80% binding, but smaller ranges can also be used). We analytically validated all assays by creating three different fecal pools (adult mixed-sex, adult male-only, adult female-only) for use in the CORT (both kits), T (both kits) and E2/P4 kits, respectively.

Serial dilutions. The first step to an analytical validation is to establish a serial dilution for the appropriate fecal pool that spans from low concentration (80% binding) to high concentration (20% binding). To accomplish this, we started with a fecal pool (neat) and diluted each step by half until it spanned the same range as the standards.

Parallelism. To test for parallelism, we ran a set of standards and a serial dilution of the appropriate fecal pool in the same plate. We then assigned the concentration from the standard binding closest to 50% to the sample from the serial dilution that was binding closest to 50%. Using this assigned concentration, we then back-calculated the "expected" values for each sample in our serial dilution based on the dilution factor. We then plotted the log of these back-

calculated values and those of our standards as a function of percent binding, and we visually inspected whether the slope of the serial dilution paralleled the slope of the standards for each of the four assays. We also established parallelism statistically, by checking if there is a significant interaction between the concentrations in a linear model (although many behavioral endocrinologists have confirmed that a visual determination of parallelism is sufficient and sometimes more conservative than statistics alone; ISWE-members listserv discussion Mar 10-25, 2021; (Ganswindt et al., 2012)).

Accuracy. To test the accuracy of each assay, we added an aliquot of each standard with a known concentration to an aliquot of our fecal pool (i.e., the mixed-sex pool for glucocorticoids, the male-only pool for androgens, and the female-only pool for estrogens and progestogens). We then calculated the expected value of these "spiked" samples (based on the known values of the standard plus the sample), and we compared the observed to expected concentrations.

Precision. Precision establishes whether the assay retrieves the same concentration when a sample is assayed multiple times. There are two different measures of precision that are required: one to measure the precision (or coefficient of variation, CV) *within* each assay (*intra-assay CV*) and one to measure the precision *across* all assays (*inter-assay CV*). Although some studies use the average of the CVs for each of their duplicate concentration measurements as an intra-assay CV, this is inappropriate. Samples are run in duplicate to identify mechanical errors in pipetting, not to establish an intra-assay CV. Many studies use kit controls (i.e., pure hormones in buffer) to calculate assay CVs which have two advantages – they are readily available for commercial kits, and they do not degrade. However, we chose to use a fecal pool because CV for these will

be closer to the true variation that we see within and across assays due to some degree of fecal matrix effects. To establish the intra-assay CV for our assays, we ran our fecal pool at a low concentration (~60-80% binding) and a high concentration (~20-40% binding) multiple times within the same assay. Our sample size for the intra-assay CV is the number of times we repeated the sample within the assay (counting each well, not each duplicate, as a separate "sample"). To establish the inter-assay CV for our assays, we ran these same low and high concentration pools as controls in each plate. We then calculated the CVs as the standard deviation for these pool concentrations divided by the mean for pool concentrations. Our sample size for the inter-assay CV is the number of plates we ran for each assay.

Biological validation

Biological validation establishes that known biological patterns for the native hormone in blood samples can be replicated with the fecal extracts using the assay components. Biological validations can include (a) hormone or behavioral challenges (e.g., for glucocorticoid concentrations, researchers often use an ACTH challenge or the addition of a known stressor (Beehner and McCann, 2008; Goymann et al., 1999; Wasserman et al., 2013; Young et al., 2017); for androgen concentrations, researchers often use a GnRH challenge or the addition of an invader male (Dloniak et al., 2004; Hirschenhauser et al., 2000; Pappano and Beehner, 2014)), (b) a comparison to serum hormone values (Capezzuto et al., 2008; Sheriff et al., 2010) or (c) comparisons across groups that *should* vary in an expected direction (e.g., adult males should have higher androgen concentrations than juvenile males (Beehner et al., 2009); for estrogens and progestogens, pregnant females should have higher hormone concentrations than lactating females (Roberts et al., 2017)).

406 Unfortunately, because we added the ISWE CORT and T assays afterwards, our
407 biological validations for these two hormones did not use the same set of samples. Therefore, we
408 are unable to directly compare performance across assay kits. However, our purpose here was
409 not to identify the "best" kit for use but rather to simply validate each kit for use on the same
410 species. Testing for the "best" assay (e.g., most sensitive, most accurate, most precise, etc.)
411 typically relies on pharmacological manipulations to assess how and when measurements using
412 each antibody respond to known changes in hormone secretion. We would pursue this route if we
413 discover later that these assays do not yield sufficient variability when applied to our various
414 research questions. All analyses were run in R (R version 4.2.0).
415
416 *Glucocorticoids* (GCs) - In line with many studies on GCs in tropical mammals where water and
417 food are restricted during the dry months (Carnegie et al., 2011b; Garber et al., 2020; Gesquiere
418 et al., 2008; Medina-Cruz et al., 2020), we expected GC concentrations in white faced capuchins
419 during the dry season to be higher than during wet season. We used samples from all three study
420 sites for this biological validation (Table 1). Based on the antibodies already in use at each site,
421 we validated the DetectX® CORT antibody using the Lomas samples (Lomas: 36 females/359
422 samples, all adults), and we validated the ISWE CORT antibody using the Santa Rosa (Santa
423 Rosa: 5 females/18 samples, 10 males/45 samples, all adults) and Taboga samples (Taboga: 22
424 females/294 samples, 29 males/272 samples, all ages). We selected samples from well within the
425 dry and wet seasons of Costa Rica (leaving approximately a one-month buffer on either end).
426 Therefore, for any given year, samples for the dry season (all sites) were from Jan-Apr, and
427 samples from the wet season (all sites) were from Jun-Nov. For the Lomas (DetectX® CORT)
428 and the Santa Rosa and Taboga datasets (ISWE CORT), we constructed linear mixed models

(LMMs) with fecal GC metabolites (log-transformed) as a function of *season* (wet/dry), with *individual ID* included as a random effect. Additionally, we included *sex* as a factor for the two datasets that were mixed-sex (Santa Rosa, Taboga) and *age* as a covariate for the dataset that contained non-adults (Taboga).

Androgens - In line with the onset of puberty, testis maturation, and the onset of testosterone production by the mammalian testes (Beehner et al., 2009; Behringer et al., 2014; O'Brien et al., 2017; Wolf et al., 2018), androgen concentrations in adults are expected to be higher than those of juveniles, with subadults somewhere in between. We therefore expected the following pattern for androgen concentrations in white faced capuchin males: adult males > subadult males > juvenile males. We used samples from Lomas (N=14 males, 14 samples) to validate the DetectX® T assay, and we used samples from Taboga (N=29 males, 308 samples) to validate the ISWE T assay. We use the following approximations for age categories: juveniles from 2.0-5.0 years, subadults from 5.0-10.0 years, and adults from 10.1 years and older. The Lomas samples used for this validation derived from juvenile males (N=5 juveniles), subadult males (N=5 males), or adult males that had achieved alpha status at the time of sampling (N=4 adult alpha males). To maximize our range for androgen concentrations in the small Lomas sample, we selected only alpha adult males for the validation because alpha males are known to have higher androgens than subordinate males in white-faced capuchins (Jack et al., 2014; Schaebs et al., 2017; Schoof et al., 2011; Schoof and Jack, 2013). For Lomas, we constructed a linear model (LM) with fecal androgen metabolites (log-transformed) as a function of age category. The Taboga samples derived from males of all ages and dominance ranks including 3 infants (13 samples), 5 juveniles (79 samples), 14 subadults (143 samples), and 10 adults (72 samples). All

452 males in the Taboga dataset < 5 years had known ages; all males >5 years had estimated ages
453 based on size and tooth wear at the start of the study (in 2017) or at the time of immigration. For
454 Taboga, we ran an LMM with fecal androgen metabolites (log-transformed) as a function of age
455 (linear) and age^2 (inverse-U shaped), with individual ID as a random effect.
456
457 *Estrogens* - In most female primates, estrogens are expected to increase across gestation
458 (Beehner et al., 2006; Carroll et al., 1990; Roberts et al., 2017). We expected the following
459 pattern for estrogens in white faced capuchin females: pregnant females > lactating females. For
460 both estrogens and progestogens, the Lomas samples used for this validation derived from
461 known pregnant females (back-dated from the birth of an infant, N=5 females) and known
462 lactating females (presence of a nursing infant, within the first year of lactation, N=4 females).
463 We ran an LMM with fecal estrogen metabolites (log-transformed) as a function of reproductive
464 category with individual ID as a random effect.
465
466 *Progestogens* - Similar to estrogens, we expected the following pattern for progestogens in white
467 faced capuchin females: pregnant females > lactating females. The sample dataset used for this
468 validation was the same as the one used for the estrogens validation. We ran an LMM with fecal
469 progestogen metabolites (log-transformed) as a function of reproductive category with individual
470 ID as a random effect.
471
472 **RESULTS**
473 **Deconjugation analysis**

Conducting solvolysis on capuchin fecal extracts released some conjugates from glucocorticoids and androgens but not from estrogens or progestogens. Where conjugates were released (glucocorticoids and androgens), we found that non-solvolysized and solvolysized samples: (1) were highly correlated with one another (~98%), and (2) maintained the same (or similar) rank order from the highest to the lowest sample (even across close-in-value samples, **Fig. 1**).

INSERT FIGURE 1 HERE

Figure 1. Hormone measures without chemical solvolysis are closely correlated with measures following solvolysis for glucocorticoids and androgens. Correlation plot between log-transformed hormone samples (in pg/ml) as a function of the same samples that have undergone chemical solvolysis for**: (a)** glucocorticoids and (**b**) androgens.

Glucocorticoids. We conducted chemical solvolysis on 20 Lomas capuchin samples (selected to represent a range of different glucocorticoid values) and compared these values to the same sample without solvolysis. Deconjugation via solvolysis produced a higher concentration of glucocorticoids as measured by the CORT assay for all samples except one (29% of the immunoreactive GCs were conjugated, IQR = 18% – 44%). However, CORT values from samples *that did not* undergo solvolysis were highly correlated with values from samples *that did* (Pearson: $r(19) = 0.98$, $p<0.001$, **Fig. 1a**); and the rank order of samples was maintained (12/20 samples had identical ranks, and 8/20 samples differed by 3 ranks or less).

Androgens. We conducted chemical solvolysis on 14 Lomas capuchin samples (selected to represent a range of different testosterone values) and compared these values to the same samples without solvolysis. Deconjugation via solvolysis produced a higher concentration of androgens as measured by the T assay for all samples except two samples (32% of the

immunoreactive androgens were conjugated, IQR = 4% – 50%). However, similar to glucocortiocids, the samples that did not undergo solvolysis had measures that were highly correlated with those from samples that did (Pearson: $r(12)= 0.98$, $p<001$, **Fig. 1b);** and the rank order of samples was almost entirely maintained (11 of 14 samples had an absolute rank difference of ≤ 1; and the other 3 samples differed by only 2 ranks).

Estrogens. We conducted chemical solvolysis on 14 Lomas capuchin samples (selected to represent a range of different estrogens values) and compared these values to the same samples without solvolysis. Deconjugation via solvolysis did not produce a higher concentration of estrogens as measured by the DetectX® E2 assay. Indeed, most samples after solvolysis were lower in value, likely due to some hormone metabolites lost during the solvolysis procedure (median decrease = -11%, IQR = -21% – -2%).

Progestogens. We conducted chemical solvolysis on 14 Lomas capuchin samples (selected to represent a range of different progestogen values) and compared these values to the same samples without solvolysis. Deconjugation via solvolysis did not produce a higher concentration of progestogens as measured by the DetectX® P4 assay. Similar to the estrogens results, all samples were lower in value after solvolysis (median decrease = -24%, IQR = -49% – -11%), likely due to the solvolysis procedure.

Analytical validation

Lomas samples were used for all four hormones using DetectX® assay kits and Taboga samples were used for ISWE CORT and ISWE T assay kits. Please refer to Table 1 for a list of which samples from which site were used to validate which hormone for which assay.

Parallelism. First, we determined parallelism for each assay both visually and by modeling the percent binding from the concentrations of a serial dilution of a fecal pool and the assay standard curve. All assays showed sufficient parallelism between the binding range of 20-80% (**Fig. 2a-f**). Additionally, there was no significant interaction between the concentrations and the type of sample (serial dilution vs. standard) for any of our assays, indicating that the slopes of these lines were not significantly different.

- *Glucocorticoids (DetectX®)* - ANOVA: F = 3.73, p = 0.085
- *Glucocorticoids (ISWE)* - ANOVA: F = 2.13, p = 0.654
- *Androgens (DetectX®)* - ANOVA: F = 2.38, p = 0.157
- *Androgens (ISWE)* - ANOVA: F = 0.351, p = 0.567
- *Estrogens (DetectX®)* - ANOVA: F = 0.09, p = 0.767
- *Progestogens (DetectX®)* - ANOVA: F = 1.99, p = 0.189

INSERT FIGURE 2 HERE

Figure 2. All assays demonstrated parallelism. Assay standards and a diluted fecal pool showed sufficient parallelism between the binding range of 20-80% for **(a)** DetectX® glucocorticoids; (**b**) ISWE glucocorticoids; (**c**) DetectX® androgens; (**d**) ISWE androgens; (**e**) DetectX® estrogens; and (**f**) DetectX® progestogens.

Accuracy. Second, we determined the accuracy for each assay by spiking the standards with a diluted aliquot of our pool and comparing observed to expected values. Mean recovery for

observed compared to expected values indicated that each assay recovers accurate fecal measurements (<15% difference between observed and expected values).

- *Glucocorticoids (DetectX®)* - 100% (range: 87 – 112%), N=6
- *Glucocorticoids (ISWE)* - 104% (range: 95 – 117%), N=7
- *Androgens (DetectX®)* - 101% (range: 90 – 112%), N=7
- *Androgens (ISWE)* - 105% (range: 103 – 115%), N=7
- *Estrogens (DetectX®)* - 103% (range: 86 – 115%), N=5
- *Progestogens (Detect X®)* - 101% (range: 87 – 110%), N=7

Precision. Third, we established the intra- and inter-assay coefficients of variation (CVs) for measuring hormone metabolites (glucocorticoids, androgens, estrogens, and progestogens) with each assay. Using a low (60-80% binding) and a high concentration pool (20-40% binding), the CVs for low and high pools for all assays were within the acceptable level of assay precision (**Table 3**).

Table 3. Hormone metabolite intra-assay and inter-assay precision (CVs) for all assays

INSERT TABLE 3 HERE

Biological validation

Glucocorticoids. We conducted a biological validation on fecal GCs by comparing concentrations for individuals during the dry and wet seasons at each site. We used the DetectX® CORT assay for the Lomas dataset (adult females only) and the ISWE CORT assay for the Santa Rosa (adult males and females) and Taboga (all ages and sexes) datasets to examine how GC

concentrations varied as a function of season (and age and sex where relevant). The Lomas samples exhibited significantly higher GC concentrations in the dry season than in the wet season (Lomas LMM: *season:* beta = -0.218, t = -2.71, p<0.01, **Fig. 3a**). The Santa Rosa samples, like Lomas, also exhibited higher GC concentrations in the dry season compared to the wet season (Santa Rosa LMM: *season:* beta = -0.384, t = -2.71, p<0.01, **Fig. 3b**). However, the Taboga samples exhibited no difference across seasons (Taboga LMM: *season:* beta = 0.058, t = 1.53, p=0.125, **Fig. 3c**).

INSERT FIGURE 3 HERE

Figure 3. The biological validation for the DetectX® and ISWE CORT assays. Applying the DetectX® CORT assay to Lomas samples revealed (**a**) higher GC concentrations in the dry season compared to the wet season. Applying the ISWE CORT assay to the Santa Rosa and Taboga samples indicated (**b**) Santa Rosa also had higher GCs in the dry than wet season, and (**c**) Taboga samples exhibited no difference across seasons. All values are shown as log-scaled hormone concentrations (pg/g).

Androgens. We compared fecal T concentrations in samples collected from 14 Lomas capuchin males classified as juveniles (5 males), subadults (5 males), or alpha adults (4 males). For Lomas, age category was a significant predictor of log androgen concentrations (Lomas LM: *age category* - F=27.12, p<0.001). Alpha adult males had higher measures than juvenile males (Z = -2.45, p < 0.05) and subadult males (Z = -2.45, p < 0.05, **Fig. 4a**). However, contrary to expectations, there was no difference between juvenile males and subadult males for this small sample (Z = -0.31, p = 0.75). The Taboga males exhibited a steady increase in fecal androgens from infant to adult males, with a leveling off at adulthood (**Fig. 4b**), with age as a significant predictor of log androgen concentrations (Taboga LMM: *age:* beta = 0.153, t = 3.07, p<0.01) and age^2 approaching significance (Taboga LMM: *age^2:* beta = -0.003, t = -1.67, p<0.1).

INSERT FIGURE 4 HERE

Figure 4. The biological validation for the DetectX® and ISWE T assays. (**a**) The biological validation for the DetectX® T assay for the Lomas males exhibited significantly higher androgens for alpha adult males compared to subadults or juveniles. (**b**) The ISWE T assay for the Taboga males showed the expected pattern with adults having higher androgens than other males. All values shown as log-scaled hormone concentrations (pg/g), rounded to the nearest age.

Estrogens. Fecal estrogen concentrations were compared in Lomas samples collected from 5 pregnant females and 4 lactating females (no female had samples in more than one state). Pregnant females had higher measures of estrogens than lactating females (Lomas LM: *state:* beta = 1.318, t = 9.31, p<0.001, **Fig. 5a**).

Progestogens. Fecal progesterone concentrations were also compared in Lomas samples collected from 5 pregnant females and 4 lactating females (no female had samples in more than one state). Pregnant females had higher measures of progestogens than lactating females (Lomas LM: *state:* beta = 1.756, t = 13.74, p<0.001, **Fig. 5b**).

INSERT FIGURE 5 HERE

Figure 5. The biological validations for the DetectX® E2 and P4 assays were successful for the Lomas capuchins. For Lomas capuchins, (**a**) pregnancy females had higher estrogen and (**b**) progestogen concentrations than lactating females. All values are shown as log-scaled hormone concentrations (pg/g).

DISCUSSION

In this manuscript, we introduce our on-site field laboratory, the Taboga Field Lab, located just outside the Taboga Forest Reserve in Costa Rica. We demonstrated that it functions under field conditions and can serve to validate and measure a wide variety of hormones and

625 other biomarkers from Costa Rican wildlife. Specifically, we validated six Arbor Assay hormone
626 assays for measuring hormone metabolites (glucocorticoids, androgens, estrogens, and
627 progestogens) in wild white-faced capuchins from three different field sites laying the
628 groundwork for future comparisons across these populations. Because the data obtained are
629 method-specific and other immunoassays may yield significantly different absolute
630 concentrations, this is one of the best ways to conduct cross-site comparisons (emplying the
631 same assay and conducting analyses in the same laboratory). Such comparative studies have the
632 potential to identify the health and environmental impacts on one population compared to
633 another that may not be identifiable with the physiology from a single population.
634 Previous research demonstrated that hydrolysis was not necessary for measuring steroid
635 hormones from white-faced capuchins (Weltring et al., 2012), and we now additionally showed
636 that solvolysis is probably also not necessary. Conjugated samples were proportional to
637 unconjugated samples for glucocorticoid and androgen metabolites, suggesting that the
638 unconjugated fraction of these hormone metabolites in white-faced capuchin fecal samples
639 appear to be biologically meaningful.
640 Both the analytical and biological validations in white-faced capuchins were successful
641 for glucocorticoids, androgens, estrogens, and progestogens (and their metabolites). Hormone
642 metabolites as measured by the two ISWE and the four DetectX® kits (all from Arbor Assays)
643 across both laboratories were parallel, accurate, and precise. Moreover, hormones measured with
644 these kits revealed the expected biological patterns. All individuals at both Lomas (females only)
645 and Santa Rosa (males and females) exhibited significantly higher concentrations of GC
646 metabolites during the dry season compared to the wet season. This common pattern has been
647 observed in many primate taxa, including white-faced capuchins (Carnegie et al., 2011b) and is

often attributed to the elevated metabolic stress imposed on animals when food and/or water are more difficult to access (Campos and Fedigan, 2009). Additionally, the dry season is much warmer than the wet season in tropical dry forests (Schoof et al., 2016; Tinsley Johnson et al., 2020), so the rise in glucocorticoids could also be due to thermoregulatory stressors similar to other studies (Wessling et al., 2018). The Taboga population showed less of a difference between GC concentrations in wet and dry seasons. However, given that Santa Rosa demonstrated the expected pattern using the same assay, we propose that this does not indicate a failed biological validation but rather a possible biological difference altogether. Taboga capuchins may be somewhat buffered from the severity of the Costa Rican dry season due to the year-round water available from irrigation canals running through the forest (Tinsley Johnson et al., 2020) – although Lomas capuchins also have ready access to rivers throughout their range. The year-round access to water (and fruit from agricultural products) at Taboga may also account for why this site has the highest density of capuchins reported (Tinsley Johnson et al., 2020).

Second, the Lomas and Taboga adult males exhibited higher androgen metabolites compared to subadult or juvenile males in the same population. Importantly, the Lomas adult males selected for this analysis were alpha males at the time we measured their androgens in order to maximize the androgen range for the validation. This result is therefore consistent with male developmental processes across age and status in vertebrates (Beehner et al., 2009; Behringer et al., 2014; O'Brien et al., 2017; Wolf et al., 2018) and more specifically with previous results from white-faced capuchins indicating that alpha males have higher androgen concentrations than non-alpha males (Jack et al., 2014; Schaebs et al., 2017; Schoof et al., 2011; Schoof and Jack, 2013). Although the Lomas dataset was too small to identify a more-detailed difference across the younger ages, the gap in ages was small compared to the age gap between

671 the alpha adults and all younger males. For the larger Taboga dataset analyzed here, males
672 exhibited a continuous rise in androgen metabolites across all ages until approximately age 10,
673 when androgens leveled off. Confirming this life history pattern of androgens, in a different age-
674 based analysis of androgen profiles in the Santa Rosa white-faced capuchins (using a different
675 androgen assay than what is presented in this manuscript), authors found higher androgen
676 concentrations in subadults than juveniles (Jack et al., 2014). A future collaborative project will
677 include an age- and status-based analysis of androgens across capuchin sites to identify the
678 normative "pivot points" for androgens in the life histories of this taxon. Moreover, although
679 white-faced capuchins do not have a strict breeding season, births do tend to be clustered during
680 half the year (Carnegie et al., 2011a; Perry et al., 2012). Therefore, similar to other androgen
681 studies (Schoof et al., 2016, 2014), seasonality needs to be taken into account for this taxon.
682 Finally, estrogen and progestogen metabolites were orders of magnitude higher in
683 pregnant than in lactating females. This pattern is not surprising given that these hormones are
684 required to maintain pregnancy in primates, and they are lowest in females who have temporarily
685 ceased ovarian cycling due to lactational amenorrhea (Ryniec and McGee, 2020). Although our
686 samples across the different trimesters of gestation in our small dataset did not reveal a steady
687 rise in these hormones from early to mid to late gestation as typically observed in catarrhine
688 primates (Beehner et al., 2006; Czekala et al., 1983; Roberts et al., 2017), previous analyses in
689 the Lomas white-faced capuchins have demonstrated overlap in progestagen values across
690 trimesters in white-faced capuchins (Godoy, 2015). It appears that capuchins may mirror other
691 platyrrhine primates with an accelerated and highly variable increase in estrogens and
692 progestogens following conception (Eastman et al., 1984; Moorman et al., 2002). Because fecal
693 hormone methods capture broad categories of downstream hormone metabolites (not the original

694 secreted hormones during gestation), we do not know the breakdown of which hormones take
695 precedence at which stage of gestation. Serum hormone concentrations will be necessary to
696 ascertain this information.
697 We were equally as successful at carrying out hormone validations in our field laboratory
698 as we were in our university laboratory. There are often logistical reasons that field laboratories
699 are not possible (i.e., no access to electricity, clean water, or access to supplies). However, where
700 these logistical problems can be overcome, we would like to highlight some of the advantages of
701 having a laboratory on site where study subjects live. The most obvious of the logistic
702 advantages is that Santa Rosa and Lomas researchers can gain quicker access to hormone
703 measures (within weeks), and Taboga researchers can gain immediate hormone measures for
704 subjects. At Taboga, for example, if we collect a fecal sample from a female in the morning, we
705 could know her hormone concentrations by as early as the afternoon of the same day. This is
706 particularly valuable for primates, like capuchins, who have concealed ovulation and extended
707 lactational ameorrhea (Recabarren et al., 2000). If we can plan our daily observations armed with
708 this physiological information about each animal, we can collect more targeted behavioral data in
709 our research endeavors.
710 On-site laboratories also have the advantage of reducing the logistics necessary to export
711 samples to an out-of-country laboratory, saving time, money, and energy on behalf of the
712 research team while ensuring minimal degradation of the samples. Additionally, because they are
713 located within the host country, on-site laboratories can foster technology and knowledge
714 transfer between all researchers involved creating more equal research partnerships that extend
715 beyond logistics planning and data collection (Minasny et al., 2020). Host-country researchers
716 can gain experience and confidence by conducting laboratory analyses, troubleshooting, and data

processing – all marketable skills that can be harnessed in other laboratories and transferred to other young researchers. Biological research stations, such as Taboga, can serve as hubs for researchers to develop collaborative networks to help facilitate in-country capacity building and encourage comparative research across sites (Beck et al., 2019). We hope that the Taboga Field Laboratory can serve as a model for future field sites with the capability to build laboratories on-site; and we look forward to future collaborative white-faced capuchin hormone studies.

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COMPETING INTEREST STATEMENT

The authors have no competing interest to declare.

DATA AND CODE AVAILABILITY

Data and code are available at: https://github.com/Capuchins-at-Taboga/Beehner-hormone.validation-2022

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Figure 1

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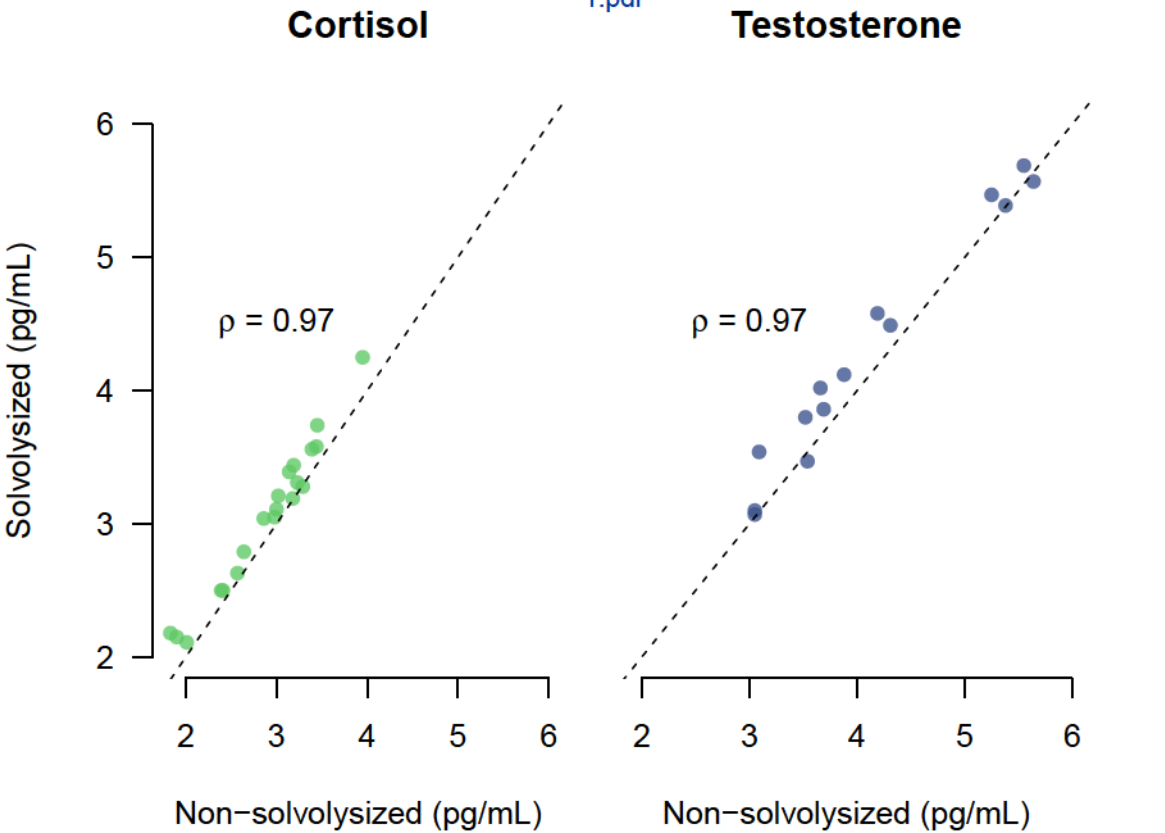

Figure 2 (panels a, b)

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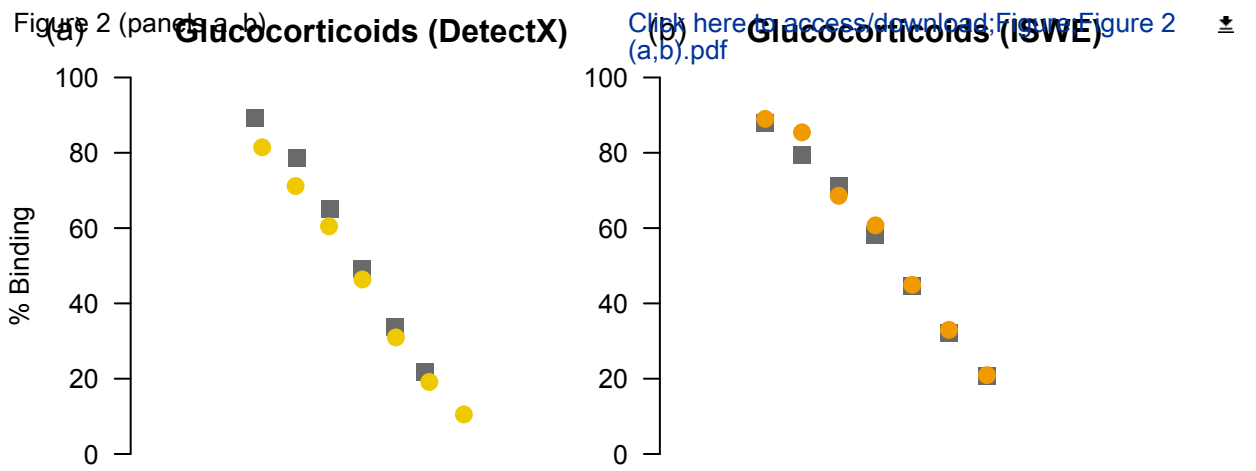

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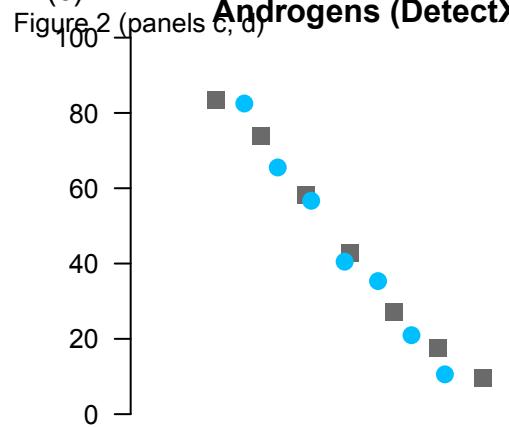

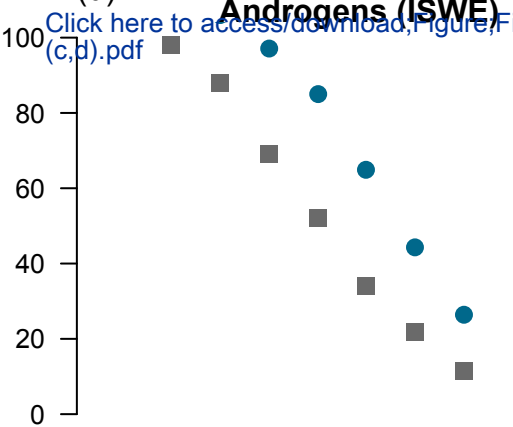

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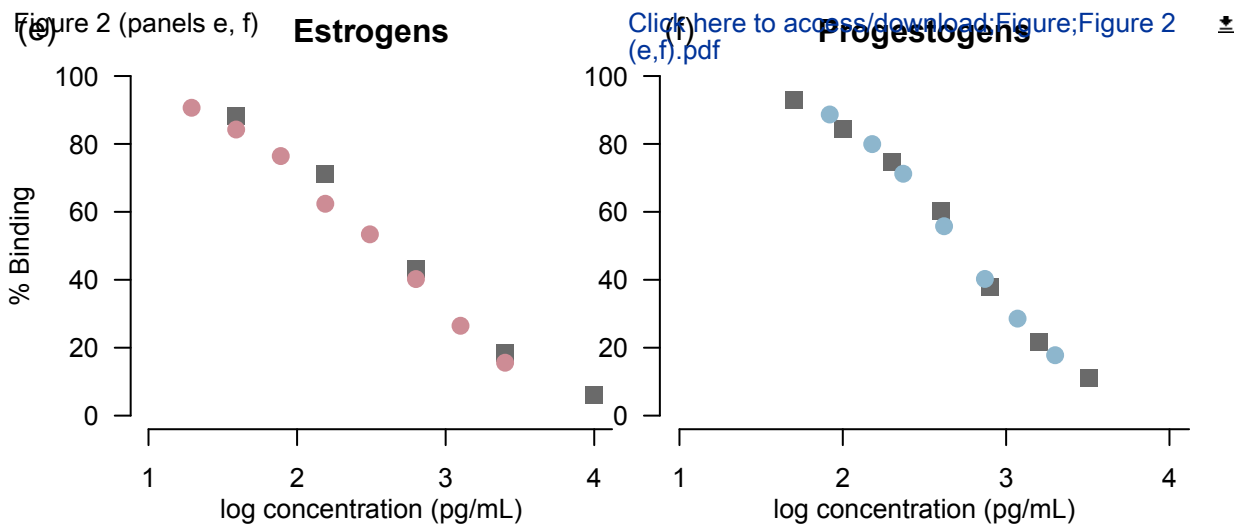

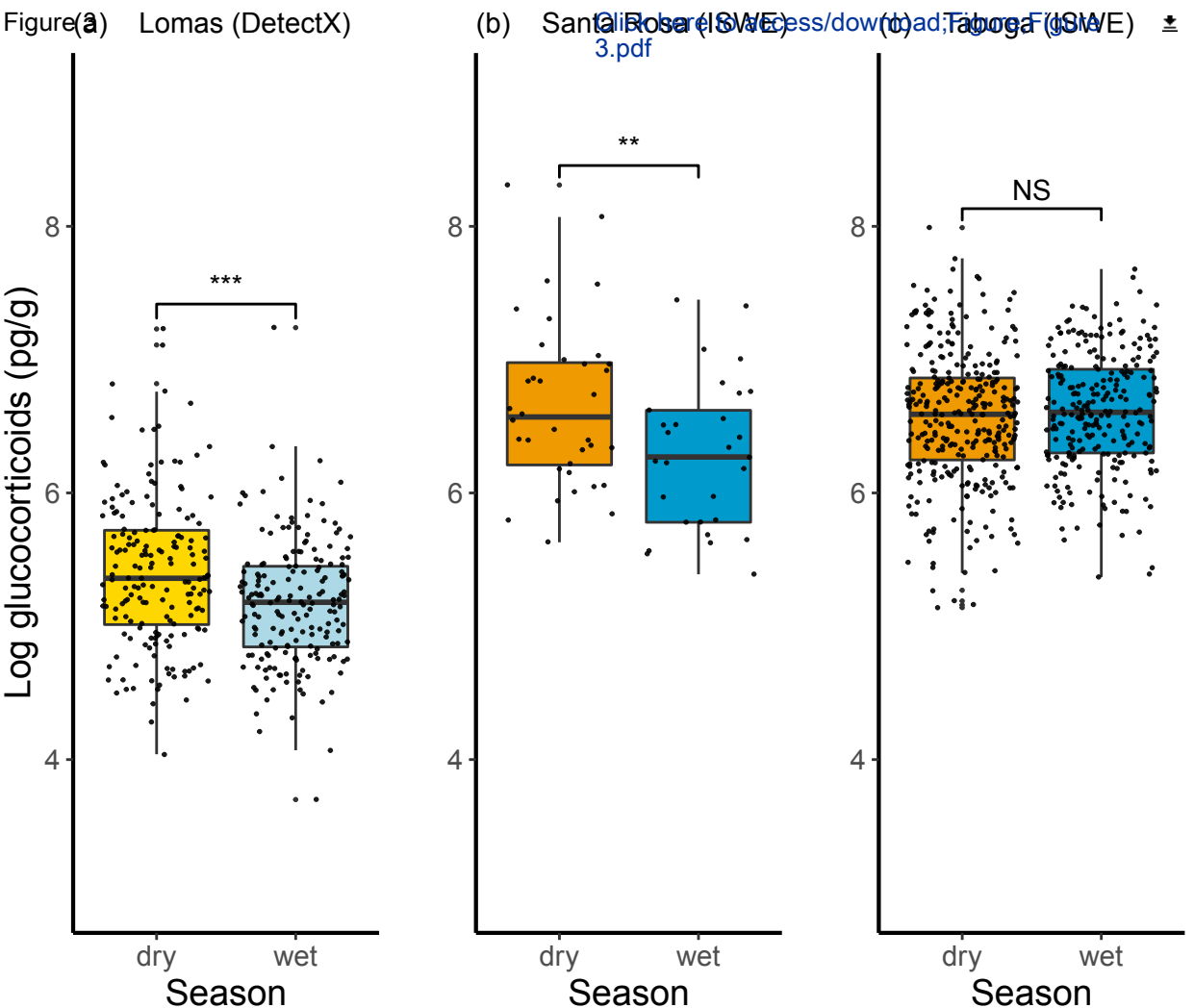

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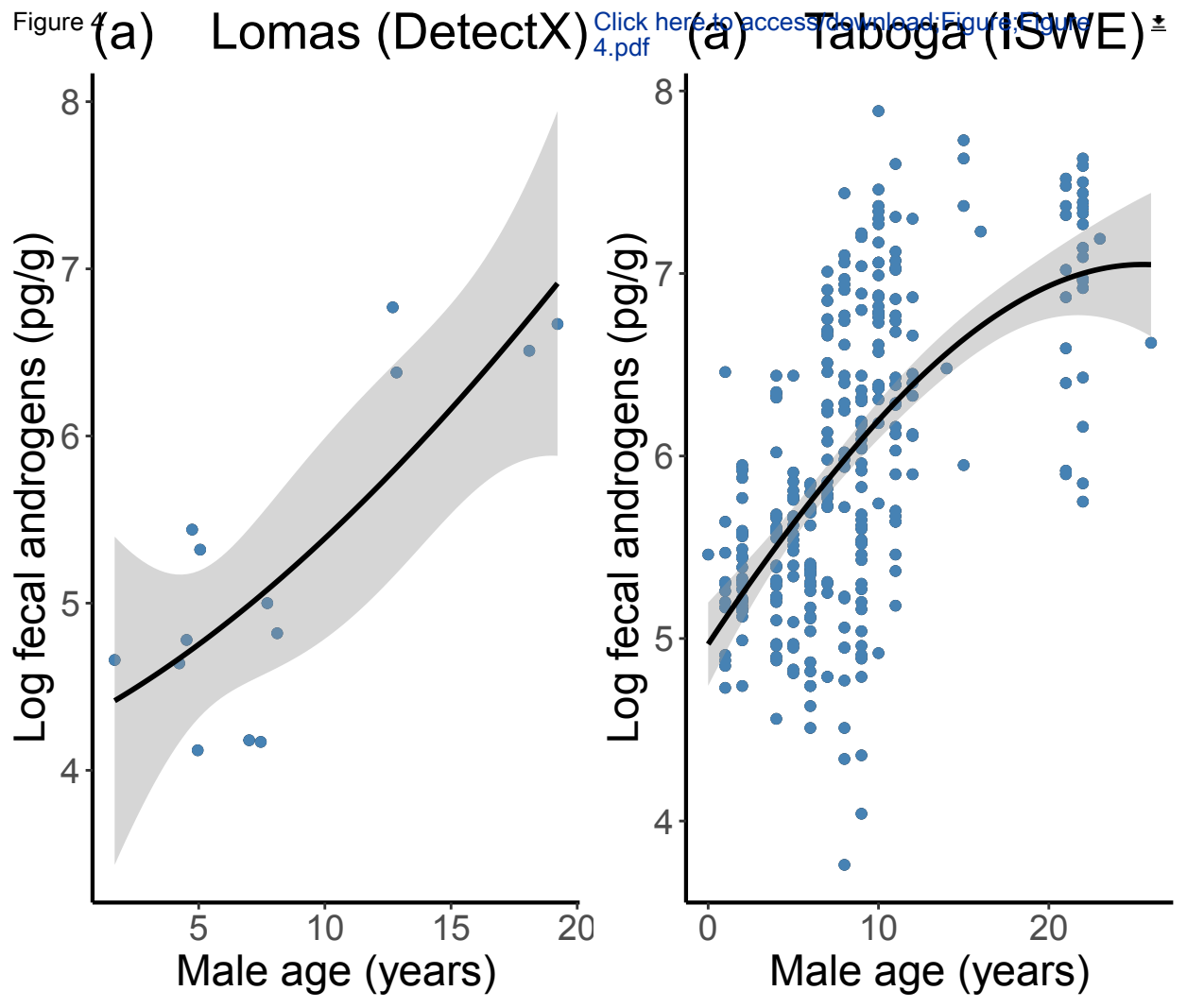

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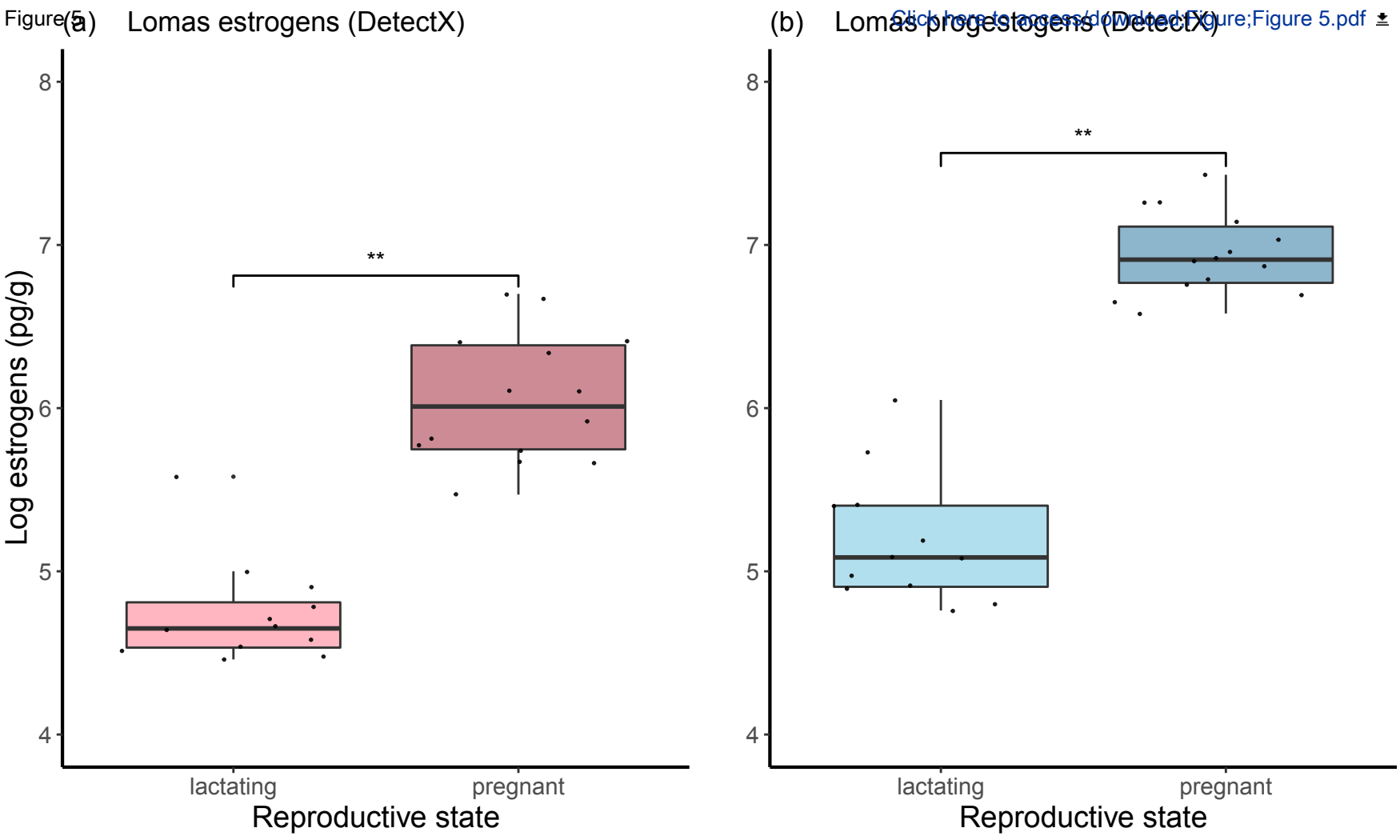

Table 1

Manuscript section	Hormone	Assay	Fieldsite	Laboratory	Test	Outcome
Solvolysis	Glucocorticoids	DetectX® CORT	Lomas	Michigan	solvolysis vs. non-solvolysis	solvolysis not needed
	Androgens	DetectX® T	Lomas	Michigan	solvolysis vs. non-solvolysis	solvolysis not needed
	Estrogens	DetectX® E2	Lomas	Michigan	solvolysis vs. non-solvolysis	solvolysis not needed
	Progestogens	DetectX® P4	Lomas	Michigan	solvolysis vs. non-solvolysis	solvolysis not needed
Analytical validation	Glucocorticoids	DetectX® CORT	Lomas	Michigan	parallelism, accuracy, precision	analytically validated
	Glucocorticoids	ISWE CORT	Taboga	Taboga	parallelism, accuracy, precision	analytically validated
	Androgens	DetectX® T	Lomas	Michigan	parallelism, accuracy, precision	analytically validated
	Androgens	ISWE T	Taboga	Taboga	parallelism, accuracy, precision	analytically validated
	Estrogens	DetectX® E2	Lomas	Michigan	parallelism, accuracy, precision	analytically validated
	Progestogens	DetectX® P4	Lomas	Michigan	parallelism, accuracy, precision	analytically validated
Biological validation	Glucocorticoids	DetectX® CORT	Lomas	Michigan	wet season vs dry season	higher in dry season
	Glucocorticoids	ISWE CORT	Taboga	Taboga	wet season vs dry season	no difference
	Glucocorticoids	ISWE CORT	Santa Rosa	Taboga	wet season vs dry season	higher in dry season
	Androgens	DetectX® T	Lomas	Michigan	across male ages	higher in adult males
	Androgens	ISWE T	Taboga	Taboga	across male ages	higher in adult males
	Estrogens	DetectX® E2	Lomas	Michigan	pregnant vs lactating	higher in pregnant females
	Progestogens	DetectX® P4	Lomas	Michigan	pregnant vs lactating	higher in pregnant females

Table 2

	Cortisol (DetectX®)	Cortisol (ISWE)	Testosterone (DetectX®)	Testosterone (ISWE)	17β-Estradiol (DetectX®)	Progesterone (DetectX®)
Cortisol	**100.00**	**100.00**	<0.02	<0.004	<0.10	
Dehydrocortisol	7.80	42.08				
Cortisone	1.20	26.53	<0.02	<0.004		
Dexamethasone	18.80	4.10				
Prednisone		3.37				
Corticosterone	1.20	0.35	<0.02	<0.004	<0.10	<0.10
Desoxycorticosterone		0.18				
Tetrahydrocorticosterone		<0.16				
Aldosterone				<0.04		
Testosterone	<0.10		**100.00**	**100.00**	<0.10	
5a-Dihydrotestosterone			56.80	35.40		
11-Ketotestosterone			2.34			
Androstenedione			0.27			<0.10
17β-Estradiol	<0.10		0.02		**100.00**	
Estrone	<0.10				0.78	
17α-Estradiol	<0.10				0.22	
17β-Estradiol				<0.004		
Progesterone	<0.10		<0.02	0.02	<0.10	**100.00**
3a-hydroxy-progesterone						188.00
3β-hydroxy-progesterone						172.00
11a-hydroxy-progesterone						147.00
5a-dihydroprogesterone					<0.10	7.00
Pregnenolone			<0.02			5.90
11β-hydroxy-progesterone						2.70

Table 3

HORMONE METABOLITE	INTRA-ASSAY CV (%) N	Low pool (60-80% binding)	High pool (20-30% binding)	INTER-ASSAY CV (%) N	Low pool (70-80% binding)	High pool (20-30% binding)
Glucocorticoids (DetectX®):	*6*	4.4	11.7	*6*	14.0	10.5
Glucocorticoids (ISWE):	*8*	4.1	4.2	*6*	6.8	6.6
Androgens (ISWE):	*6*	9.7	6.7	*18*	13.2	11.7
Androgens (DetectX®):	*6*	5.5	7.0	*3*	5.3	4.6
Estrogens (DetectX®):	*6*	13.6	7.5	*3*	1.2	7.9
Progestogens (DetectX®):	*5*	12.2	8.4	*3*	16.0	15.3

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