ESTRADIOL EXTRACTION AND QUANTIFICATION IN MACAQUES: DEVELOPMENTAND EVALUTION OF SAMPLING METHODS

MICHAEL LUBINSKY

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ABSTRACT

Ovarian hormones exert a strong influence on the brain; for example, estradiol (E2) has been found to modulate neuronal plasticity measured in vitro. To develop an understanding of how in vivo neuronal changes relate to the human brain, the non-human primate represents the closest animal model; however, no methodology is currently available for the continual, daily monitoring of E2 using non-human primates. The purpose of this thesis is to develop such a method. Daily sampling from 2 consecutive cycles in each of 3 rhesus macaques indicate that saliva can be used to detect the mid-cycle peaks in E2. Saliva-serum correlations were low, suggesting that further refinement will be needed to increase this method's applicability to measures that require off-peak daily E2 fluctuations. An example includes the measurement of E2 concentration in conjunction with electrophysiology, to monitor the influence of estradiol levels on neural activity.

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INTRODUCTION

Estradiol is the most potent and pervasive member of a class of steroid hormones called estrogens. In females, estrogen plays a significant role in the development of sexual secondary characteristics, regulation of gonadtropin secretion, maintenance of bone mass, regulation of lipoprotein synthesis, aversion of urogenital atrophy and regulation of insulin responsiveness. Recently, estradiol has been implicated in the maintenance of a variety of cognitive functions as well as neuroprotection and neural plasticity (Cagnacci et al., 1992). The neural mechanisms underlying these events have yet to be fully understood as a result of limitations in the ability to directly manipulate neural activity in humans, and in the differences in ovarian function across species. As I will describe, the macaque is an important animal model for understanding the influence of ovarian hormones on the human brain. Whereas in humans, several different methodologies have emerged for measuring ovarian hormones, such as saliva collection and dried blood spots (Lewis, 2006), currently there is no available method for the continual daily sampling of ovarian hormones using rhesus macaques. In what follows, I will describe estradiol, how it contributes to ovarian and brain function, and issues surrounding the sampling of estradiol.

Traditionally, estrogen has been cast as a "female hormone" most commonly associated with ovarian function, despite there being several extraglandular sites capable of synthesizing this hormone (Nelson and Bulun, 2001). These extraglandular regions include adipose, skin fibroblasts, bone and several different brain regions (Nelson and Bulun, 2001). These alternate sources may explain why estrogen has been implicated in physiological and patho-physiological roles in both men and women (Simpson et al., 1999).

Of the major classes of estrogens (estradiol (E2), estrone, estriol), E2 is the most dominant throughout a women reproductive life; Estradiol (E2) is a C18 steroid hormone that is approximately 10 times as potent as estrone and about 80 times as potent as estriol making it the predominant estrogen during a woman's reproductive years, both in terms of absolute serum concentrations and estrogenic activity (Cornil et al., 2006). Moreover,

during menopause when circulating E2 is no longer the most prevalent, the change in steroid concentrations occurs as a result of diminished E2 synthesis and not as a result of an increase in alternative estrogen synthesis (Simpson et al., 1999). In males, estrogen is produced as an active metabolic product of testosterone and plays a critical role in spermatogensis and preserving bone density and mineralization (Simpson et al., 1999). Taken as a whole, the influence that estradiol has on the brain and body can be characterized by the source of estrogen synthesis and the reproductive state of the body.

Systemic estradiol production through the HPG axis and the ovaries:

The hypothalamic-pituitary-gonadal axis is primarily responsible for the systemic production of estradiol. The hypothalamus releases gonadotropin-secreting hormone (GnRH), which stimulates the anterior pituitary to release both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (General Biology. 2009). These hormones arrive at the ovaries, which stimulate the production of steroid hormones such as estradiol. Depending on the phase of the menstrual cycle (or the amount of E2 present) the ovaries either stimulate or inhibit the response of the hypothalamus in a dynamic feedback loop thereby regulating estrogen production (Reproductive Biology. 2006). During the follicular phase of menstruation, FSH secrets increasing amounts of estrogen which in turn suppresses LH, resulting in the formation of a new layer of endometrium (Reproductive biology. 2006). During ovulation (when the egg has matured) estrogen begins to stimulate LH; these two opposing processes are thought to be mediated by two classes of different estrogen receptors, alpha and beta respectively, with the former controlling the negative feedback loop and the latter the positive one (Reproductive Biology, 2006). The increase in LH causes the release of the egg from the wall of the follicle, which eventually becomes the ovum (Reproductive biology. 2006). At the time of the luteal phase, large an amount of progesterone is secreted, causing a secondary rise of estrogen in terms of absolute serum concentrations (Reproductive biology. 2006). It is the fall of progesterone during this phase that ultimately triggers menstruation.

Estradiol's production in the ovaries begins in the internal cells of the ovaries (General Biology. 2009). After a side chain cleavage and using the delta-5 or the delta-4 pathway, synthesis of androstenedione occurs from cholesterol (Simpson. 1999). This compound crosses the basal membrane into the surrounding granulosa cells, where it is converted to estrone or estradiol, either immediately or through testosterone (Simpson. 1999). The conversion of testosterone to estradiol, and of androstenedione to estrone is catalyzed by the enzyme aromatase (Simpson. 2009).

Local estrogen biosynthesis:

I. Adipose and skin

A key factor in the local production of estrogen relates directly to the availability of precursor C19 steroids for the aromatization to estrogens in peripheral tissues (Labrie et al. 1998). Aromatase expressions in adipose and skin tissue are capable of producing estrogen in large enough concentrations to be measured in circulating blood (Nelson and Bulun. 2001). Hemsell et al (1996) were the first to demonstrate human adipose as a major source of estrogen production. They showed that age-related increases in estrogen concentrations were associated with increases in aromatase and mRNA activity in adipose tissue, specifically fibroblasts (Nimrod et al. 1975). Aromatase expression in adipose is under the control of promotor I,4 which is regulated by cytokines and glucocorticoids (Agarwal et al. 2006). Specific increases in aromatase expression were the result of increases in aromatse expression per fibroblasts that occurs with increasing age.

The skin also synthesizes estrogen. Aromatase in the skin is expressed predominantly in hair follicles and sebaceous glands, however the exact origin of cell types is not known (Sawaya et al. 1992). Furthermore, the expression of 17-b hydroxysteroid dehydrogenase type 1, the enzyme responsible for catalyzing the conversion between estrone to estradiol has been found in skin providing further support that both skin and adipose are significant sites of peripheral estrogen production (Nelson and Bulun. 1999).

II. Bone and Brain

Local estrogen production in bone plays a significant role in maintaining bone mineralization and the prevention of pathological conditions such as osteoporosis in both men and women (Simpson et al. 1999).

Estrogen can reach the brain via two pathways: the blood brain barrier or through aromatase found locally within the brain (Bake and Sohrabji. 2004). Enzymatic precursors necessary for estrogen production have been found in several different brain regions including the hypothalamus, prefrontal cortex, amygdala and hippocampus (Cornil et al. 2006). Estrogen effects in the brain would appear to underlie several notable changes in cognition and behavior. For example, ERT (estrogen replacement therapy) significantly improves cognition, reduces the risk of developing progressive dementia and decreases the severity of preexisting dementia (Yaffe et al. 1998).

Ovarian Hormone Sampling

Saliva:

Saliva is a variable and complex fluid playing a role in a plethora of biological functions ranging from oral health to homeostasis (Lewis. 2006). Saliva is not a simple ultrafiltrate of plasma; it has an active component requiring the formation of energy (Vining and McGinley. 2010). Salivary indexes of bioactivity depend critically on prior knowledge of salivary gland physiology, fluid composition and mechanism of formation. There are three major salivary glands that predominate the production of saliva; the paratoid, submandibular and sublingual (Simpson. 1999). Smaller portions of saliva are produced by buccal glands, which are numerous in the inner lining of the mouth (Vining and McGinley. 1987). Salivary flow rate can affect the nature of the compounds found in saliva. Differences are evident in terms of ionic concentrations and protein quantities but not certain hormones; because of the rapid passive diffusion of *steroid* hormones into the saliva ducts, steroid hormones themselves are not different depending on salivary flow

rate (Kalk et al. 2002). Moreover, not all saliva is produced through glands; some fractions of saliva may also contain fluid of a non-glandular derivation originating from the oropharingeal mucosa, upper airway secretions and gastrointestinal pathways (Naglar. 2002). Furthermore, salivary content and composition can show a significant discrepancy depending on whether or not saliva collection is basal or unstimulated with the latter being more similar in composition to plasma (Kalk et al. 2002; Chiappin et al. 2007). Stimulated saliva contains more calcium and bicarbonate, and has a higher pH than unstimulated saliva (Kalk et al. 2002; Chiappin et al. 2007).

Of critical importance, saliva also contains variable quantities of plasma exudates resulting from oral abrasions and gingival crevicular fluid, which can interfere with hormone assay quantification (Chiappin et al. 2007). In healthy individuals, quantities of these exudates are low (0.5%) but can be significantly increased in persons with oral disease such as gingivitis (Chiappin et al. 2007). Saliva may contain food debris, blood derived molecules such as proteins and erythrocytes and leucocytes in the cases of oral disease (Aps et al. 2005). There are a variety of mechanisms allowing compounds to reach the salivary fluid. These include ultrafiltration through gap junctions whereby small molecules with low molecular weight such as water, ions and steroid hormones are permeable and their salivary concentrations generally are 300-3000 times lower than those observed in plasma (Chiappin et al. 2007). Plasma contents such as albumin can reach the oral cavity through the cervicular fluid or directly from the oral mucosa (Chiappin et al. 2007). 3) Transport through cellular membranes through diffusion (passive) or actively through protein channels can also account for the presence of various compounds found in saliva (Chiappin et al. 2007). Saliva is composed of six principle compounds: inorganic, organic, protein/polypeptide, lipids and hormone (Chiappin et al. 2007).

Inorganic compounds:

The composition of saliva is predominately made up of water and weak/strong ions. The principal secretion of fluid by salivary glands is plasma ultrafiltrate but in the salivary ducts themselves there is an active energy dependant recycling of Na+ and Cl resulting in fluids with a significantly lower ion concentrations relative to plasma. The presence of mineralcorticoid receptors in salivary ducts results in higher levels of salivary K+ ions and lower levels of Na+ ions compared to plasma (APS, JKM. 2005). Ions found in saliva include Na+, K+, Cl, Ca2+, Hco3, Mg2+, Nh3 and their concentration is dependant on whether or not saliva collection was basal or stimulated (Agha-Hosseini. 2006).

Organic compounds:

Non-proteins such as uric acid, bilirubin and creatinine are also present in saliva in small quantities. Lipids such as cholesterol and fatty acids such as alpha-linoleic and archidonic acid can also be detected. The concentration of fatty acids is positively correlated with dietary fatty acid intake (Agha-Hosseini. 2006).

Protein/polypeptide compounds:

Numerous different proteins have been identified in whole saliva; the most readily identified proteins are amylase, PRPs secretory IgA, and albumin (Chiappin et al. 2007). Functionally, proteins such as lysozyme, lactoferrin, lactoperoxidase, immunoglobins and mucins collectively protect the oral cavity; other proteins containing bacteria killing properties such as histatins and defensins play a broader role in maintaining overall health. The physiological circumstances in which saliva is collected can impact the concentrations of these proteins especially the immunoglobins.

Hormones:

Significance of hormone detection in saliva is completely dependant on the correlation between circulating levels in plasma and that found in the mouth. A-priori knowledge of the connection between free unbound and total-circulating fractions together with their specific binding proteins is needed before any salivary index of bioactivity can be established. Steroid hormone detection is the most common salivary application as several studies have previously found high correlations between fractions found in both serum and saliva (Chatterton et al. 2005). The most frequently studied salivary hormones include cortisol, testosterone, DHEA progesterone and estradiol (Chiappin et al. 2007).

Steroid hormones in saliva: Estradiol (E2)

For almost 30 years salivary methods have been used to assess ovarian hormone function. Of critical importance for hormone application is the "bioavailibility" of that compound. The majority of steroid hormones in blood (95%-99%) are bound up by binding proteins such as sex hormone binding globulin (SHBG), cortisol binding globulin (CBG), and albumin. The remaining unbound fraction (1-5%) is commonly referred to as "free" hormone fraction, and is generally regarded as "bioavailable" (Read et al. 2006). Estradiol has been one of the most researched hormones to date and several studies have consistently demonstrated that estradiol can be precisely quantified in saliva and that salivary concentrations show high correlations with circulating serum fractions (Belkien, Bordt, Moller, Hano, and Nieschlag, 1985; Choe, Khan-Dawood, and Dawood, 1982; Lipson and Ellison, 1996; Lu, Bentley, Gann, Hodges, and Chatterton, 1999; O'Rourke and Ellison, 1993; Worthman, Stallings, and Hofman, 1990). For example, relatively high correlations between E2 in saliva and xxx (Shirtcliff et al., YEAR). Saliva and serum fractions show correlations as high as r=0.76 (Worthman et al 1990). Others have

reported salivary and serum correlations between 0.70-.80 (Lu et al., 1999). Salivary E2 concentrations are considerably lower than serum concentrations and are estimated to be between 0.20 and 7.90% of that in the blood circulation (Lu et al., 1999). The difference in concentrations may lead to some of the unexplained variance in the sample correlations, if saliva concentrations border the lower limits of detection. Although concentrations in saliva are lower, the estrogenfound in saliva is unbound or "free" and is reflective of the bioavailable portion found in general circulation (Lu et al., 1999). Whereas this may make the saliva measurements more relevant, it could further introduce variability between serum and saliva sampling methods.

The range of salivary estradiol concentrations is 0.8-14.3 pg/ml compared to the luminescent assay sensitivities which have lower detection limits of 0.3 pg/ml of E2.No salivary E2 index is avaialble for non human primates but given the similarities in reproductive features there is no reason to believe that non human primates will differ from humans with regard to salivary E2 concentrations.

Dried blood spots: Estradiol

Dried blood spots (DBS) was first introduced in the 1960's by Dr. Robert Guthrie who collected heel-prick blood spots in infants as a way of detecting phenylketonuria (Guthrie et al. 1963). Since its introduction DBS has been used in a variety of clinical settings to study various proteins and hormones (McDade et al., 2007). Similar to saliva, DBS has the advantage of being minimally invasive and having a repeatable nature of sampling relative to venipuncture. Whole blood collected from DBS is similar to blood collected via venipuncture with some notable exceptions: Firstly, whole blood is composed of a variety of liquid and cellular components, and centrifugation of these samples after venipuncture removes cellular components to produce serum. Whole blood dried on filter paper contains these components, which rupture upon reconstitution. This requires that prior information regarding the sensitivity of interference of the compound in question is required before clinical usefulness can be established. DBS is relatively

straightforward and sample collection can be done with minimal training unlike venipuncture which requires advanced skill in phlebotomy. Blood spot sample collection, has been used to reliably measure thyroid hormones (Waite, Maberly, and Eastman, 1987), prolactin (Bassett, Gross, and Eastman, 1986; Worthman, Stallings, and Gubernick, 1994), androstenedione (Thomson, Wallace, and Cook, 1989), cortisol (Kraiem, Kahana, Elias, Ghersin, and Sheinfeld, 1980), and steroid hormones including estradiol (Shirtlcliff et al. 2000 and (Worthman and Stallings, 1997). Similar to salivary indices of bioactivity, for DBS to have both research and clinical applications it must show high correlations with that of serum/plasma, which are the most common fluids used to quantify hormones. Worthman and Stallings (1997) report an extremely high correlation between estardiol collected from DBS and that from venipuncture (r=0.98); however, the results have been criticized on the account that scant amounts of information were available to guide researchers wanting to replicate their results. Shirtlcliff and colleagues (year) more recently replicated these results and demonstrated that E2 can be accurately measured and quantified using DBS reporting a correlation of r=0.96.

Given that estradiol has been measured using saliva and DBS of humans, we hope to apply these methods to non-human primates for the daily monitoring of ovarian hormones across the menstrual cycle. The purpose of this study is to apply both salivary and dried blood spot methods to non-human primates.

Non-human primates represent the animal model that most closely matches humans both in terms of neural structures, biochemistry, and reproductive system. Female rhesus macaques have comparable reproductive cycles to human females with a mean cycle length of 33 days (McDade 2007). Given the similarities in reproductive anatomy and nervous system structure, and their regular use as animal models in studies of neural function, rhesus macaques are the ideal animal models when trying to uncover the role steroid hormones are likely to have on the human brain. The majority of literatures pertaining to ovarian hormones have predominately used rodents as the chosen animal

model. Given the drastic differences between the reproductive systems of rodents and primates (rodents have estrous cycles) and as a result little extrapolation to humans can be done. Rehsus macaques like humans have 30 day menstrual cycles and comparable reproductive features making them far more similar then the nearesy common animal model. To my knowledge no methods are currently available for the continuous daily sampling of ovarian hormones *in vivo* using rhesus macaques. The gold standard for quantifying hormones using non-human primates (NHPs) is venipuncture. Given the number of viable veins available for sampling, bruising associated with the procedure and the use of sedatives (which can confound the biochemistry) venipuncture isn't a reasonable option for the daily monitoring of ovarian hormones *in vivo* over any extended period of time. This poses a significant barrier for physiologist's observing neural recordings daily over the course of months who are interested in monitoring hormone levels during recordings. The development of both salivary and dried blood spot methods would significantly benefit physiologists, making it possible for the first time to simultaneously monitor how day-to-day fluctuations at the endocrine level influence the neural signal and consequent behaviour.

MATERIALS AND METHODS

Subjects

Three adult female rhesus macaques were tested (LU, AN, RI, all 6 years old). Procedures were approved by the York University Animal Care Committee and were performed in accordance with the Canadian Council on Animal Care.

Training: Saliva

Salivary habituation was performed incrementally through increased levels of contact with the NHP. Initially, the first level consisted of the animal becoming accustomed to variable epochs of direct human contact. The macaque was seated comfortably with only the back portion of the NHP chair (Crist Instruments) opened; this allowed the NHP to move their hands and feet and rotate or make postural adjustments while at the same time restricting locomotion. The animal was trained to present their hands and feet and hold that position for 5-60 seconds in order to receive a fruit reward. At first, the animal was required to have her limb extended for a minimum of 5s without retraction. Retractions are defined as any pulling back against the contact, but the same procedure hold for sudden movements of the hands or limbs including swatting and grabbing before the command to release contact. During limb presentation, finger manipulations and hand/foot massages were given to accustom the NHP to direct human physical contact. With each successive trial, an additional 5 seconds was added until a maximum of 60 seconds was reached without any retractions. If errors occur, the trial begins from the previous success trial and no reward is given. For example, if the macaque behaves well for 45s then decides to retract, the previous 40s trial is repeated. Once the animal was able to passively present their limbs for a total of one minute without retraction habituation ended and the next level of contact was pursued. The next stage involved head and face habituation.

Direct head/face manipulation was accomplished without the use of a salivary collection device. Following a similar protocol used for hand/foot contact, I would touch

the head with both my hands for 5s and immediately provide a fruit reward if no aggressive gestures were made. Aggressive gestures were defined as attempted biting, open mouth threats and sudden head jerks. Once the NHP was accustomed to contact around the head and mouth region the salivary collection device was introduced. Initially, the device was not put into the primate's mouth; habituation involved placing the device on the outside of the mouth while simultaneously grasping the head for support. For each trial the primate received a fruit reward for good behavior.

Once the animal was comfortable with the saliva collection device on the exterior, attempts were made to insert the collection device into the interior pouch of the primate's mouth. Habituation began by grasping the NHP head for support and inserting the absorbent foam material directly inside the deep pouch for 5s after which a fruit reward was immediately given. Both sides of the mouth were sampled (5s each side) after which a short 3 minute break was given to accustom the NHP to the time needed to centrifuge the absorbent material between saliva collections. As was the case for the previous protocols, an additional 5 seconds was added each time the animal was successful until a maximum of 60s was reached without any retractions or aggressive gestures. During the course of saliva collection the animal needs to learn to behave in the absence of an immediate fruit reward. During the collection of saliva, no food or drink is given to the animal to avoid sample contamination. The same protocol was repeated with the exception that a fruit reward was not given immediately after each insertion; a large fruit reward was given each 15s that the absorbent material was inside the NHP mouth until a maximum of 60s was reached without the need for reward. This was repeated 4 times consecutively allowing the animal to get accustomed to the length of time needed to obtain the desired quantity of saliva. By the end of training the NHP did not receive any immediate rewards rather received a "lump sum" reward consisting of an entire fruit bowl.

Collection of saliva

Previously unused 5ml polyethylene tubes were labeled and marked with a 1ml fill line; this ensured that the desired volume required for assaying samples in duplicate was obtained. Saliva was collected using specialized salivary absorbent material (SUPERSAL, Oasis Diagnostics Corporation, Vancouver). Absorbent material was attached to a 5-inch metal rod and securely tied using dental floss (Figure 1). Subjects received 5ml of cold water prior to saliva collection that was orally administered via a 5CC syringe to remove any particles of food left over from the previous night. Supersal absorbent material was inserted into the deep interior pouch of the NHP for 1-minute intervals. Both the left and right pouch was sampled in duplicate (total of 4 insertions) to obtain the required 500ul-1ml volume of saliva. After each trial the absorbent foam was removed from the animal's mouth and immediately centrifuged at 2000 x g for 4 minutes, to remove the saliva contents from the absorbent material. Saliva samples were collected daily for the duration of an entire menstrual cycle (~28 days). No samples were processed fresh; instead they were all immediately frozen and subsequently thawed for assay. On the day of the assay, samples were centrifuged at (2000 x g, 15min) in order to break down the mucopolysaccharides that can impede accurate pipetting.

Saliva assay procedure

Extraction of salivary hormone estradiol (E2) was accomplished using a commercially available human estradiol luminescence assay (AFFINITY Diagnostics Corp., Toronto) without the use of any modifications. Prior to assay, all materials, chemicals and reagents were allowed to reach room temperature (25 degrees Celsius) and vortexed to ensure sufficient mixing. All samples were processed in duplicate to identify potential pipetting errors. The wells of the microtiter plate are coated with donkey anti-sheep antibody. 50μl of the standards, controls and saliva samples were pipetted into their respective wells. 50μl of freshly prepared enzyme conjugate (containing alkaline phosphatase conjugate, NaN3) is then added to each well in addition to 50μl of estardiol antiserum (containing 17-β-estradiol sheep antibodies, NaN3) before being covered by an

adhesive foil. The wells are incubated on an orbital shaker (400-600 rpm) at room temperature for 4 hours to allow for maximal binding. Incubation solution is then discarded and the plate is washed X4 with 250μl of wash buffer (containing Tris buffer, Tween, NaN3). Excess solution is additionally removed by tapping the inverted microtiter plate on a lint free paper towel. 50μl of chemiluminescence reagent AP (acridan based substrate) was added into each well following a specific order and time delay. The relative luminescence unit (RLU) was then measured with a luminometer within 10 minutes of substrate addition, following the same specific order and time delay. The obtained RLU of the standards (y-axis, linear) are plotted against their concentration (x-axis, logarithmic) using prism (figures 1-6). The concentration of the samples can be read directly from the standard curve. The amount of hormone in the sample is inversely proportional to the RLU detected by the plate reader.

Collection of serum: venipuncture

The collection of whole blood was carried out by a licensed veterinary technician. All blood was collected via the femoral vein located on the animal's thigh. The NHP was sedated using dexdomitor (dexmedetomidine) and α2-agonist, administered according to the manufacturer's preset standards (based on the animals weight), in addition to 0.2 ml of ketamine (well below actual calculated dosage). 2-3 ml of whole blood was collected and stored in a 5ml red-topped BD vacutainer with no additional additives. The animal was given antisedan (atipamezole) α2-antagonist in an equivalent dose as the dexdomitor to assist with the animal's recovery. Whole blood was then allowed to sit at room temperature for 10 minutes in order to allow for clotting. Blood was then immediately centrifuged for 15 min at (2000X g, 15min) to allow for serum separation. Serum was then extracted using a micropipette and stored at -20 degrees Celsius until day of assay. Animals were sedated semiweekly for one month yielding a total of 8 serum samples. No samples were processed fresh; instead they were all frozen and thawed on day of assay.

Serum assay procedure

Extraction of serum hormone estradiol (E2) was accomplished using a commercially available human estradiol ELISA (enzyme linked immunosorbent assay) provided by (Calbiotech Inc, Spring Valley CA) without the use of any modifications. Prior to assay, all materials, chemicals and reagents were allowed to reach room temperature (25 degrees Celsius) and vortexed to ensure sufficient mixing. All samples were processed in duplicate to be able to identify potential pipetting errors. 25μl of standards, controls and samples were allocated into the microtiter plate coated with goat anti-rabbit IgG antibodies. 100μl of estradiol-HRP conjugate and 50μl of rabbit anti-estradiol reagent were also added to each well before being covered by an adhesive foil and incubated at room temperature for 90 minutes. Incubation solution was then discarded and the plate was washed X3 with 300μl of wash buffer. Excess solution was additionally removed by tapping the inverted microtiter plate on a lint free paper towel. After incubation, 100μl of TMB reagent was added to each well and gently mixed for 10 seconds and incubated for 20 minutes at room temperature. The reaction was stopped by the addition of 100μl of stop solution to each well. The absorbance was measured spectrophotometrically at 450 nm within 15 minutes of applying the stop solution. Similar to the salivary assay, a standard curve was obtained by plotting the concentration of the standard (x-axis) versus the absorbance (y-axis). The amount of hormone in the sample is inversely proportional to the optical density detected by the plate reader.

Training: DBS

Salivary methods were established first, eliminating the need for incremental levels of contact needed to accustom the animal to direct human contact (refer above). The macaque was seated comfortably with only the back portion of the chair opened. The primate was trained to present her hands and feet initially for 2 min; a single digit is isolated and cleaned with alcohol. On a “count of 3”, the digit is then gently pinched (to simulate the lancet device) after which a fruit reward is immediately given. The next stage involved training the primate to keep their limbs extended after delivery of the

noxious stimulus. After the pinch, the animal is trained to keep their limbs extended without retraction for a total of 2 minutes during which the finger is manipulated above a protein saver card that stores the blood drops. This was achieved by positively reinforcing the animal with a fruit reward for every successful 30s that the limb was extended without retraction. To accustom the macaque to the lancet device, variable needle depths were used (figure 2). The procedure is then repeated with the notable difference that the pinch is now substituted for the real lancet device. At first a small 28G needle depth (Unistik 2 – yellow) is used; this is not adequate for drawing blood but was used to habituate the primate to the lancet device (table 1). This procedure was repeated until the animal was able to successfully complete the 4 min requirement needed to fill each blood spot. Next a 23G lancet was introduced (Untistik 2 – blue) which was successful in drawing small amounts of blood. The same procedure was repeated until the primate was able to complete the entire 4 minutes without any retraction. Next, a 21G lancet (Surgilance – Pink) was used to accustom the animal to a deeper lancet device before using the 18G lancet which was used for sample collection (see Table 1).

Table 1: Lancet device technical specifications

Supplier	Gauge	Depth	Color	Blood Flow
Unitsick 3	28	2.2	Yellow	10-20ul
Surgilance	21	2.8	Pink	40-60ul
Surgilance	18	2.3	Blue	150-200ul

Collection of whole blood: Dried blood spot (DBS)

Dried blood spot samples are collected on specialized filter paper cards (e.g., Whatman Protein Saver Cards). The cards are standardized to ensure blood is absorbed in a homogenous manner. This guarantees that all identical punches (3.5 mm whole punch) will provide an equal amount of blood. The subject's digit is sterilized using both a sterile cotton gauze and alcohol. The finger is then pricked using an automated glucose lancing device designed specifically to minimize pain and discomfort. When the blood begins to flow the first drop is wiped away as it may contain possible sources of contamination such as tissue fluids. The remaining drops are then allowed to freely drop onto the protein saver card. It is important not to physically touch the digit to the filter paper. Only one drop of blood is collected per dry blood spot. Protein saver cards are then placed in a gas impenetrable bag with a desiccant. Samples are then stored at -20 degrees Celsius until assay. Analysis of DBS samples was beyond the scope of this thesis; here, I established that collection procedures for DBS are a viable alternative to saliva collection procedures.

Analysis

Standard curves were generated individually for both saliva and serum assays (12 in total). A two-phase exponential decay was used to best fit the data and to extrapolate samples values from the standard curve using Graphpad (Prism). The precision of the assays was calculated using both the inter/intra coefficients of variation (see Table 2). Peaks would be considered consistent if they occurred within a few days of the same date following the onset of the last cycle. Pearson's correlation coefficient was then used to determine the degree of correlation between saliva and serum samples that were collected on the same days.

RESULTS

Saliva samples were collected daily for the duration of two consecutive menstrual cycles. Each menstrual cycle consisted on average of a 30-day cycle followed by menses. In total 180 salivary samples and 48 serous blood samples were analyzed and quantified for E2 (see table 1). Standard curves were highly repeatable, yielding a mean correlation of r=0.98 for saliva and r=0.99 for serum respectively (see figures 1-7). Serum-saliva correlations varied depending on the individual; NHP 1 and 2 (AN and RI) showed the highest serum-saliva correlation r=0.53 and r=0.49 respectively, whereas NHP 3 (LU) showed the lowest correlation r= 0.19. Despite weak correlations, E2 peaks were consistently observed mid-cycle for all three non-human primates (see Figure 8). The precision of the assays can be seen in the inter/intra coefficients of variation (see Table 2).

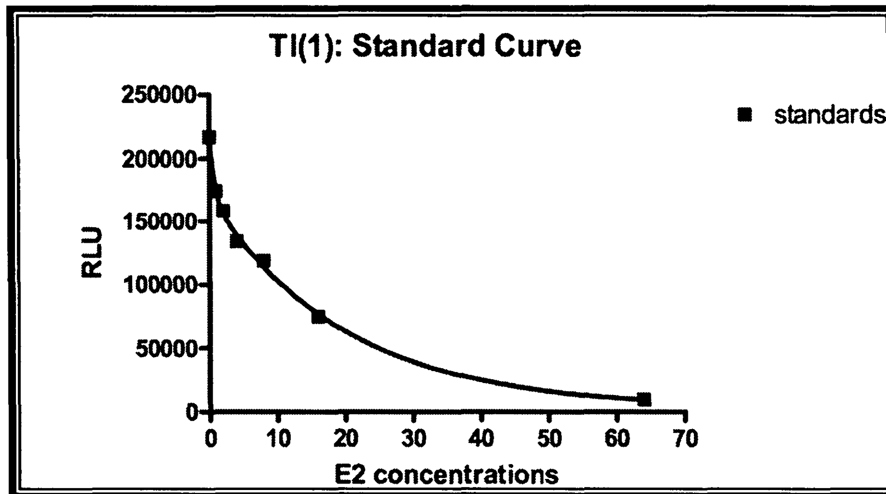
Table 2: Salivary and serum assay precision. CV: coefficient of variation, (%).

NHP	Inter-assay CV	Intra-assay CV
NHP 1 (TI)	11.7	8.9
NHP 2 (RI)	22.4	5.7
NHP 3 (LU)	15.3	6.0

Table 3: Saliva-Serum Sampling Dates

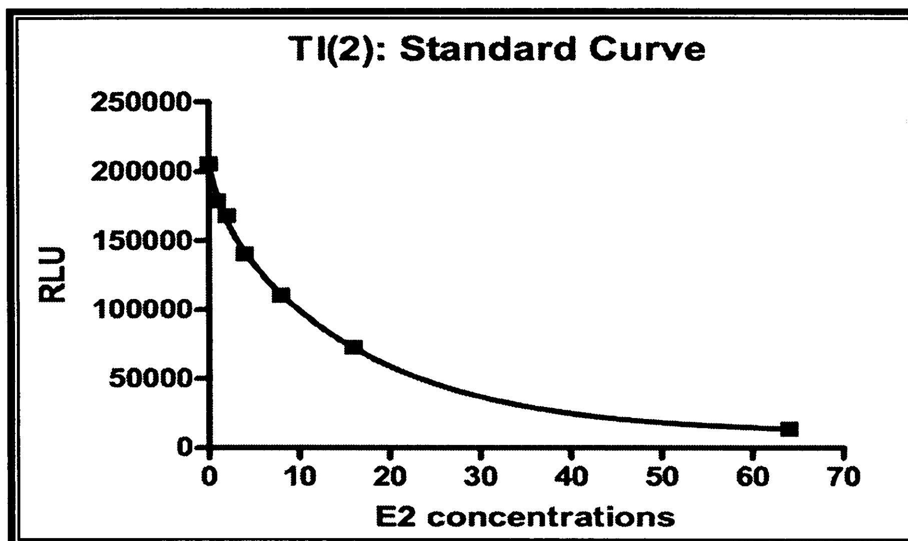
NHP	Saliva start/end date	Serum sample	DBS start/finish
Antigone:(NHP1)	Start: January 25th 2011 Finish: December 25th 2011	Semi-weekly	Start: January 25th 2011 Finish: December 25th 2011
Ripley: (NHP2)	Start: January 25th 2011 Finish: December 25th 2011	Semi-weekly	Start: January 25th 2011 Finish: December 25th 2011
Lucy: (NHP3)	Start: January 25th 2011 Finish: December 25th 2011	Semi-weekly	Start: January 25th 2011 Finish: December 25th 2011

Figure 1. Salivary standard curve for the first cycle of NHP1(Antigone). (Top) The standard curve depicting the relative luminance units observed as a function of standardized estrogen concentration, in pg/ml. (Bottom) curve fit results.



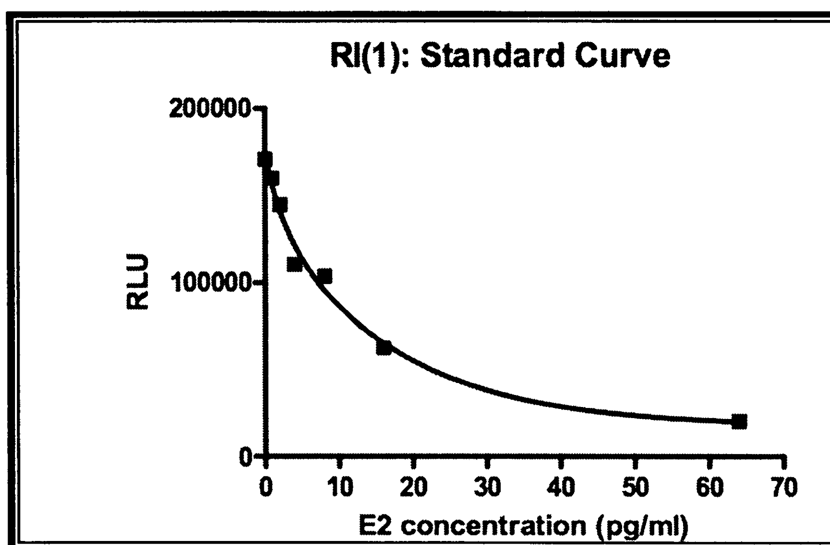
Two phase exponential decay	
Best-fit values	
SPAN1	166774
K1	0.05094
SPAN2	46884
K2	1.438
PLATEAU	2962
Halflife_1	13.61
Halflife_2	0.4819
Std. Error	
SPAN1	8812
K1	0.008580
SPAN2	9930
K2	0.7080
PLATEAU	7603
95% Confidence Intervals	
SPAN1	128858 to 204690
K1	0.01402 to 0.08786
SPAN2	4154 to 89614
K2	0.0 to 4.485
PLATEAU	-29750 to 35677
Halflife_1	7.889 to 49.45
Halflife_2	
Goodness of Fit	
Degrees of Freedom	2
R^2	0.9980

Figure 2. Salivary standard curve for the second cycle of NHP1(Antigone). (Top) The standard curve depicting the relative luminance units observed as a function of standardized estrogen concentration, in pg/ml. (Bottom) curve fit results.



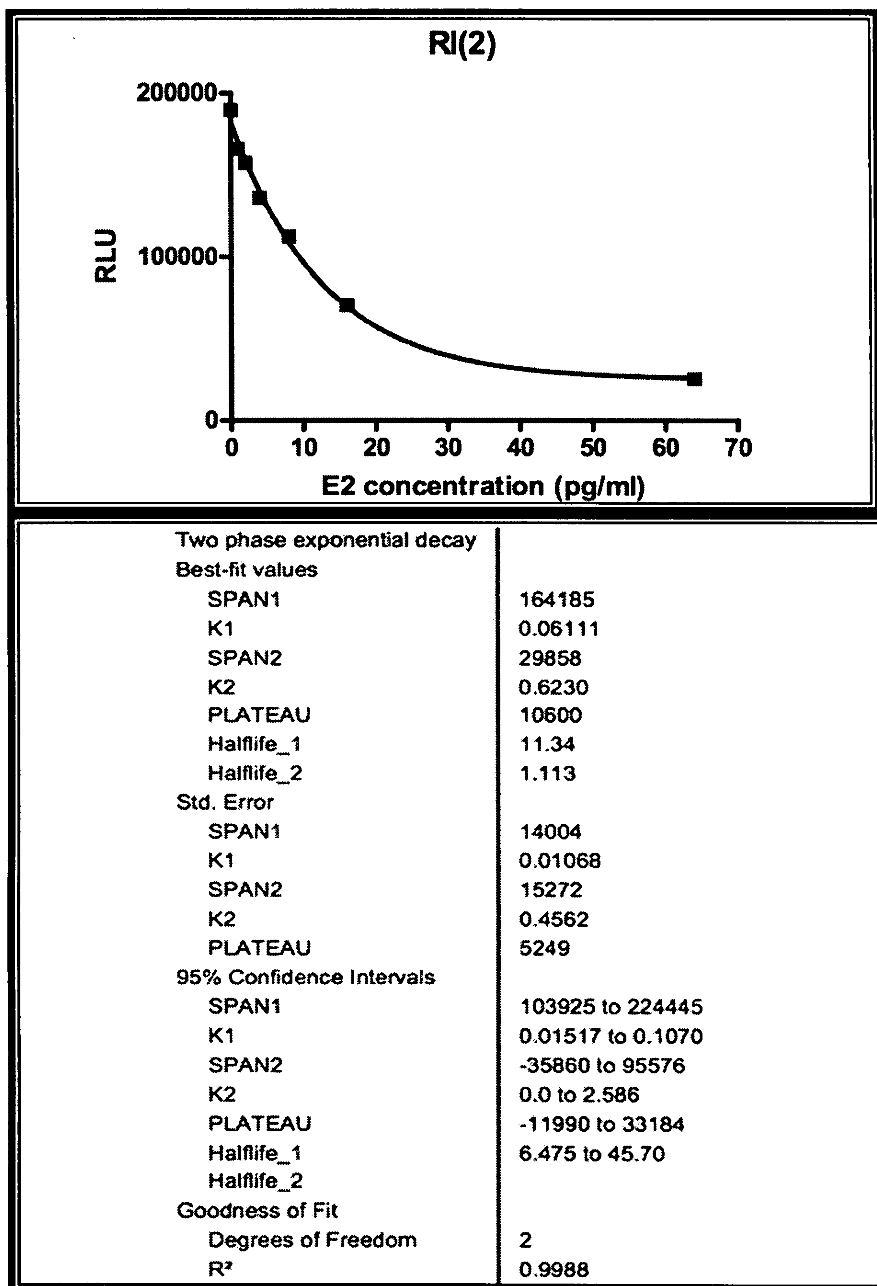
Two phase exponential decay	
Best-fit values	
SPAN1	164185
K1	0.06111
SPAN2	29858
K2	0.6230
PLATEAU	10600
Halflife_1	11.34
Halflife_2	1.113
Std. Error	
SPAN1	14004
K1	0.01068
SPAN2	15272
K2	0.4562
PLATEAU	5249
95% Confidence Intervals	
SPAN1	103925 to 224445
K1	0.01517 to 0.1070
SPAN2	-35860 to 95576
K2	0.0 to 2.586
PLATEAU	-11990 to 33184
Halflife_1	6.475 to 45.70
Halflife_2	
Goodness of Fit	
Degrees of Freedom	2
R^2	0.9988

Figure 3. Salivary standard curve for the first cycle of NHP2 (Ripley). (Top) The standard curve depicting the relative luminance units observed as a function of standardized estrogen concentration, in pg/ml. (Bottom) curve fit results.



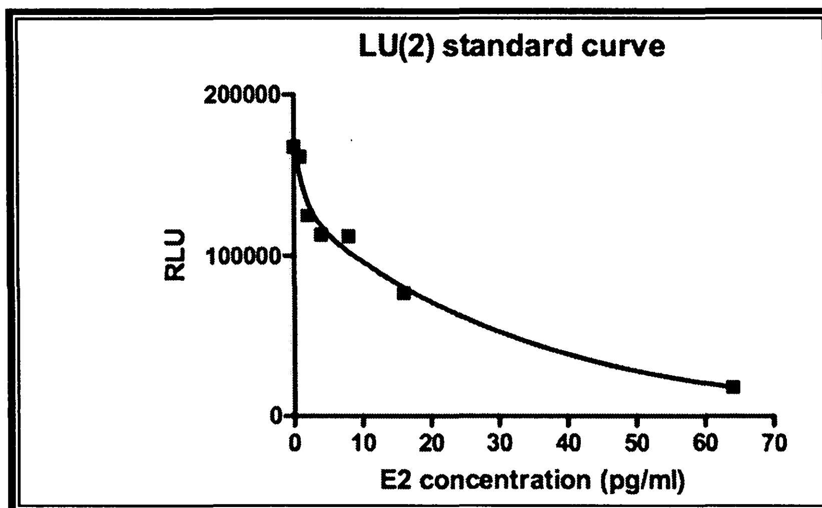
Two phase exponential decay	
Best-fit values	
SPAN1	125251
K1	0.06018
SPAN2	30825
K2	0.4393
PLATEAU	17455
Halflife_1	11.52
Halflife_2	1.578
Std. Error	
SPAN1	59890
K1	0.04906
SPAN2	64649
K2	1.074
PLATEAU	14628
95% Confidence Intervals	
SPAN1	-132500 to 382958
K1	0.0 to 0.2713
SPAN2	-247400 to 309009
K2	0.0 to 5.062
PLATEAU	-45490 to 80401
Halflife_1	
Halflife_2	
Goodness of Fit	
Degrees of Freedom	2
R^2	0.9876

Figure 4. Salivary standard curve for the second cycle of NHP2 (Ripley). (Top) The standard curve depicting the relative luminance units observed as a function of standardized estrogen concentration, in pg/ml. (Bottom) curve fit results.



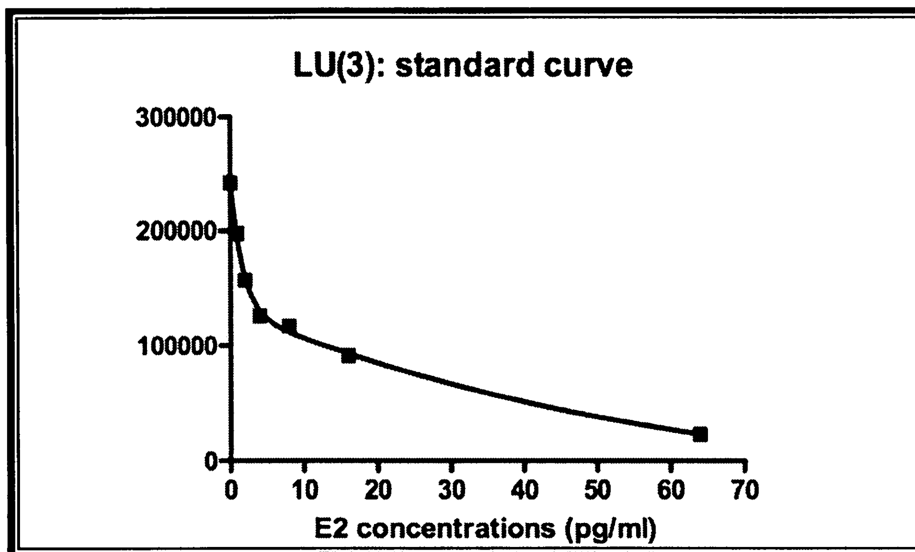
Two phase exponential decay	
Best-fit values	
SPAN1	164185
K1	0.06111
SPAN2	29858
K2	0.6230
PLATEAU	10600
Halflife_1	11.34
Halflife_2	1.113
Std. Error	
SPAN1	14004
K1	0.01068
SPAN2	15272
K2	0.4562
PLATEAU	5249
95% Confidence Intervals	
SPAN1	103925 to 224445
K1	0.01517 to 0.1070
SPAN2	-35860 to 95576
K2	0.0 to 2.586
PLATEAU	-11990 to 33184
Halflife_1	6.475 to 45.70
Halflife_2	
Goodness of Fit	
Degrees of Freedom	2
R^2	0.9988

Figure 5. Salivary standard curve for the first cycle of NHP3 (Lucy). (Top) The standard curve depicting the relative luminance units observed as a function of standardized estrogen concentration, in pg/ml. (Bottom) curve fit results.



Two phase exponential decay	
Best-fit values	
SPAN1	132737
K1	0.02919
SPAN2	41849
K2	0.6505
PLATEAU	-2639
Halflife_1	23.74
Halflife_2	1.066
Std. Error	
SPAN1	39816
K1	0.04194
SPAN2	38399
K2	0.9589
PLATEAU	65672
95% Confidence Intervals	
SPAN1	-38590 to 304063
K1	0.0 to 0.2097
SPAN2	-123400 to 207078
K2	0.0 to 4.777
PLATEAU	-285200 to 279946
Halflife_1	
Halflife_2	
Goodness of Fit	
Degrees of Freedom	2
R^2	0.9769

Figure 6. Salivary standard curve for the second cycle of NHP3 (Lucy). (Top) The standard curve depicting the relative luminance units observed as a function of standardized estrogen concentration, in pg/ml. (Bottom) curve fit results.



Two phase exponential decay	
Best-fit values	
SPAN1	167070
K1	0.01644
SPAN2	112376
K2	0.6167
PLATEAU	-35250
Halflife_1	42.17
Halflife_2	1.124
Std. Error	
SPAN1	98782
K1	0.02077
SPAN2	16782
K2	0.1602
PLATEAU	113500
95% Confidence Intervals	
SPAN1	-258000 to 592130
K1	0.0 to 0.1058
SPAN2	40165 to 184587
K2	0.0 to 1.306
PLATEAU	-523600 to 453138
Halflife_1	
Halflife_2	
Goodness of Fit	
Degrees of Freedom	2
R^2	0.9974

Figure 7.Serum estradiol concentration as a function of cycle sample number. Results are shown from NHP1 in green, NHP2 in blue and NHP3 in red.

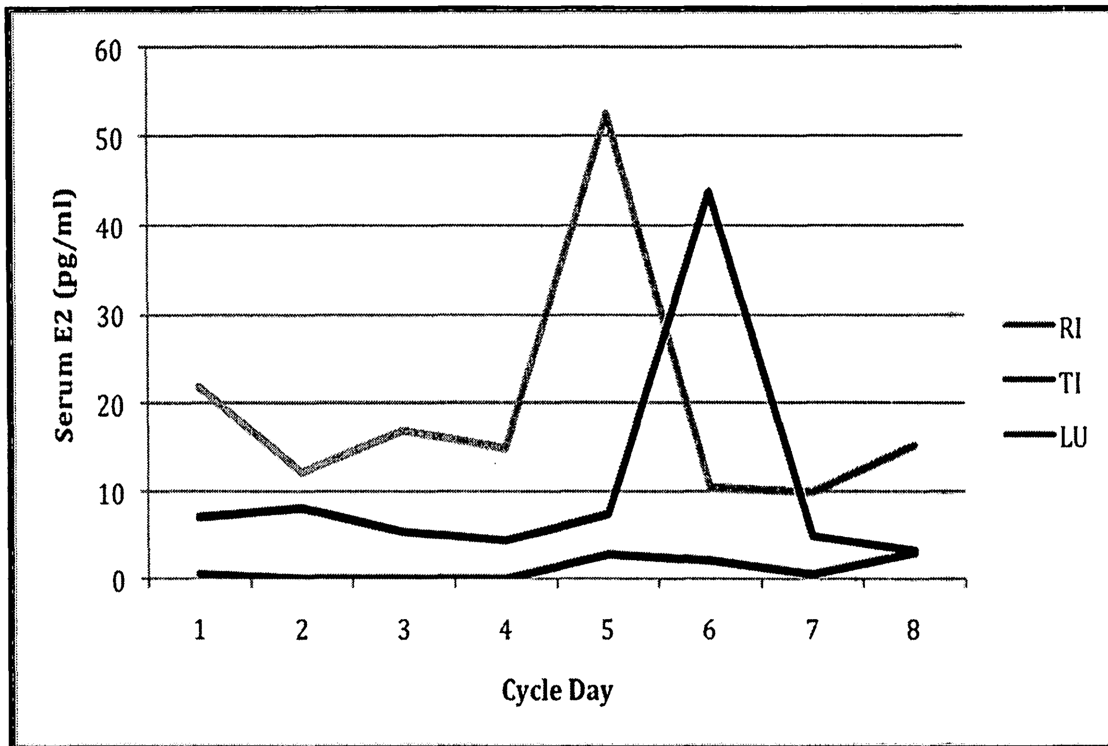
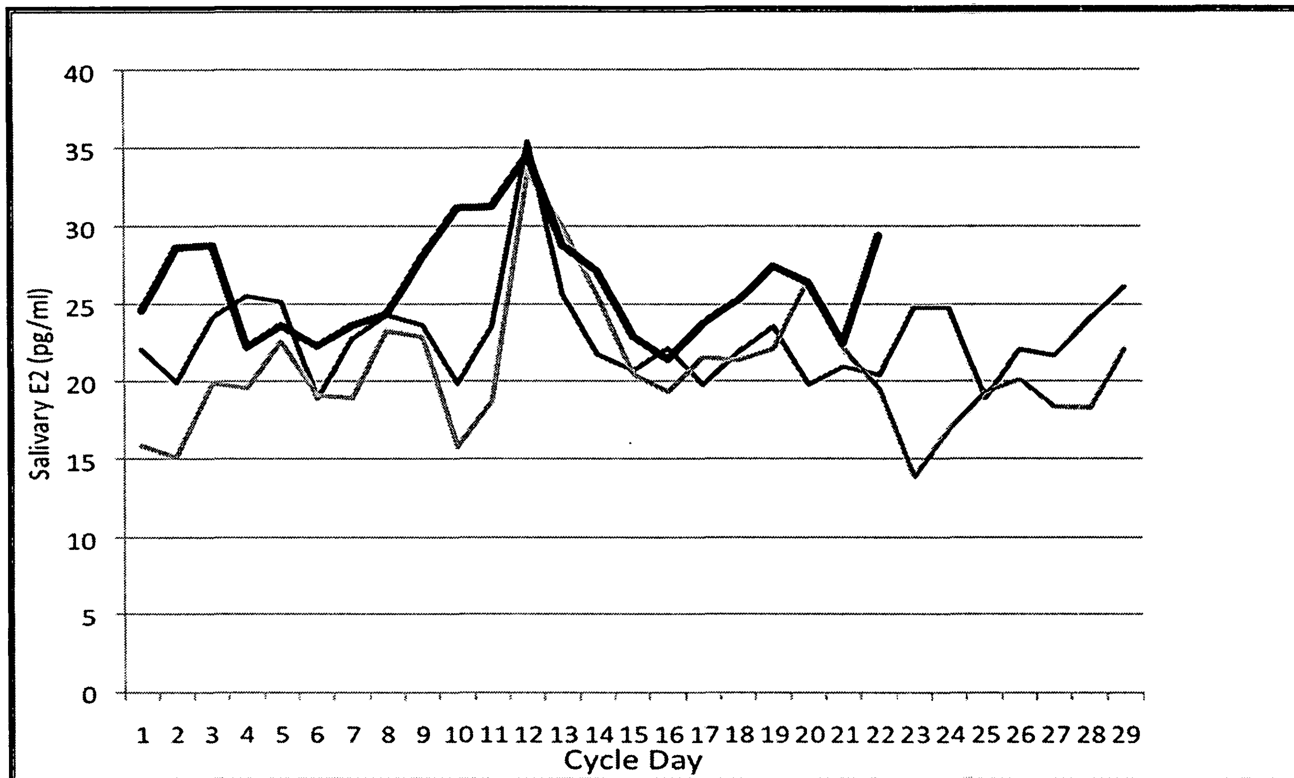


Figure 8: Average salivary estradiol concentration as a function of cycle day, aligned to E2 peak. Results are shown from NHP1 in green, NHP2 in blue and NHP3 in red.



DISCUSSION

The present investigation is the first methodological study in which ovarian hormones in macaques have been collected from biological media such as saliva and dried blood spots (DBS). Three rhesus macaques were trained for both salivary and dried blood spot collection. Saliva was successfully extracted from the inner portions of the non-human primate's mouth and quantified using a luminescent-based immunoassay. Salivary estradiol did not correlate strongly with serum samples collected via venipuncture. Despite low correlations with serum samples, salivary E2 peaks were visible in all three non human primates during an appropriate time window for ovulation, suggesting that the assay was able to detect biological fluctuations when concentrations were at their highest (see Figure 8). In this field, blood serum is considered to be the 'gold standard' for the quantification of E2. To date, only one published study (Thomspon, et al. 1999) has successfully employed daily E2 measurements in macaques for the purpose of quantifying ovarian hormones. The authors successfully trained non-human primates to voluntarily present their hands for venipuncture without restraints or medical sedation. The duration of Thompson's study was a single menstrual cycle, therefore the sample collection period lasted no more than 30 days. Although Thompson and colleague's innovative approach introduced a viable method for daily quantification of E2 levels, this methodology is still unsuitable for many types of neural and behavioural experiments in macaques, which often extend beyond one-month durations. Use of such a procedure daily for long durations would be difficult given the limited number of veins available for sampling, particularly following bruising associated with the process of venipuncture. Moreover, drawing blood directly from a vein of a non-sedated animal adds additional hazards on the part of the phlebotomist and requires considerable training to avoid hemolysis of the serous contents. Luminescence based assay kits for the quantification of estradiol are extremely sensitive to extraneous blood components and can void the results if efforts are not in place to prevent any form of blood contamination during the separation of whole blood to serum. Taken as a whole, venipuncture is an unattractive method for the daily sampling of ovarian hormones in vivo using non-human

primates involved in tasks that last longer than one month. Other methods for observing ovarian hormones include temperature monitoring and changes in perineal tumescence; however, studies have failed to consistently demonstrate both the reliability and validity of such approaches (Watman. 2006).

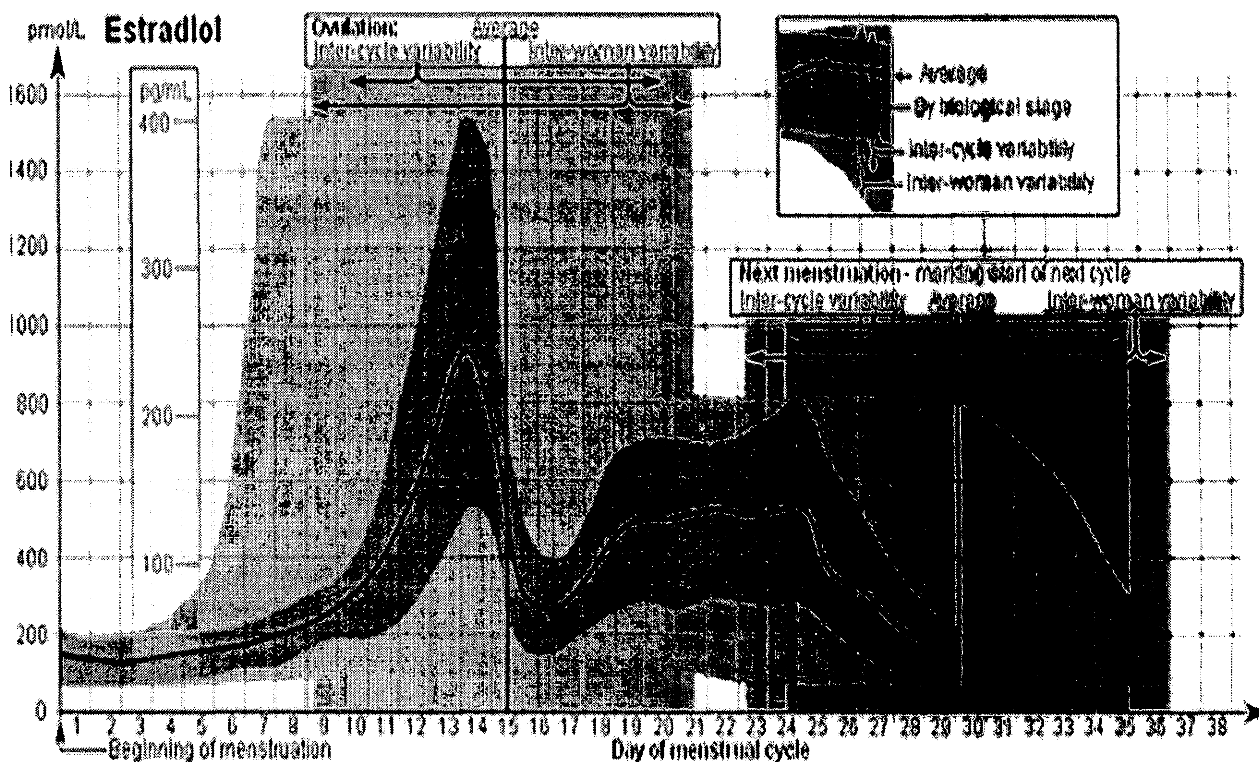
Bridging the methodological gap, in particular, measuring ovarian hormone levels in macaques, is warranted in light of recent discoveries linking ovarian hormones to numerous aspects of neural function. Several lines of research in humans have demonstrated that estrogen levels can have significant effects on perception and memory (Esposito et al. 2011) and may be neuro-protective against pathologies associated with aging and stroke (Sherwin et al. 2007). Moreover, dramatic changes in brain anatomy and function are associated with ovarian hormones applied in slice in rodent models. Because several avenues of neural investigation rely on techniques that record neural responses continually over the course of weeks to months, any ovarian hormone sample collection method that cannot be successfully administered on a *daily* basis over the course of several months' limits the scope of prospective studies.

Saliva collection summary

All 3 non-human primates showed similarities in terms of absolute salivary E2 concentrations (Figure 7). Moreover, all three showed the prototypical peaks characteristic of estradiol during both the ovulatory and luteal phase respectively, as measured using regularly cycling human females (figure 9) (Chiappin et al. 2007).

Figure 9: Human saliva estradiol concentration as a function of cycle

Establishment of detailed reference values for luteinizing hormone, follicle stimulating hormone, estradiol, and progesterone during different phases of the menstrual cycle on the Abbott ARCHITECT analyzer. Reto Stricker, Raphael Eberhart, Marie-Christine Chevailler, Frank A. Quinn, Paul Bischof and Rene′ Stricker. Clin Chem Lab Med 2006;44(7):883–887 PMID: 16776638.



Both the consistency of E2 values across animals, in addition to the biphasic peaks seen throughout the cycle, suggest that saliva methods were reflective of true underlying biological hormonal activity (figure 7). As previously mentioned, this was first time that salivary methods have been applied to a non-human primate population; as a result no prior range of expected concentrations could be used as reference, and this work

contributes to our understanding of expected fluctuations in salivary estrogen in macaques. Salivary estradiol concentrations were significantly higher than values reported in the human literature (figure 5 and figure 9). Despite detecting estradiol peaks from saliva samples, salivary E2 values did not correlate with serum fractions collected throughout the cycle, therefore saliva samples cannot be fully verified as a useful index of ovarian hormone function. The lack of a strong correlation with serum samples may be due to several non-mutually exclusive factors. First, luminescent-based immunoassays are extremely sensitive to blood contamination, even at miniscule quantities. Non-human primates pouch large quantities of food and play with sharp plastic toys, which over time may have resulted in small unnoticeable abrasions inside the oral cavity. No empirical method was in place for quantifying blood found in saliva samples and so microscopic contaminations could not be objectively ruled out and may have contributed to the elevated saliva values and low serum-saliva correlations seen across all three non-human primates. Second, only one type of commercially available kit was used for assay and differences in antibody selectivity and sensitivity may have been a contributing factor. For example, no method was in place to quantify organic contents found in saliva and there may have been other compounds present, which interfered with the precision of the assay. Two out of the three macaques used for this study showed remarkably consistent menstrual cycles. One had highly abnormal cycles and heavy uterine bleeding associated with menses that were clearly different from the other two monkeys. Interestingly, this animal (LU) showed a remarkably different serum hormone profile relative to the other two individuals.

Salivary methods offer at least three major benefits over previous sampling procedures: repeatability, non-specificity, and accuracy. One, the present design is repeatable. When using blood samples, daily venipuncture over the long term induces hematomas, which in turn is likely to result in hemolysis (the release of erythrocytes), which contaminates the sample upon separation of whole blood to plasma. By contrast, saliva sampling can be repeated daily – or several times per day - throughout several consecutive menstrual cycles without the need for separation, thus simplifying the

experimental procedure. Two, saliva sampling is non-specific. Saliva contains a diverse array of compounds such as lipids and proteins making this method applicable to several lines of investigation extending beyond estradiol sampling. Three, having the ability to conduct multiple determinations from a single subject statistically enhances the accuracy of corresponding results. An additional benefit is the minimal invasiveness of the procedure, which does not require the use of any restraint, or sedation, which has the potential for confounding the animal's biochemistry. All of these advantages make salivary sampling a viable candidate for the daily sampling of ovarian hormones in primates, though the accuracy of off-peak values needs to be addressed. Ultimately, this method can still be used for non-specific quantification of E2 as mid-cycle peaks were consistently detected; for example, if an underlying hormonal imbalance or pathology is suspected samples can be collected to determine if the profile differs from other individuals in the group and thus serving as a diagnostic function

DBS collection

One of the advantages of training non-human primates for saliva sampling procedures is that once successfully completed, the animal will have become sufficiently habituated to human contact such that both blood and saliva sampling is possible. Hormones collected via saliva and dried blood spots (DBS) reflect different circulating fractions; salivary hormones are thought to represent free unbound hormones while DBS reflect fractions found in circulating blood where up to 97% is inactively bound to sex binding globulin hormone (SBGH) (Kreizman et al. 2010) therefore each method can be used selectively depending on the research question at hand. Given that venipuncture is the gold standard for E2 quantification it should be noted that DBS shows great potential in being both highly specific and accurate in terms of quantifying E2. DBS uses serum for assays (like venipuncture) however obtaining adequate sample volume (50 ul) from a blood spot has proven to be difficult especially given the padding on the non human primates hands and feet, Further work is needed to develop methods to obtain larger aliquots of blood during the procedure.

Future Directions

Salivary and DBS hormone collection in macaques are novel techniques that show potential for daily hormone sampling. Although nearly half of the population is subject to fluctuating ovarian hormones, almost no research has investigated how these hormones change neural function in the brains of behaving animals, much less in non-human primates, which are the research model that most closely matches humans. The development of this method will allow for the first time daily saliva sampling of circulating hormones, which can then be used simultaneously in supplement with other physiological methods such as EEG and electrophysiology providing a more comprehensive picture on the question at hand. Although one of the methods isn't yet suitable for daily measurements, the other method (DBS) may be a good alternative, and saliva sampling may be useful for other uses of E2 measurements such as cycle regularity and hormonal disorders. The paucity of data on hormone sampling in non-human primates and the far-reaching health implications for human and non-human primates, alike, suggest that this is an important technique to pursue.

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