Ethnic variation in correlations of salivary and serum reproductive steroid hormones: a comparison of Bangladeshi and British women

Lauren C. Houghton

Masters Thesis M.Sc.

Ethnic Variation in Correlations of Salivary and Serum Reproductive Steroid Hormones: A Comparison of Bangladeshi and British Women

Lauren C. Houghton

15 June 2008

University of Durham
Department of Anthropology

Abstract

Matched samples of saliva and blood have yielded significantly correlated hormone concentrations. However, these verified correlations are restricted to healthy women of European descent in more developed countries. This relationship does not appear to have been extensively explored for non-White women. This study examined the correlation between progesterone and oestradiol concentrations in matched saliva and serum samples among sedentee women living in Sylhet, Bangladesh, and migrant Bangladeshi and British women of European descent in London, England. Participants were aged 19-42, regularly menstruating, free from exogenous steroids with no history of thyroid or fertility problems. Thirty-nine women provided matched blood and saliva samples during the luteal phase of their menstrual cycle at least one hour after consuming food or drink. Concentrations of progesterone and oestradiol were measured by direct radioimmunoassay and examined in relation to demographic groups. The study subjects were also examined in relation to age. Bangladeshi migrant women matched British women of European descent in age (mean= 33), but sedentee women were significantly younger than both groups. Serum and salivary progesterone concentrations were highly and significantly correlated in the British group of European descent (rho=0.879, n=10, p<. 0005) and the Bangladeshi sedentees (rho=0.700, n=11, p<. 0005), but not in the Bangladeshi migrants (rho= 0.191, n=18, p= 0.448). Serum and salivary oestradiol were significantly positively correlated in the Bangladeshi sedentee group (r=0.678, n=11, p<. 0005) but not in either the British group (r= 0.431, n=10, p= 0.214) or the migrant Bangladeshi women (r=-0.178, n=18, p= 0.479). With inconsistent correlations across the three study groups, these findings do not clarify whether hormone measurements in saliva or serum best reflects ovarian function. The current study prompts further research with a larger sample size and refined assay procedures.

Word Count
Total: 18,302

Acknowledgements

I am grateful to all women who willingly participated in this study and shared a part of their lives with me. I wish to express special thanks to: Taniya Sharmeen, Nazneen Choudhury, Tonu and Wendy for help with translation, recruitment and data collection; Prof Osul Choudhury for his help and hospitality in Bangladesh; Prof Lynnette Leidy-Sievert for her wise words and encouragement; Kesson Magid for his advice and friendship; Mito Habe-Evans and Rick for teaching me the ropes in the lab; Prof Chatterton for his expertise and guidance, Dr Tessa Pollard for her support; Prof Gillian Bentley for her continual supervision, keen eye and being just a phone call away at those heightened moments; Martyn Lawrence-Bullard for his generous financial support; Mum, Dad and Jamie for their undying support and love; and, Greg for being by my side no matter the distance.

I was supported by a bursary from the Biosocial Society and an Ustinov Scholarship from Durham University.

CONTENTS

List of Tables

List of Figures

Preface

Beginning in the 1980s, an endocrinological body of work emerged using salivary testing to compare reproductive function among women. Salivary sampling of hormones revolutionised the field of reproductive ecology because collection is easy and tends to be free from most ethical and religious taboos. Compared to blood, collection of saliva avoids the physiological stress caused by venepuncture and is more favourable for long-term monitoring of large sample sizes. Saliva sampling is pain-free, and cost effective, and samples can be collected and stored in the comfort of one's home, wherever in the world that home may be. Most importantly saliva steroids consist only of the free portion of hormone; so, saliva samples allow for direct measurement of hormones that are bioactive.

Peter Ellison was the first to apply salivary sampling (originally developed by the Tenovus Group for Cancer Research in Cardiff) to biological anthropology. Subsequently his work broke new ground in the area of reproductive ecology. For example, studies in reproductive ecology have demonstrated that there is great variation in ovarian function among women in Bangladesh, Bolivia, Nepal, Poland, and Zaire (now Democratic Republic of Congo). In order to demonstrate that variation in ovarian function is not dysfunctional but an adaptive response to ecological factors, previous research utilised saliva sampling across anthropological populations. Yet, saliva sampling has not been validated against serum samples specifically for such populations.

In developing methodologies for salivary collection, matched samples of saliva and blood have yielded significantly correlated hormone concentrations. However, verified correlations were restricted to healthy women of European descent in more

developed countries. It was presupposed that correlations of saliva/serum found in these women apply to all women. This relationship does not appear to have been explored for non-Europeans.

More recently, Chatterton et al 2006 found markedly different ratios of salivary to serum progesterone in Bolivian women compared to US women of European descent. This discrepancy begs the questions: using salivary assays, how can we interpret gonadal function in different populations? Does saliva or serum better reflect the *availability* of steroid hormones to target organs? Can we make assertions that ovarian function in different populations is reduced or enhanced based on data from saliva alone?

Salivary steroid analyses needs to be further assessed in specific populations. The current study therefore investigates correlations and ratios of saliva/serum progesterone and oestradiol in Bangladeshi and British women. Previous research conducted among these same populations demonstrates that Bangladeshi sedentee and adult migrant women display similarly lower salivary hormone profiles when compared to British women of European descent. In order to verify the salivary assays used in the prior study, the current study consists of British and Bangladeshi women divided into three groups according to their migrational status. Sedentee women and British women of European descent were born and live in Bangladesh and England respectively, while Migrant women migrated from Bangladesh to London as adults.

It is essential that saliva be more extensively validated as a reliable diagnostic tool because 30 years of research in reproductive ecology has relied primarily on salivary hormone profiles. If further investigation in a variety of populations consistently shows a

lack of uniform correlations between salivary and blood hormone concentrations, many of the findings in reproductive ecology may need to be re-evaluated.

Hypotheses

Hypothesis 1: Among all groups there should be a high, positive correlation between matched saliva and serum samples for both progesterone and oestradiol since these correlations have been established previously for European women.

Hypothesis 2: Based on previous research (Tenovus Group 1982), the ratio of saliva: serum progesterone and oestradiol during the luteal phase should be between 1-2% for all groups.

Hypothesis 3: Hormone levels should follow trends for inter-populational differences that have been demonstrated by previous studies (Núñez de la Mora et al 2007a, 2008). Specifically, the British group should display the highest levels, while Bangladeshi sedentees and migrants should be lower yet similar to each other.

Chapter 1: Background

The purpose of this chapter is to discuss methods assessing ovarian function as background to the current study that tests correlations of salivary and serum hormone assays in Bangladeshi and British women. The chapter begins with a description of the menstrual cycle, its physiology and the role of steroid hormones. It is followed by a brief summary of the diagnostic fluids used to measure steroid hormones. The history of radioimmunoassay describes the assay development specific to steroid hormones followed the advancements in sample collection that were necessary for salivary assays to be used among a range of populations. With the prevalence of salivary assays growing, clear advantages and disadvantages deemed from the reviewed literature are then outlined marking the point at which salivary assays and their applications now lie. The "Current Debate" section expands on the theories that have been created as a result of salivary sampling. However, the "normal" levels among European and US women would be pathological for a clinician looking at other populations. Therefore, the next section entitled 'Reproductive Ecology' summarises the studies anthropologists have conducted using salivary assays and how such studies have challenged the conventional understanding of ovarian function and reproductive variation. The cross-cultural and migrant population studies (direct predecessors of the current study) specifically point to the contributions of reproductive ecology to human biology. However the chapter ends cautioning against these large claims, returns to questioning the methodology used in such studies, and then summarises the current study.

1.1 Physiology of the Menstrual Cycle

Along the hypothalamic-pituitary-ovarian (HPO) axis reproductive hormones regulate ovarian function. External cues from visual, auditory, tactile, olfactory senses (Karsch 1984) are processed at the hypothalamus, transformed into hormonal signals and sent to the anterior pituitary gland. Here, the information is further processed and transmitted as gonadotrophin hormones heading for the ovaries through the blood supply. The ovaries react by secreting steroid hormones which act on target tissues including the brain and pituitary.

Progesterone and oestradiol, two steroid hormones, are synthesised on demand by steroid-secreting cells in the ovaries; progesterone is secreted primarily from the corpus luteum during the luteal phase of the cycle. Progesterone synthesis changes from the corpus luteum to the placenta during pregnancy. Oestradiol is the predominant oestrogenic hormone produced by growing follicular cells of the ovary. Like other steroids, progesterone and oestradiol are lipophilic; they pass easily through cell membranes and do not bind to cell surface receptors. Therefore, to circulate in the blood, progesterone and oestradiol bind to carrier proteins for transport: both bind to albumin and respectively to corticosteroid-binding globulin (CBG) and sex-hormone binding globulin (SHBG).

Menstrual cycle length varies among women although a 28-day cycle considered the most. Variation in cycle length occurs primarily during the follicular phase as the luteal phase (constrained by the finite maintenance of the uterine lining) is consistently between about 9-15 days, whereas, the follicular phase can last sometimes for weeks. Keeping in mind these are norms, as with any bodily system, there are certain disturbances that can occur in the endocrine system along the HPO axis including

amenorrhoea (the absence of menstruation), oligomenorrhoea (an irregular cycle), and anovulatory cycles (normal cycle length, menstruation, yet no ovum is released). Healthy women are assumed to be reproductively normal and their ovulatory cycle to be fertile as long as their cycles are within normal cycle length and occur regularly (Walker et al 1984).

1.2 Blood, Saliva or Urine: Which fluid is better?

It is known that hormone levels reflect organ function but, for more than 50 years, researchers have investigated how best to detect hormone concentrations in human biological fluids. In clinical studies, hormones have been assayed in blood (Yalow and Berson 1960), urine (Van der Molen 1968) and saliva (Katz and Shannon 1969). As assays developed, a pattern seemed to emerge: to detect smaller amounts of specific hormones in particular relationships to time (acute or longitudinal). Determining hormone levels in urine allowed for large volumes of biological fluid with relatively high concentrations of hormone metabolites to be collected (Van der Molen 1968, Stancyzk 1997). However, urine reflects the metabolic end products of once active hormones rather than the concentration of active hormone present at the time of collection. Metabolites can yield a baseline measure for hormones that were present over a 24-hour period. This can be useful for certain studies exploring how diet affects the metabolism of hormones (Adlercreutz 1995). Conversely, blood detects hormone levels in smaller but potent quantities at the actual time the sample is taken and, depending on the nature of the study, has been preferred over urine particularly for studies detecting the circadian rhythm of hormones. Saliva contains the smallest concentration of hormones since only the bioactive or free hormone diffuses into this medium, but it can be easily collected

both frequently and longitudinally given the non-invasive nature of saliva collection. While much research has been dedicated to creating immunoassays to measure hormones in relation to women's reproduction, the question remains which biological fluid best reflects the availability of steroid hormones to target organs.

1.3 History of Radioimmunoassay

In 1960, Rosalyn Yalow and Soloman Berson developed the first radioimmunoassay (RIA) for hormones measured in plasma. The creation of RIA revolutionized endocrinology because this new method could measure minute amounts of a single substance in blood. Immunoassays work using antibodies that recognise and bind to hormones at specific antigenic sites (Brook and Marshall 1982). Basing their technique on the principle called saturation analysis (advocated by Roger Ekins 1960) and, building upon their own prior work with insulin antibodies, Yalow and Berson (1960) used these antibodies as binding agents for proteins in plasma. While the earliest RIA methods relied on standard immunological procedures, they were replaced by methods using radioactively labelled hormones that could trace very low concentrations of hormone. Radioactive labelled hormone is mixed with both antibody and unlabeled hormone from the sample. The labelled and unlabelled hormones compete for antibody binding sites and the displaced hormone is measured (Wright and Taylor 1967). Nowadays varying types of immunoassays -- such as enzyme-linked immunosorbent assays (ELISA), which eliminate the hazards associated with radioactive materials -- are used in medical and cognate sciences including endocrinology, biochemistry, physiology, applied nutrition and clinical practice.

1.3.1 RIA for Steroids in Blood

Once Berson, Yalow and Ekins established the basic techniques for RIA, it was feasible for an analytic method to be devised for any hormone of interest (James and Jeffcoate 1974). Murphy (1964) first applied Yalow and Berson's findings to developing an assay for steroid compounds by using naturally occurring plasma proteins, such as transcortin. Similarly, once SHBG —the protein in human plasma that has a high affinity for oestradiol and testosterone — was discovered, it opened up the possibility of using it to measure these very hormones in blood (James and Jeffcoate 1974). While blood is still used in clinical settings, during the 1980s endocrinologists started advocating saliva as another medium for the measurement of steroids.

1.3.2 Salivary Progesterone RIA

In 1982 a group of cancer researchers called the Tenovus Group working in Cardiff, UK, published a volume called "Immunoassay of Steroids in Saliva" which emerged from the proceedings of the Ninth Tenovus workshop (Read, Riad-Fahmy, Walker, Griffiths 1982). Since earlier studies showed that: a) adrenal function could be assessed by measurements of salivary cortisol, and b) treatment of children with congenital adrenal hyperplasia could be monitored by salivary 17a-hydroxyprogesterone, these authors tested if ovarian function could also be investigated by measuring concentrations of progesterone in *saliva* rather than plasma (Walker et al 1979). Salivary assays directly measure unbound hormone, while serum assays require the separation of unbound from bound hormone through time-consuming and expensive techniques such as membrane dialysis. Therefore, the Tenovus Group saw an advantage to exploring salivary steroid assay and they were successful in developing an assay with a high saliva/blood correlation.

1.3.3 Refining Assay Elements

Developing an assay for salivary steroid hormones was a gradual process because the assay needed to demonstrate high sensitivity (ability to detect low amounts of the hormone in question), specificity (ability to discriminate between different hormones) and precision (reproducibility within and between assays). Since hormones exist in saliva in relatively low concentrations compared to plasma, the amount of radioactively-labelled hormone added must be comparably minute to be considered sensitive. A labelled hormone of high specific radioactivity is therefore required. Simultaneously, the labelled hormone must remain immunologically identical with the unlabeled hormone (Wright and Taylor 1967). The Tenovus group were the first to create an RIA for salivary progesterone that was sensitive as well as specific to unbound progesterone without significant cross-reactivity with other steroid forms. Their method was precise with acceptable inter- and intrassay coefficients of variation.

Since then other endocrinologists have tested correlations of blood and saliva progesterone levels in regularly menstruating women in order to support and enhance the use of salivary sampling in research and practice (Walker et al 1979, Shah and Swift 1982, Choe et al 1983, Read et al 1984, Tallon et al 1984, Bourque 1986, Evans 1986, Lipson and Ellison 1992, Lu et al 1997). Table I summarises the research design and results of numerous studies that have investigated the correlations of salivary and serum progesterone. All of these studies were conducted with women of European origin.

Table I. Correlations of matched salivary and serum progesterone among women of European descent comparing sample sizes, study design and whether assay procedure was direct or extracted across studies.

Study	Matched Sample Size (N)	Study Design	R value	P value	Direct or extraction
Walker et al 1979	112	4 women, luteal phase	0.91,0.92,0.93,0.97	x	extraction
Shah & Swift 1982	50	50 women at random throughout menstrual cycle	0.89	x	x
Choe et al 1983	x	4 women throughout menstrual cycle	0.58	0.001	x
Read et al 1984	15	Women over 40 yrs, luteal phase	0.9	<.01	extraction
Tallon et al 1984	56	11 women during menstrual cycle	0.85; range 0.95,0.96, 0.96, 0.88	<.001	extraction in blood, direct in saliva
Borque et al 1986	76	14 women	0.78	<.001	x
Evans 1986	x	27 women once during cycle; 4 women multiple days during cycle	0.9	x	extraction
Lipson and Ellison 1992	x	3 women	.80-.97	x	extraction and centrifugation
Lu et al 1997	48	4 women aged 20-40, luteal phase	0.75	<.001	direct

1.3.4 Salivary RIA Alternative Procedures

Development of salivary RIAs led to methodological alternatives that reduced the amount of steps or time associated with assay procedure. Endocrinologists developed alternatives for making salivary assays more efficient in the lab. Rapid precipitation can replace centrifugation (Read et al 1983), I^{25} can replace H^3 as the tracer (Lu et al 1997),

and direct assays can replace extracted assays (Bourque et al 1986). Many of the studies exploring progesterone correlations differed in direct and extracted procedures. An extracted RIA removes conjugated steroids from the analyte (Stanczyk 2004). Chromatographic purification then isolates progesterone for measurement. These preparatory steps could be omitted with the development of more specific antisera that displayed little cross-reaction with other hormones. Direct assays made assay procedures more manageable for laboratory technicians; they could assay more samples in less time, simultaneously improving assay sensitivity.

1.3.5 Direct versus Extracted RIA

With the development of direct RIAs, new issues arose. Because of the low concentrations of progesterone in saliva, direct assays required highly selected antisera to measure progesterone in picomole units (Riad-Fahmy et al 1987). Stanczyk (2004) notes that there are major differences between direct and extraction RIAs. He prefers the latter because extracted assays: 1) achieve high specificity through separating out conjugated steroids and unconjugated metabolites, 2) yield high sensitivity due to larger sample volumes, and 3) eliminate matrix problems since both purified sample and standards are dissolved in buffer (Stanczyk 2004). Corrie (1982) compared results from a direct iodine-based RIA with an extracted, tritium-based assay using the same samples. The direct assay yielded lower values than the extracted assay, particularly pertaining to those samples containing high levels of progesterone. Corrie posited that these unexplained differences could be due to unexplained dietary factors, yet concluded that the direct assay was a satisfactory method for tracking ovarian function during the luteal phase.

Nonetheless salivary assays, either extracted or direct, offer a simplified methodology compared to serum assays for measuring free hormone.

1.4 Advancements in Sample Collection

The elements of an accurate assay -- specificity, sensitivity, and precision -- yield accurate results in the lab; yet, salivary sampling in anthropological field sites, called for changes in sample collection. Realising the versatility of salivary sampling, Peter Ellison expanded the study of human reproductive function from clinical settings to anthropological field settings. Ellison embraced the advantages of salivary sampling; it is non-invasive, allows for serial sampling, can be used on young children, avoids physiological stress caused by venepuncture and, most importantly, it directly reflects the free hormone levels found in plasma (Ellison 1988). Methodological advances, such as salivary stimulation and preservation enhanced sampling techniques. The effects of certain salivary stimulants were explored including: wax (Luisi and Franchi 1984), a specific sugarless gum (Ellison 1988 Lu et al 1997), and citric acid (Bourque et al 1986 Vining and McGinley, 1986), none of which significantly interfered with assay results. Still, other studies recommended rinsing the mouth with water before sample collection rather than using stimulants, in order to eliminate any risk of interference (Read et al 1984, Riad-Fahmy et al 1987). Differential methods for storage and preservation of samples were also explored. It was agreed that samples could be stored frozen from between 6-9 months (Walker et al 1979, Sufi et al 1985, Lu et al 1997), but samples could also be preserved without refrigeration over several days (Banerjee et al 1985). Ellison (1988) and Lu et al (1997) found sodium azide to be an acceptable salivary preservative for unfrozen samples. More recently Chatterton et al have been using

Merthiolate (thimerosal) as a preservative. Lipson and Ellison (1989) investigated the extent to which progesterone binds to polystyrene tubes since Banerjee et al (1985) found polyethylene tubes to absorb steroid from samples; polystyrene tubes did not interfere (Lipson and Ellison 1989).

Ellison further developed sampling protocols conducive to field situations. If procedures were not carefully monitored, possible caveats to salivary sampling become problematic. Based on Ellison's (1988) recommendations, Table II lays out clear ways to correct for possible areas of error including assay protocols, sample collection and quantitative analysis.

Table II. Summary of Ellison's Sample Collection (1988)

Caution	Correction	Further recommendation
Assay protocols		
Absence of standardised laboratory procedures	Accuracy, precision and sensitivity of assay require greater quality controls	
Sample Collection		
Stimulation	Stimulants such as parafilm and certain sugarless gums were tested and can be used to collect instantaneous or high volume samples	Stimulants should be tested whenever a new one is used for potential cross-reactivity with assay
Contamination-minor blood or food contaminates can distort results	Participant should rinse mouth with cold water and wait 30 minutes after eating	Saliva only collecting capsule can also avoid contamination
Repeated Samples- salivary samples detect the transient pulses of steroid secretion. This can cause problems if only few samples are taken.	Sampling regimes should be designed to increase the likelihood of observing characteristics as temporal pattern, thus repeated samples should be taken of the pattern of interest	
Preservation and storage of sample	Using sodium azide (0.1g/L) at ambient temperature allows storage up to 6 months.	At lease one cycle of freezing and thawing allows better separation of saliva from mucins before assayed
Collection tube material	Polystyrene collection tubes are a validated substitute for borosilicate glass tubes	Any potential collection tube should be tested before it is used in the field
Quantitative Analysis		
Data Alignment - menstrual profiles depend on how data is aligned based on day of period	Menstrual onset alignment is better than midcycle alignment for ambulatory subjects	
Definitions of subject populations- selected studies for clinically normal may bias sample	Eligibility criteria should be clearly stated and comparative data sets should also be collected	
Data Grouping: grouping of data can significantly affect reported means and ranges	Age, weight, weight history, and exercise habits need to be controlled for	
Determining ovulatory cycles- incomplete data may fail to show an functioning corpus luteum	Samples taken over 3-4 day intervals seems to classify ovulatory cycles,	PULSAR analysis software and following quantitative model set out by Wood could also help analyze data

1.5 Advantages of Salivary Sampling

While urine, blood, and saliva all have been proven to quantify steroids using RIAs, it is arguable which method is the best for reflecting ovarian function. Much of this debate revolves around balancing the needs a) to decipher between free and total steroid forms, b) to use the least invasive methods for women, and c) to develop accurate methods conducive to repeated sampling.

1.5.1 Free Hormone Only

Saliva is supposedly more accurate in measuring steroid hormones compared to blood and urine because it reflects the *free* hormone that is bioavailable to a target organ. When progesterone and oestradiol are assayed in plasma, the results reflect the total concentrations (steroids bound and those unbound to carrier proteins). Salivary assays are generally believed to only measure the free (unbound) faction of steroid hormone because the protein carriers to which the bound steroid is attached are too big to pass through salivary glands

The free faction reflects the hormone available to target organs because it diffuses to tissues more readily through the lipid-rich membrane of target cells (Riad-Fahmy et al 1987). As changes in the concentration of binding proteins occur, total and bound concentration of hormones can also change quite drastically. However, there is supposedly only a small change in free hormone concentrations (Brook and Marshall 1982, Goldsworthy 1981*)*. Therefore the most important advantage to salivary sampling is that salivary diffusion should naturally filter out bound steroids.

1.5.2 Convenience and Versatility

Collecting saliva to measure a woman's reproductive hormone levels was readily tested and accepted initially because this technique is convenient and more participant

friendly than venepuncture. Saliva can be collected at home by the participant herself and stored in home refrigerators or on the shelf with preservatives until transferred to the laboratory. There is no requirement for skilled personnel to take samples as with blood. Furthermore, Walker et al (1984) stressed that salivary sampling was a preference of volunteers. Salivary sampling is also conducive to women's different lifestyles. Riad-Fahmy et al (1987) demonstrated that saliva samples were successfully collected from Cardiff school children, Thai women, British women, and Bangladeshi women attending fertility clinics. In Bangladesh, saliva sampling was described as a social event where the women competed in filling up their tubes (Riad-Fahmy et al 1987).

While the Tenovus Group focused mainly on cancer research and monitoring healthy and unhealthy women, they suggested that assays for salivary progesterone could also monitor changes in ovarian activity during adolescence. In 1984, Read et al utilized salivary sampling as part of larger research that investigated the possible correlation of ovarian dysfunction in maturing girls at risk of later breast cancer. Collecting salivary samples from girls bypassed the possible ethical constraints of drawing blood from minors. Read et al (1984) found the incidence of luteal phase progesterone was positively correlated with chronological and gynaecological age, and postmenarcheal girls showed similar patterns of salivary progesterone when compared with premenopausal healthy women This study added to the growing body of literature on salivary progesterone because it assessed luteal phase concentrations with regard to ovulatory cycles and gynaecological age in healthy adolescent girls (Read et al 1984).

1.5.3 Longitudinal Monitoring

Saliva is preferred over blood and urine as a longitudinal measure of ovarian function. The pulsatile production of hormones by GnRH is problematic for the timing of hormone sampling because an isolated sample may yield an extreme peak or lull. In addition, one sample is not particularly useful given variation in secretion from day-to-day or even hour-to-hour even though many clinical studies still rely on this kind of sampling technique. Matched saliva and plasma samples suggest that salivary progesterone concentrations are better indicators of average hormone levels than rapidly fluctuating plasma values (Shah and Swift 1982). Thus the ability to conduct longitudinal sampling is paramount. Riad-Fahmy et al (1983) validated longitudinal salivary sampling by collecting daily saliva samples over entire menstrual cycles and verifying these levels with weekly blood draws. Lipson and Ellison (1992) conducted one of the largest studies of age-related variation in progesterone levels by collecting saliva samples every day over one menstrual cycle; they found a consistent pattern of age-variation in relation to luteal function. Such serial sampling from healthy women established a normal pattern of salivary progesterone concentrations for the menstrual cycle.

Women who were not menstruating regularly due to pregnancy or infertility were also subjects of investigation, and their profiles were compared to established normal patterns. It was evident that the ability to easily collect serial samples of saliva over a period of time provided greater accuracy in detecting pathophysiological changes (Luisi et al 1982). For example, saliva samples detected the period of transition from luteal secretion to placental secretion of progesterone during pregnancy (Shah and Swift 1982). The transition was tracked by a transient dip in saliva levels which then later continued to rise as the pregnancy progressed. On the other hand, serum levels from the same cycles

varied and were relatively low until week 8 of pregnancy. Thus longitudinal saliva collection was better at tracking the switch between ovarian to placental secretions of progesterone, and was argued to have a greater diagnostic value (Luis et al 1982).

In 1982, the Tenovus Group stressed that salivary sampling could aid reproductive ecology research in developing countries (Walker et al 1982). Three years later and funded partially by the WHO, Sufi et al (1985) organized a multicenter evaluation of salivary assays. Following the same assay protocol and using centrally provided reagents, five labs in five different countries performed salivary assays for progesterone and oestradiol. Quality control results were compared from each site; between batch and between-laboratory results were comparable with those reported for salivary assays performed in other centres (Sufi et al 1985). Salivary sampling was thus being advocated as the sampling method of choice for endocrinologists worldwide.

1.6 Disadvantages of Salivary Sampling

Ellison relied heavily on standardising salivary sample collection as a means to eliminate error, stating that there are few physiological factors that interfere with salivary/serum correlations. However, despite the record of good correlations, there are discrepancies in salivary assay research including: differences in absolute values, varying ratios, inconsistent reporting and aasay interference.

1.6.1 Differences in Absolute Values

While salivary progesterone profiles among different studies followed similar biphasic patterns, with peaks occurring in the luteal phase, there are differences in absolute values. Studies undertaken on Italian and US women revealed salivary levels above normal ranges of progesterone for the luteal phase (Riad-Fahmy et al 1987). It is

possible that these exceptions to the normal ranges could simply be due to differences in assay accuracy or laboratory methodology. Read et al (1984) have pointed out that the bias introduced by antiserum cross-reactivities can produce an apparent lack of parallelism between salivary and plasma levels.

1.6.2 Varying Ratios

More troubling than discrepancy in absolute values among studies is the fact that salivary/serum progesterone ratios appear to vary throughout the menstrual cycle (Luisi et al 1981, Walker et al 1981, Luisi et al 1982, Choe et al 1983, Donaldson et al 1984, Tallon et al 1984, Zorn et al 1984, Butt et al 1984, Evans 1986, Chatterton et al 2006). Luisi and colleagues (1981) showed a 10-fold increase from a 1% ratio of salivary to serum progesterone in the follicular phase to a 10% ratio during the mid-luteal phase, whereas Walker and colleagues (1981) reported no change in ratios over the cycle. The latter group concluded that the percentage of 'free' progesterone in plasma is constant throughout the normal cycle (Walker et al 1982). But Evans (1986) also found that, while plasma and salivary progesterone showed similar patterns across the cycle, the ratio between them changed over the menstrual cycle. Although, in this study, the correlation of saliva and serum progesterone was good (r =0.9), the concentration of salivary progesterone varied from being either higher or lower than that in plasma depending on the cycle phase (Evans 1986). The ratio increased by 2.5 from the follicular to the luteal phase (Evans 1986) compared to a 10-fold increase found by Luisi et al (1981). However, in additional studies that also showed a change in saliva/plasma ratios across the cycle, there were lower ratios during the luteal phase than the follicular (Table III).

To explain these findings, Evans (1986) suggested that different proportions of total progesterone transfer from blood to saliva at different stages of the menstrual cycle. Explanations for why or how this might occur have yet to be proven. Furthermore, there is a lack of consensus on the actual percentage of progesterone circulating in the unbound state in blood alone. There are different reports ranging from 2% (Westphal et al 1977, Rosenthal et al 1969) to 5% (Tulchinsky and Okada 1975), and even 8-10% (Yannone et al 1969). Even if saliva is understood to reflect the unbound concentration in plasma, there is limited evidence of what the "normal" percentage of unbound to bound hormone should be in blood, or whether this can vary substantially across individuals or even different populations of women. Methodological limitations of separating unbound hormone from bound in serum samples account for this lack of evidence.

Table III. Summary of studies reporting salivary and serum concentrations of progesterone as ratios.

Study	Matched Sample Size (N)	Study Design	Ratio in Luteal Phase	Ratio in Follicular Phase	Direct or extraction
Luisi et al 1981	x	29 women (unspecified but assumed to be Italian)	10.2%	0.9%	extraction
Walker et al 1981	x	x	1.2%	2.3%	x
Luisi et al 1982	15	29 women	7.52 (H^3) 8.46 (I^{25})	1.37 (H^3) 4.83 (I^{25})	extraction
Choe et al 1983	x	4 women throughout menstrual cycle	4.0%	16.0%	x
Donaldson et al 1984	x	2 women	1.0%	x	direct
Tallon et al 1984	56	11 women during menstrual cycle	1.7%	7.5%	extraction in blood, direct in saliva
Zorn et al 1984	32	luteal phase	1.0%	5.1%	x
Butt et al 1984	x	x	0.6%	x	x
Evans 1986	x	27 women once during cycle; 4 women multiple days during cycle	115 (plasma/saliva) .87% (saliva: blood)	47.1 (plasma/saliva) 2.13% (saliva: blood)	extraction
Chatterton et al 2006	46	26 Bolivian women; 20 American women	.83% (Bolivian); 3.4% (American)	x	direct

Therefore the issue at hand comes full circle: the same limitations associated with assaying free concentrations in plasma samples acted as motivation to investigate saliva sampling as a viable alternative, but saliva sampling is difficult to verify without a having

a good idea of the normal ratio of unbound to bound hormone in blood and whether this ratio itself varies across the cycle.

1.6.3 Inconsistent Reporting

There has been a tendency in the literature to acknowledge but then dismiss studies that showed a weaker relationship between salivary and plasma levels of hormones (Luisi et al 1982, Choe et al 1983). There is also a mismatch in methodology between studies that reported the hormonal relationship between saliva and serum as a correlation (Shah and Swift 1982, Read et al 1984, Bourque et al 1986, Lipson and Ellison 1992, Lu et al 1997) as opposed to a ratio (Zorn et al 1984, Butt et al 1984, Chatterton et al 2006). Among the three studies that reported the progesterone relationship between saliva and blood as both a correlation *and* a ratio (Choe et al 1983, Tallon et al 1984, Evans 1986), those studies that had higher ratios found a lower correlation between saliva and serum progesterone. Furthermore, as discussed above, some studies demonstrated that ratios of saliva to plasma steroids differed during phases of the cycle. However, those studies that reported high correlations between plasma and salivary hormone concentrations seemed to validate the sampling method regardless of discrepancies in the ratio reports. Nonetheless, as D.B. Ferguson warns, there is danger in assuming that methodological error accounts for variations in these ratios when biological factors may be at play (Ferguson 1984).

1.6.4 Assay Interference

Complicating factors could be present when using saliva as a diagnostic tool. Plasma binding proteins, such as albumin present in salivary gland tissues, could skew results (Riad-Fahmy et al 1987). Dietary or other masticatory habits such as chewing

betel nut, coca leaf or drinking stimulants such as coffee could affect concentrations of salivary steroids (Vitzthum 2005, Núñez de la Mora 2007b). While specific salivary stimulants such as particular gums or parafilm apparently do not interfere with assay results, there is still debate about whether varying rates of salivary flow might interfere with assay analyses (Ellison 1988). Metabolism of steroids within the salivary gland itself could also disrupt the correlation between saliva and blood steroid levels (Ellison 1988).

Contrary to earlier thinking, there is a possibility that target cells could also use the bound fraction of hormones. Ekins (1982) suggested that bound hormones could affect the rates of steroid delivery to tissues with a high clearance rate such as the salivary gland. Rapid transfer of steroids into saliva could depend on both free and bound steroid concentrations.

There are other drawbacks when saliva sampling is conducted with different populations. In populations prone to chronic gingival inflammation, small abrasions or infections in the mouth could contaminate saliva with blood or gingival fluid. This would account for significant increases in measures of progesterone and oestradiol concentrations in saliva (Sufi et al 1985). These issues are argued to be "unlikely" to cause direct metabolic changes in salivary steroid concentrations (Walker et al 1982:24). However, significant serum and saliva hormone correlations have not been verified for a range of populations. Bound versus unbound hormones and their role in ovarian function has primarily been deduced by testing people derived from one ethnic group. There might be variation in the concentrations of unbound hormones among different

populations, or variation in the proportion of binding proteins. Riad-Fahmy et el (1987:257) cautioned:

> The assessment of salivary steroid assays should rely not on some presupposed theoretical advantages but on a practical demonstration of their value in providing reliable indices of endocrine function.

1.7 Current Debate: What is normal ovarian function?

Some endocrinologists have used "normal" women as participants. Here, "normal" women are defined clinically as regularly menstruating, with cycles between 26-30 days and having no gynaecological complaint (Walker et al 1979, 1984). But, other endocrinologists have started to question the values that these studies yielded. In one study, Walker et al (1984) found that while, most women had salivary progesterone profiles showing the typical biphasic pattern, there was great variability between cycles. Cycles in women classified as "normal" could vacillate from anovulation, luteal-phase insufficiency to normal luteal function over a 6-month period.

Furthermore, there was variation in the ovarian activity of "unhealthy" women attending fertility clinics. Even if their hormonal levels were out of the "normal" range, conception still occurred with treatment (Walker et al 1984). It was becoming more difficult to diagnose and treat unhealthy women when hormonal ranges varied and a normal hormone threshold remained unclear. Endocrinologists, however, were keen to find "normal" thresholds with which to diagnose unhealthy women. The comparative studies conducted by Walker et al (1982) using longitudinal salivary samples revealed that a definition of "normal" could be problematic.

The findings from hormonal profiling over *whole* cycles shifted the paradigm from medical standards of ovarian function (usually based on single blood samples *within* a cycle) to a continuum of ovarian function. In relation to studies on menstruation and

exercise, Jerilynn Prior (1985) suggested that a step-wise progression occurs along the HPO axis from cycles with a normal luteal phase, to short luteal phase, to anovulation, to amenorrhoea. Ellison (1991) used a similar model and provided more evidence across populations to support it. He sees ovarian function as a graded continuum degenerating from fully competent cycles to luteal phase suppression, ovulatory failure, oligomenorrhoea, and ultimately amenorrhoea. Of these gradients, only extreme disruption of the menstrual cycle (amenorrhoea) is observable at the clinical level. All other disruptions in ovarian function are considered "moderate suppressions" that are likely to go unnoticed by both a woman and her physician because she continues to menstruate regularly. Ellison extended what was considered a regular cycle length to 22-38 days to include a broader perspective of women in reproductive ecology studies (Lipson and Ellison 1992). For women, it may be reassuring to menstruate monthly since this seems normal; however, in case of problems, the physiologist wants to know what is occurring at a more refined level which is not visible using cycle lengths alone.

There are many physiological factors at play beneath the surface of what is observable in a cycling woman. This variability of ovarian function revolves around the body and its systems' ability to read the environment, which is evident in seasonal birth patterns. Thus it becomes very important for the HPG axis to be able to decipher what its target tissue really needs as opposed to interference or 'background noise' (Brook and Marshall 2001). It appears ovarian function along the HPO axis can sustain fecundity by ignoring background noise or can inhibit fecundity when ecological, behavioural or constitutional stressors become long term (Ellison 1991). Moreover, the "normal" levels

found among women of European descent would be diagnosed as pathological for a clinician looking at other populations such as Nepal.

1.8 Reproductive Ecology

Since the early 1980s biological anthropologists have travelled to various countries to collect frequent and repeated saliva samples from populations traditionally inaccessible to medical researchers. Reproductive ecologists and anthropologists have compared average hormonal profiles across groups of women and found that there is great variation in ovarian function between populations. Natural fertility populations (women who do not use contraception) who also live in ecologically stressful environments demonstrate lower ovulation rates, longer birth intervals, and presence of birth seasonality compared to western women who have higher base-line levels of reproductive hormones (Ellison 1990). Such research supports life-history theory, which posits that the body must decide between three competing physiological processes – growth, maintenance and reproduction—to allocate limited energetic resources (Ellison 2005). Following life-history theory, reproductive ecology has contributed evidence that humans (as with other species) possess adaptive strategies that enable their bodies to decide whether to invest energy in reproduction or other bodily functions.

From studying anthropological populations, reproductive ecologists have been able to determine ecological, behavioural and constitutional factors that affect fecundity. Age, a constitutional factor, is a consistent marker of fecundity. Factors that contribute to age-related changes in fecundability include coital frequency, age of the partner, ability to conceive and carry to term, physiology, ovulatory frequency, and endocrinological characteristics of the menstrual cycle (Lipson and Ellison1992). The last three factors

can be measured using salivary assays making it possible to compile luteal progesterone profiles for women of different ages (Lipson and Ellison 1992). Progesterone profiles for three populations living in Boston, Democratic Republic of Congo (DRC) and Nepal match in pattern, show parabolic trajectories of progesterone in relation to age, but lower hormonal profiles for women from DRC and Nepal. It appears that women have the highest levels of mid-luteal progesterone between 25 and 34 years (Lipson and Ellison 1992). While levels are progressively lower among populations in more stressful environments, the relative patterning by age is highly consistent across all populations (Ellison 1990).

With regard to ecological and behavioural effects on fecundity, there is a strong relationship between energetics -- energy intake, energy expenditure and net energy balance -- and ovarian function. For women from DRC, Nepal and Poland, ovarian function showed a similar response to energetic stress (Bentley et al 1999, Panter-Brick 1994, Jasienska 1998). In all three populations, groups experiencing higher stress appeared to have suppressed ovarian function. With field studies contributing such evidence via salivary hormone profiles, interpretation of human reproductive function has changed.

1.8.1 Bolivian versus US women: a cross-cultural example:

A cross-cultural study found that progesterone in ovulatory cycles was significantly lower in better-off and lower still in poor Bolivian women compared to women from Chicago (Vitzthum et al 2002). This study suggested that levels of salivary progesterone in Western women could significantly exceed concentrations necessary for

successful conception and gestation when compared to women in other populations who conceived at much lower levels.

1.8.2 Ovarian Function Variation Among Migrant Populations

Studies of salivary hormones among populations in transition have been used to show how developmental factors can affect ovarian function. A migrant study of Bangladeshi women by Núñez de la Mora et al (2007a) showed significant differences between Bangladeshi migrant groups and sedentees in adult levels of progesterone. While British women of European descent had the highest adult hormone levels, Bangladeshi women who migrated before 8 years old had significantly higher levels of progesterone than their sedentee counterparts and other women who migrated later in life. These findings, based on one-month menstrual salivary hormonal profiles, suggest a critical developmental window influencing later reproductive function.

Variation in reproductive function has been connected to early life determinants in additional ways. There is an inverse relationship between menarcheal age (age at first menstruation) and adult hormone levels (Apter et al 1984 and Vihko et al 1989). Women living in more developed countries reach menarche earlier, and children from less developed countries living in Western countries have been shown to reach menarche earlier than children in the country of origin (Dunger et al 2005). All of these studies suggest that, at some point during childhood, the body is able to adjust its reproductive trajectory. Ellison (1990) refers to this adjustment as a possible "bioassay of prevailing conditions of energy availability" and writes:

The rate of childhood growth and adolescent maturation should provide a bioassay of environmental quality, particularly the overall level of energy above

maintenance costs that is likely to be available for reproduction in later life (Ellison 1996).

1.10 New Questions for Salivary Assays

While reproductive ecologists were devising revolutionary models to explain inter-populational differences in hormonal levels, reproductive endocrinologist Dr. Robert Chatterton raised questions about these differences. Differences in salivary hormone levels may not reflect adaptive variation but be due to potential methodological issues. Chatterton explored steroids in matched saliva and blood samples from Bolivian women previously studied by Vitzthum et al (1998). While the original cross-cultural study emphasized variation in ovarian function, it assumed that saliva was accurately assessing bioavailable hormone levels. Chatterton et al (2006) questioned whether saliva was capturing the true physiological picture and looked to blood for confirmation.

Twenty six Bolivian women and 20 American women with regularly occurring cycles between 26-32 days provided matched blood and saliva samples during the self-reported mid-luteal phase. Saliva samples were stimulated with sugarless chewing gum and were preserved with 0.5mg sodium azide (NaN3). Chatterton et al (2006) found that, in contrast to the lower concentrations of salivary progesterone, mean serum progesterone was significantly higher in the Bolivian women ($p<.01$). However, it is not clear if it is bound or unbound progesterone which is higher in the serum among Bolivian women because total hormone concentrations were being measured in the serum samples. The mean salivary progesterone concentrations in the Bolivian women was 48% that of the US women ($P<.001$). Moreover, salivary progesterone in Bolivian women was only 0.83% of the concentration in serum, whereas in US women it was 3.4%. Serum

oestradiol levels were not different between the groups; therefore, discrepancies around steroid transfer from blood to saliva revolve around progesterone only in this study.

Chatterton looked at other compounds in the samples to attempt to explain the findings. It is possible that stress levels for women are higher in Bolivian than US women, which might suppress levels of salivary progesterone. But cortisol levels for both groups were similar and showed no stress-related affects. There were also no differences in serum CBG or SHBG levels between the Bolivian and US women, so these binding globulins did not account for transfer differences. However progesterone also binds to albumin, another binding protein that Chatterton et al (2006) did not measure. There were also no differences in cholesterol levels between the two groups, indicating that there was no limitation in steroid biosynthesis. Chatterton et al (2006) concluded that the differences between concentrations of salivary P in these two population groups are not indicative of the serum concentrations of these steroids. Thus the original hypothesis set out by Vitzthum et al (1998) that Western women conceive at higher levels of hormones than Bolivian women cannot be confirmed unless it is certain that only the free portion of the hormone (as measured in saliva) matters. These findings led Chatterton et al to ask: Does saliva or serum better reflect the availability of progesterone to target organs? (It is important to note that this study did not isolate free progesterone or oestradiol in serum, therefore these findings cannot be fully interpreted until a direct comparison of free salivary free serum hormone is made). Nonetheless, the discrepancy in salivary and serum progesterone among Bolivian and American women remains to be explained.

Chatterton's findings lead to many questions for studies in reproductive ecology. There are many results that rely on salivary steroid assays for assessing ovarian function, but have these results accurately reflected the unbound hormone available to the ovaries in specific populations? How can the differences in ratios across the menstrual cycle be reconciled to support salivary sampling? Can conclusions about ovarian function be drawn from salivary steroids alone? Or, was it erroneously assumed that high correlations in European and US women meant high correlations for all women?

1.11 Summary of Study

Examining the correlation between plasma and salivary steroids in other populations will help resolve this paradox. Exploring saliva to serum ratios across populations may also illuminate the issues of free versus total hormone concentrations. The present study presented here attempts to extend our knowledge of the salivary to serum ratio of reproductive steroids by analysing the correlation between plasma and salivary progesterone and oestradiol in Bangladeshi women whose salivary steroid levels were examined earlier in Dr Chatterton's laboratory using similar assays to the Bolivian study undertaken by Vitzthum et al (1998). Analyses conducted in the same laboratory will reduce methodological error.

Chapter 2: Methods

2.1 Study Population

Bangladeshi women were targeted as a study population since previous research has explored their salivary steroids levels (Núñez de la Mora et al 2007a, b, 2008). These prior studies involved a migrant study of Bangladeshis to London mentioned already on page 28. Because the majority of British Bangladeshis migrate from the Sylhet District in the northeast of Bangladesh and have low rates of intermarriage with other ethnic groups, they lend themselves to a naturally controlled experiment. Núñez de la Mora et al (2007a) found there were no significant differences in salivary progesterone profiles between Bangladeshi women living in Bangladesh and those who migrated to England as adults. However, their study found marked differences when comparing these two groups with second generation Bangladeshi and women of European descent. The Bangladeshi and British women born in London had higher hormone levels. The current study followed an abbreviated version of the same study design. Recruits for the study here were drawn from 3 different groups:

- Sedentees- 12 Bangladeshi women who were born, raised and resident in Bangladesh.
- Migrants- 19 first generation migrants born in Bangladesh but who moved to England as adults (after menarche).
- British- 10 women of European descent born in England and living in London.

Participants were healthy, regularly menstruating women (cycles between 25-32 days), with no clinical history of diabetes, thyroid diagnoses, polycystic ovaries or other

infertility problems. All women had not breastfed within the past six months or taken hormonal birth control within the past three months. Participants were between the ages of 19 and 42 years. The study was designed to: a) target women of reproductive age with robust hormone levels, and b) to screen out those whose steroid levels could be influenced by certain pathologies, reproductive conditions, or exogenous hormones. The sample was not representative of the general population because those with conventional irregular menstrual cycles were excluded.

2.2 Recruitment

Participants were recruited from March through June 2007 in two field sites: Sylhet, Bangladesh, and London, England. The study initially aimed to recruit 20 women from each group with fieldwork scheduled to begin in January 2007. However, field conditions hampered complete collection. Political unrest in Bangladesh at that time caused the Bangladeshi government to declare a State of Emergency in early January 2007 delaying the onset of fieldwork in Bangladesh to March and restricting time for data collection in Sylhet. In London, data collection was also hampered because the (Bangladeshi) phlebotomist initially recruited to help with the study was held up in Sylhet waiting for a new passport to be issued by the interim government, and a new phlebotomist had to be found. Given the one-year time frame of a Masters by research, it was not possible to extend recruitment beyond a three-month period. The sample size is thus reduced from the original study design.

In Bangladesh, all but one participant was born in the city of Sylhet in Sylhet District. The remaining sedentee was born in a nearby rural village. All sedentees were contacted through networking and word of mouth by a bilingual research team stationed

in the district. One researcher was a Bangladeshi trained medical doctor who drew all blood samples at a local hospital. While this research team member was able to convince women to provide blood, many Bangladeshi women were unwilling to volunteer due to fear and cultural constraints.

In London, some Bangladeshi migrant and British women were contacted and recruited through established networks. In addition, a bilingual (Bangla-English) research assistant recruited migrants from Bangladeshi neighbourhoods in east and north London, primarily within the London boroughs of Camden, Hackney and Tower Hamlets. While all the migrants came from Sylhet District, the majority were born in rural villages. The migrants were recruited from local schools, community centres and markets. British women were recruited using networking, word of mouth and advertisements posted at universities and shops. Fifty percent were from the boroughs of Camden and Hackney and the remainder lived in Greater London. Bangladeshi migrant women preferred the research team to travel to their homes rather than meet them elsewhere. The European women also found it more convenient to provide blood samples in their homes.

Ethical permission for the study was obtained from the Ethics Committee of Durham University, Department of Anthropology and data were stored in compliance with the Data Protection Act (UK). Written informed consent was obtained from all participants after the study was explained in the participant's first language. Volunteers were compensated for their participation.

2.3 Data Collection

A screening questionnaire was administered to all women to ascertain whether they were eligible to participate in the study using the eligibility criteria outlined earlier (page 37-38). (See Appendix for Screening Questionnaire).

Questionnaires- Reproductive and migration (where applicable) histories were collected via structured questionnaires. These questionnaires also included questions adapted from the previous study that concerned demographic, reproductive, socioeconomic, and energetic status data. Reflection questions were asked after all data was collected to compare the participant's opinion of providing saliva and serum samples in order to gain an emic view of sampling procedures and to investigate prior claims that saliva collection is preferred over blood. (See Appendix for Structured Questionnaire).

To obtain peak progesterone levels, sample collection was targeted towards the mid-luteal phase of the menstrual cycle (Day -7 or +21). As part of the screening questionnaire women were asked to recall the date of their last menstrual period (LMP) and report their usual cycle length (from the first day of menstrual bleeding to the last day preceding the next day of menstrual bleeding). From these dates, it was determined when the mid-luteal phase could be targeted for sample collection. During the structured interview, women were asked to mark on a calendar their last menstrual period (LMPcal) and when they expected their next period to begin (ENMP). Follow-up calls were made to women about a week after sample collection to verify the start of their next menstrual cycle (VNMP). If a woman had not commenced her period, follow-up was continued until it was clear whether samples had in fact been inadvertently collected during the follicular phase.

Anthropometrics- Anthropometric data was collected by myself in order to avoid potential differences between participants that might act as confounds. Indices included height, weight, mid-triceps skinfolds, waist and hip circumferences. The body mass index (BMI) and waist to hip ratio were calculated from these data.

Saliva and blood samples- All samples had comparable collecting, handling, storage and assay conditions. Participants were asked not to consume food, drink, or to chew betel nut for at least one hour prior to collection (Núñez de la Mora *2007b).* All saliva was collected within 15 minutes *before* blood was drawn to avoid physiological stress possibly caused by venepuncture and to be classified as matched samples.

Saliva- 5 ml of saliva was collected in polystyrene tubes pre-treated with merthiolate as a preservative to a final concentration of approximately 0.1%. This preservative was tested to ensure it does not interfere with assay results. A particular brand of sugarless, spearmint gum was provided to stimulate saliva; this gum has been extensively tested in the laboratory and does not interfere with assay results (Ellison 1988; Lu et al 1997). Vials were stored at room temperature until transported to the laboratory, where they were centrifuged, and stored at 8° C. It has been shown that steroid levels remain stable in saliva samples collected and stored in this manner (Ellison 1988, Lu et al 1997).

Blood- A certified phlebotomist drew 10 ml of blood from each participant. The blood samples were centrifuged to separate the plasma before freezing. Serum was stored at -20° C. Samples collected in

Bangladesh were transported to London on dry ice, stored at -20° C and then shipped on dry ice to Chicago using a courier.

Assay procedures- All assays were performed in Professor Robert T. Chatterton's laboratory at the Department of Obstetrics and Gynaecology, Northwestern University, Chicago, USA. I performed one set of progesterone assays during the month of July and laboratory assistants conducted subsequent assays in November.

Salivary progesterone was, in fact, assayed three times due to discrepancies in the initial results. The first assay used 3h-progesterone and the last two used I25-progesterone as the tracer for a more sensitive assay. Initially, salivary progesterone was assayed using a direct radioimmunoassay (RIA) according to published procedures with minor modifications (Lu et al 1997). The principle of this assay is that a competitive equilibrium for specific antibody sites is established between standards of progesterone and antibody and 3H-progesterone in pH 7.56 PBS-BSA-EDTA buffer. Unbound 3H-progesterone is separated by addition of dextran-coated charcoal.

The tracer, 3h-progesterone, was used at a concentration of 10,000 cpm/0.1ml in the assay tube. The buffers were PBS-bovine serum albumin (BSA) buffer (0.1 M NaH2PO4 pH 7.56), steroid buffer with merthiolate, and steroid buffer without merthiolate. Dextran-coated charcoal (DCC) was prepared by adding 50mg of dextran and 500mg of activated charcoal to 100 ml of gelatin buffer. The standard progesterone was obtained from Sigma Chemical Company, St Louis, MO and a stock solution of 1.0 mg/ml was prepared in methanol and stored at 4° C. The antiserum (which cross reacts12.12% with 5B-pregnandione, 5.5% with 5a-pregnanedione, 4.22% with deoxycorticosterone, 1.56% with 20-B-OH-P, 2.32% with 5B-pregnenolone, 1.6% with

corticosterone, 0.55% with pregnenolone, and <0.1% with 2-a-OH-p) was prepared at Northwestern University and used at a dilution of 1:280 to achieve 40% binding.

Two subsequent progesterone assays used the DSL-3400 Progesterone RIA kit made by Diagnostic Systems laboratories, Inc. All samples were included in one assay at a time. The intrassay CV was < 15%. All measurements of salivary progesterone are in pg/mL. Serum progesterone was measured without extraction and all samples were assayed together. The intrassay CV was <15%. Serum progesterone was measured in ng/mL. Salivary and serum oestradiol were assayed using a kit (DSL-4800) and both analytes were measured in pg/mL. Salivary oestradiol was not run in duplicates due to low volumes of sample.

2.4 Data Analyses

Menstrual dating- The last menstrual period marked on the calendar for women (LMPcal) was compared to the date originally reported during screening (LMP). The estimated next menstrual period (ENMP) was compared to the verified next menstrual period (VNMP). The correspondence between day of sample collection and day of menstrual cycle was verified by participants reporting their next menstrual period. Cycle length reported during screening was compared to the cycle length calculated from the last menstrual period and the verified next menstrual period. The success rates of each group in estimating their next cycles accurately are reported as percentages below.

Determination of cycle day- For each sample, the day of the cycle on which the sample was collected was determined. The day of the next menstrual onset (VNMP) was identified as Day 0, the preceding day identified as -1 and so on. Days were counted back until the day of collection was reached.

Assay levels- The calculations for the results of the first progesterone assay were carried out by myself. The non-specific binding value (NSB) was determined by taking the average of all samples that neared 0. NSB was subtracted from each count and the result was divided by the total to determine the percent binding. Counts were converted to percent bound and were plotted on a linear logit-log scale. Counts were done in duplicates and none was excluded because all had a CV< 15%.

Five percent of the total sample was lacking because of missed or improper collection. The sample size was reduced to include only those cases with matched saliva/plasma values. Out of 41 samples, there were 39 samples that contained both serum and saliva values. Since there could be bias/error in self-reporting of recent menstrual histories, it was problematic to restrict samples only to those reported from the mid-luteal phase. The results reported here for luteal P levels are therefore based on values for reported days -23 to -2. In this instance, the absolute concentrations of hormones are more applicable than the self-reported supporting data. For this study, matched samples that yielded detectable levels were included, except for one sample which was an extreme outlier.

Statistical analyses- SPSS 13 for Mac OS X was used to carry out all statistical analysis. Sample sizes varied from model to model depending on the available data for each covariate. Preliminary statistics explored whether progesterone and oestradiol concentrations and ratios were normally distributed. Where the Kolmogorov-Smirnov statistic was significant ($p<0.05$) indicating a violation of the assumption of normality, non-parametric tests were conducted. Kruskal-Wallis tests were used to compare differences between groups in relation to age, marital status, work, schooling,

reported age at menarche, and parity. Mann-Whitney U tests were conducted for post-hoc analysis. Non-parametric analyses were used for all tests that included serum progesterone since none of these data were normally distributed.

To validate whether saliva is an accurate reflection of serum sampling among the different groups, the relationship between salivary progesterone and serum progesterone concentrations was investigated using Spearman's rho correlation coefficient. Oestradiol correlations were assessed using Pearson's correlation. Since prior research has reported results as either correlations or ratios, this study reports results as both for cross-comparison. Hormone concentrations and ratios were compared between groups using Kruskal- Wallis analysis of variance, and post-hoc analysis was conducted via independent 2-tailed Mann-Whitney-U tests using the Bonferoni adjustment of .05/3= 0.017.

Chapter 3: Results

3.1 Participant Characteristics

Table IV lists the descriptive statistics for the three groups under study. None of the groups differed significantly in reported age at menarche. The sedentee women were significantly younger (on average by 9 years) than the migrant women (Mann-Whitney U, U= 17, z= -3.5, $p<0.001$, r= -0.78) and the British women (Mann-Whitney U, U=0, z= -3.8, $p<0.001$, r= -0.72). None of the sedentee women was married or had ever been pregnant, and all held undergraduate degrees. The majority were currently university students. In contrast, the migrant women were in their mid-30s, all were married with two or more children, and only 20% held undergraduate degrees. The British women were also in their mid-30s, of whom 33% presently or in the past had had a partner, while 90% held undergraduate or higher degrees, and 60% had been pregnant and/or had children.

Table IV. Comparison of Group Characteristics

		Cycle Day	Age	Education Level	Age at Menarche	Pregnancies	Children	BMI	Waist: Hip Ratio
Sedentees	Mean	-7.7	23.7	6.1	12.3	0	0	22.2	.76\
	SD	4.3	1.6	.54	1.6	0	0	4.1	.035
	Range	-17/ -2	22/ 25	5/ 7	9/ 15	0	0	17.5/ 29.6	.69/ .81
	Median	-7	23.5	6	12	0	0	21.8	.76
Migrants	Mean	-10.2	33.5	3.4	12.5	3.2	3	28.5	.84
	SD	5.6	5.7	1.8	1.5	1.2	.97	4.1	.06
	Range	23/ -2	19/ 42	1/ 6	9.5/ 15	1/ 6	1/5	22.6/ 38.7	.71/ .88
	Median	-10	35.5	3	12	3	3	27.1	.84
British	Mean	-7.9	32.6	6.1	13.1	1	.50	23.0	.74
	SD	2.8	3.3	1.2	1.6	1.2	.71	3.4	.04
	Range	--15/ -5	28/ 36	3/7	10/15	0/3	0/2	17.4/ 29.6	.69/.83
	Median	-7	33	6	13.5	1	0	22.7	.74

3.2 Cycle Day

Available dates of verified next menses (VNMP) determined what day of the menstrual cycle the samples were collected from each woman. Table V shows each woman's cycle day at the time of sample collection and also denotes from which phase the sample was taken. Forty one percent of all samples were estimated to be collected from the mid-luteal phase. This percent is broken down by group in Table VI, along with other data on accuracy of self-reporting cycle phases. Overall, the British women were the most accurate in identifying mid luteal-phase samples.

Table V. Participants' Cycle Day at Collection

Groups	Collection Day	Phase
Sedentees		
1	-7	Mid-Luteal
2	-3	Luteal
3	-7	Mid-Luteal
4	-8	Mid-Luteal
5	-2	Luteal
6	-7	Mid-Luteal
7	-10	Luteal
8	-11	Luteal
9	-3	Luteal
10	-17	Follicular
11	-10	Luteal
British		
1	-7	Mid-Luteal
2	-7	Mid-Luteal
3	-7	Mid-Luteal
4	-5	Luteal
5	-7	Luteal
6	-9	Luteal
7	-15	Follicular
8	-7	Mid-Luteal
9	.	Detectable
10	-7	Mid-Luteal
Migrants		
1	-7	Mid-Luteal
2	.	Detectable
3	-5	Luteal
4	-2	Luteal
5	.	Detectable
6	.	Detectable
7	.	Detectable
8	-23	Follicular
9	-12	Luteal
10	-9	Luteal
11	-13	Luteal
12	.	Detectable
13	-11	Luteal
14	.	Detectable
15	.	Detectable
16	-9	Luteal
17	-11	Luteal
18	.	Detectable

Key

Mid-Luteal = samples collected between days -7 and -8

Luteal = samples collected between days -2 and -13 but not including -7 and -8

Follicular = samples collected between days -14 and -23

Detectable = samples collected without verified next menstrual period yet detectable by assay procedure

Table VI. Percent Accuracy of Self-Reported Menstrual Dates by Group

Group	Matched Last Menstrual Period and Last Menstrual Period calendar	Matched Estimated Next Menstrual Period and Verified Next Menstrual Period	Matched Cycle Length Reported and Cycle Length Calculated	Mid-Luteal phase (Day -7 +/- 2)
Bangladeshi Sedentees	0%	67%	64%	36%
Bangladeshi Migrants	50%	50%	50%	22%
British	78%	83%	83%	80%

3.3 Sampling Preference

Table VII tabulates women's reactions to the different sampling techniques used. Overall, about 45% of women expressed negative reactions towards giving blood, referring to feelings of fear and pain. Thirty percent of women did not like giving saliva samples expressing it was awkward, embarrassing and caused nausea. British women had the highest preference for blood sampling while Bangladeshi sedentees expressed the highest problem with blood sampling.

Table VII. Sampling Method Preference

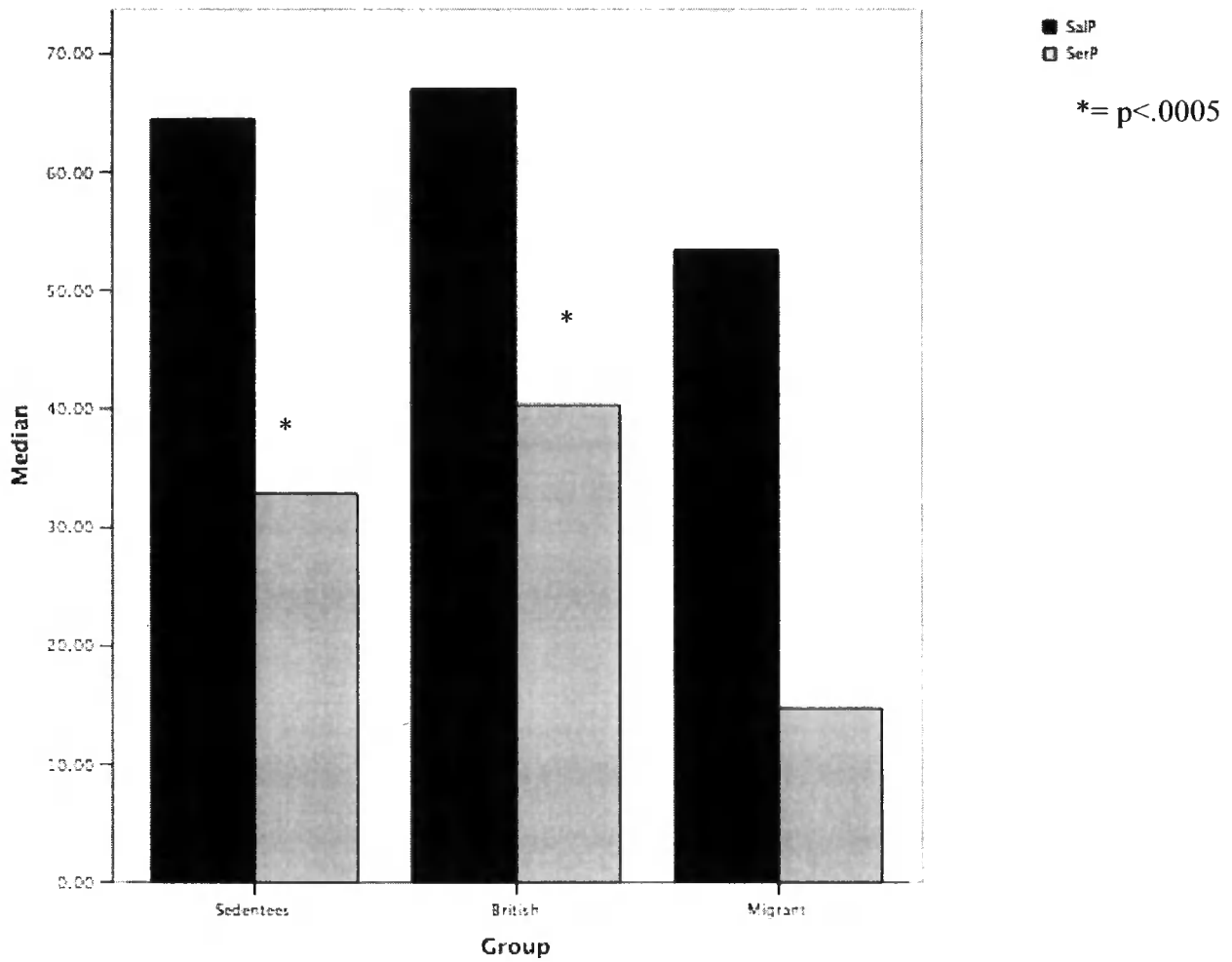
Group	Neutral	Expressed Problem with Blood Sample	Expressed Problem with Saliva Sample
Bangladeshi Sedentees	33%	50%	17%
Bangladeshi Migrants	28%	44%	28%
British	10%	40%%	50%

3.4 Correlations of Progesterone and Oestradiol

Progesterone non-parametric correlations - Serum and salivary progesterone concentrations were highly significantly correlated in the British group (rho=0.879, n=10, p<. 0005) and the Bangladeshi sedentees (rho=0.700, n=11, p<. 0005). There was no significant correlation between salivary and plasma progesterone among the Bangladeshi migrants (rho= 0.191, n=18, p= 0.448).

FIGURE 1. MEDIAN PROGESTERONE BY GROUP

British (n=10) and Bangladeshi Sedentee (n=11) groups demonstrate significantly correlated salivary and serum progesterone.



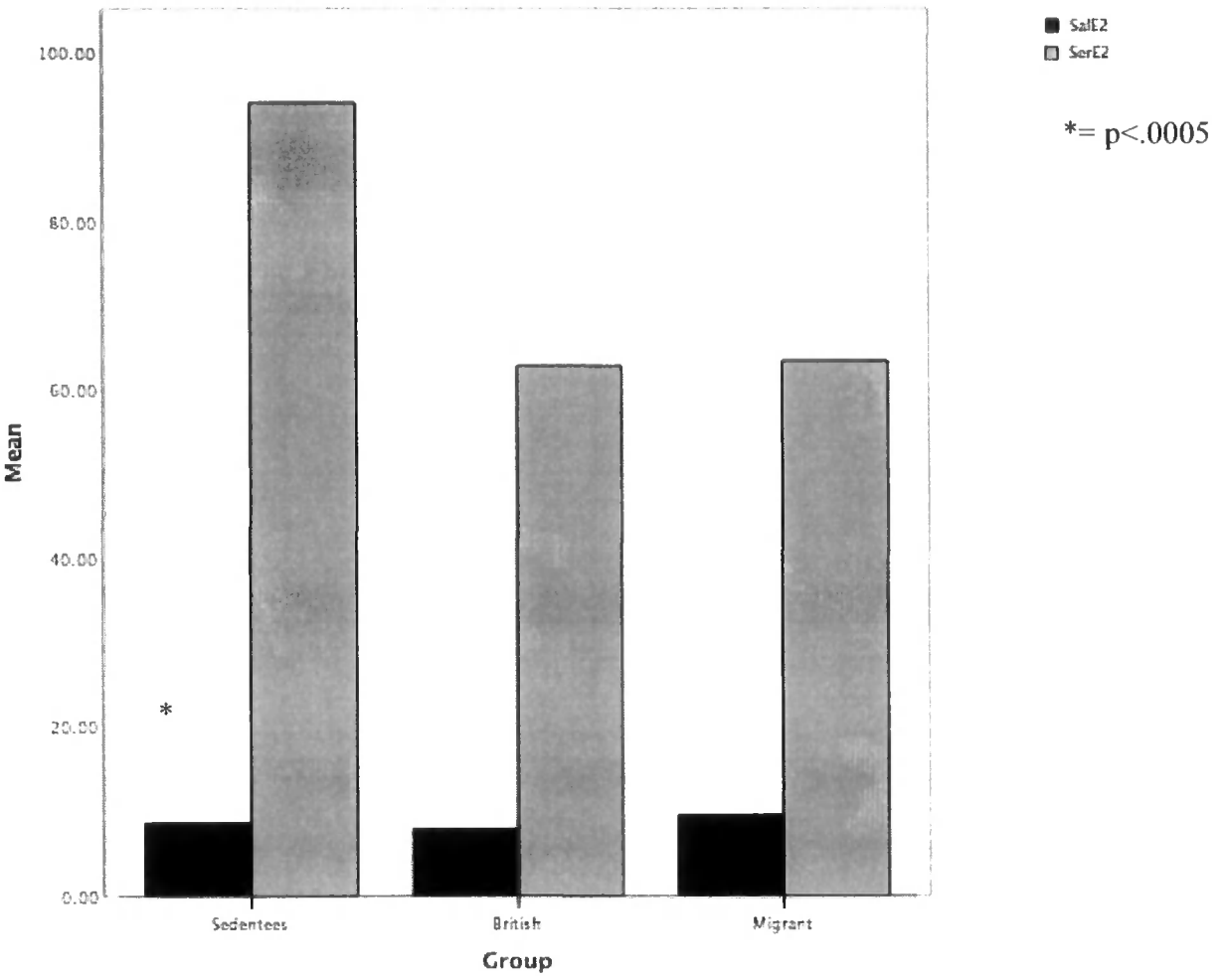
Salivary P is measured in pg/mL

Serum P is measured in ng/mL

Oestradiol parametric correlations - Serum and salivary oestradiol were significantly positively correlated in the Bangladeshi sedentee group (r=0.678, n=11, p<. 0005). There was no significant correlation between salivary and plasma oestradiol in either the British group (r= 0.431, n=10, p= 0.214) or the migrant Bangladeshi women (r=-0.178, n=18, p= 0.479).

Figure 2. Mean Oestradiol By Group

Bangladeshi Sedentees demonstrate significantly correlated salivary and serum oestradiol.



*= p<.0005

Salivary E2 is measured in pg/mL

Serum E2 is measured in ng/mL

3.5 Group Comparisons of Hormone Concentrations

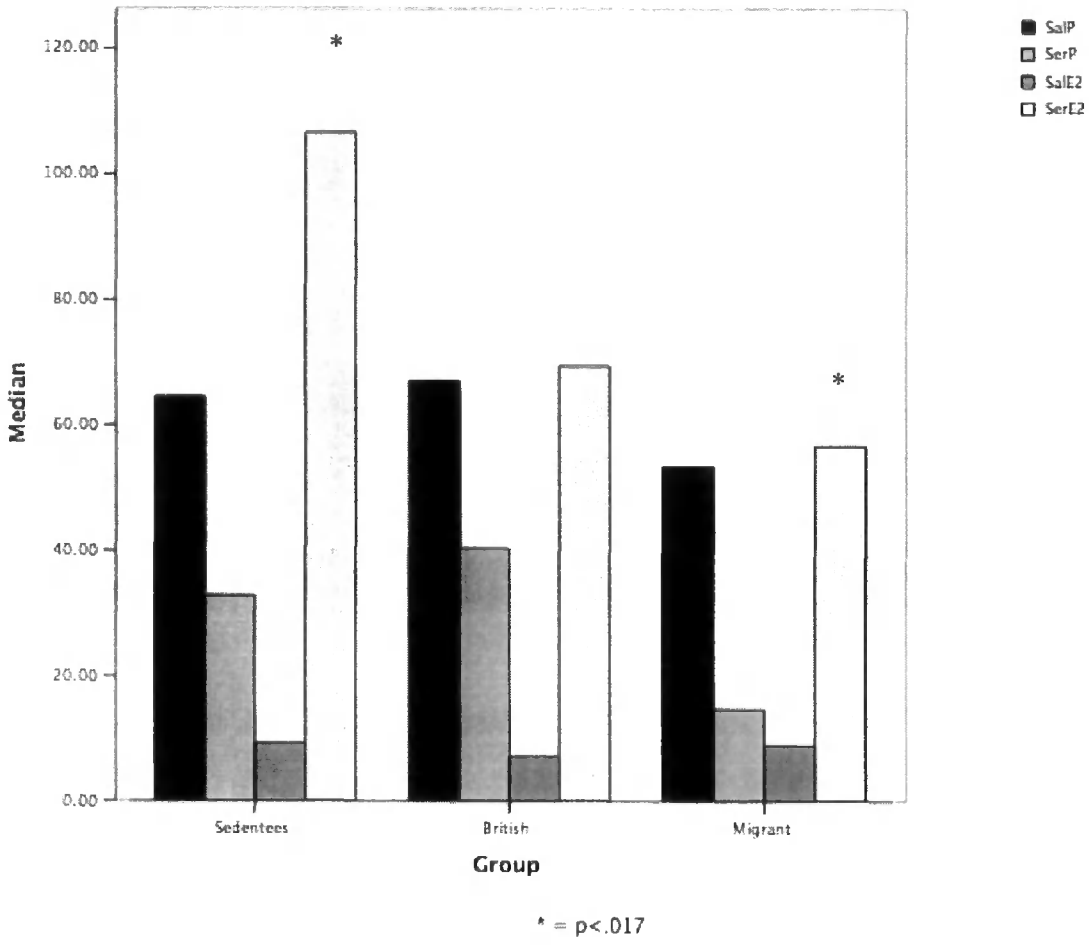
Median salivary and serum concentrations of progesterone and oestradiol from Bangladeshi sedentee, Bangladeshi migrant and British women are shown in Figure 3. The salivary progesterone levels reported in this study are higher than those reported in Núñez de la Mora et al's (2007a) study (1.6 times, 2.6 times and 2.5 times for British, migrants and sedentees respectively). The levels reported for salivary oestradiol are similar yet slightly higher than those reported in the previous study (about 1.4 times for British and Bangladeshi migrants and 1.3 for sedentees).

A Kruskal-Wallis one-way analysis of variance revealed a statistically significant difference only in serum oestradiol across the three different groups (sedentees n= 11, migrants n=18, British n=10), X^2 (2, n=39) = 6.73, p =.035. The sedentees had higher median oestradiol (Md= 106.6) than the both the British and Migrant groups, which had medians of 69.4 and 56.7 respectively.

Since the Kruskal-Wallis test for oestradiol resulted in a significant difference, it was necessary to conduct a post-hoc analysis. Mann-Whitney *U* tests revealed significant difference between serum E2 levels of sedentees (*Md*=106.6, *n*= 11) and migrants (*Md*=56.7, *n*=18), *U*=46, *z* =-2.382, *p*=.01, *r*=0.442). Using the Bonferoni adjusted significance level p<.017, there was no significant difference in E2 levels between British *(Md=69.4, n= 10)* and sedentees (Md=106.6, *n*= 11) *U*= 25, z =-2.113, p=0.035, r=0.461*)*.

FIGURE 3. GROUP MEDIANS OF HORMONE CONCENTRATIONS

There is a significant difference between serum E2 levels of sedentees and migrants via Mann-Whitney *U*.



3.6 Group Comparison of Hormone Ratios

Table VIII summarises the mean ratio of salivary to serum progesterone and oestradiol for sedentee Bangladeshi, migrant Bangladeshi, and British groups.

Table VIII. Saliva to Serum Percent Ratio Means

Group	Progesterone	Oestradiol
Sedentee	.25 +/- .23	9.9 +/- 3.5
Migrant	1.10 +/- 2.2	14.1 +/- 5.8
British	.30 +/- .18	18.7 +/- 10.5

A Kruskal-Wallis test revealed a statistically significant difference only in saliva/serum oestradiol ratio across the three different groups (sedentees n= 11, migrants n=18, British n=10), X^2 (2, n=39) = 6.67, p =.036. The sedentees recorded a lower median score (Md=8.5) than the two other groups, white (Md= 13.5) and migrants (Md=18.6). However, post-hoc analysis via Mann-Whitney *U* test revealed no significant difference in saliva/serum E2 ratio between groups at the adjusted significance level p=.017.

Chapter 4: Discussion

4.1 Salivary and Serum Correlations of Progesterone and Oestradiol

I collected saliva and blood samples from 39 women from three different populations to test whether progesterone and oestradiol concentrations correlated significantly in matched samples. Based on previous studies conducted on women of European descent, there should be a significant, positive correlation between matched samples for both hormones among all groups. Results from this study report otherwise. For progesterone, there was a positive, significant correlation for both Bangladeshi sedentees and British women. However, Bangladeshi migrants did not show a significant correlation. For oestradiol, only the Bangladeshi sedentees showed a significant, positive correlation.

In the study comparing Bolivian and US women discussed on page 29, Chatterton et al (2006) found differences in progesterone levels across the two groups and fluids. While concentrations of salivary progesterone were lower in Bolivian women, serum progesterone was higher. On the other hand, there were no differences between Bolivian and US women in levels of serum oestradiol; therefore, only progesterone seemed to be different.

Among groups of Bangladeshi women and British European women, neither progesterone nor oestradiol followed such consistent patterns when comparing mean values between salivary and serum samples. The inconsistency in results for the British and sedentee women is puzzling and may point not to real differences in values but more to assay variability or interference. Progesterone correlations were significant for British

women and Bangladeshi sedentees, but oestradiol correlations were not significant for British women. Following Chatterton's reasoning that only progesterone seems to have a low transfer rate of steroid into saliva, most likely matched oestradiol should have also shown a significant correlation for British women in the current study.

It is interesting that neither progesterone nor oestradiol samples correlated for migrant Bangladeshi women. There could be environmental, dietary or behavioural factors that affect the concentration of steroid hormones in saliva. Metabolism could be affecting salivary assays both at the biological and laboratory levels. The level of progesterone in blood is determined by the rate at which it is being produced and entering the circulation, and by the rate at which it is leaving circulation due to excretion or tissue metabolism (Fotherby 1979). Perhaps the metabolism of migrant Bangladeshis differs from that of British women due to dietary factors in their new environment in London. While migrant Bangladeshi women do not suffer from food insecurity (Núñez de la Mora 2007a), their diets could differ in fibre content, fat consumption, and hydration levels—all factors that impact hormone metabolism. It is apparent that women with a diet higher in fibre excrete more faecal matter along with higher faecal oestrogen, decreasing the plasma concentration of this hormone (Goldin et al 1982). Furthermore, Adlercruetz et al (1995) suggest that higher oestrogen levels in Finnish compared to Asian women result from a higher fat diet and lower faecal oestrogen excretion. However it is inconclusive whether a low fat, high fibre diet reduces ovarian hormone levels in premenopausal women (Gann et al 2003).

Additionally, activity in the salivary gland could affect the metabolism of steroids which would later disrupt correlations between salivary and blood steroid levels (Ellison 1988). Enzymes present in the salivary gland such as 11-beta-hydroxysteroid

dehydrogenase and 5-alpha-reductase could be acting upon steroid hormones in saliva. However, direct evidence of such activity is lacking (Ellison 1988). Furthermore, enzymes present in contaminants of mixed whole saliva could also metabolise salivary steroids (Riad-Fahmy et al 1987). Betel nut, which Bangladeshis commonly chew, is a possible contaminant, but it does not affect salivary assays if consumption ceases at least an hour before sample collection (Núñez de la Mora et al 2007b). All participants in this study who chewed betel nut restrained from chewing before sample collection, therefore any interference was avoided. But perhaps there are effects of long-term chewing on salivary diffusion or other factors that might affect the migrant Bangladeshi women's saliva. This could then explain the weak correlations observed. The migrant women were significantly older than the sedentees and therefore much more likely to have been long-term users of betel nut. Bangladeshi women may have lower hormone levels than British European women due to immunological stress more so than energetics or dietary factors (Núñez de la Mora et al 2007a, 2008). Mouth infections have also been shown to affect salivary sample results because oral bleeding contaminates saliva samples with blood hormone concentrations. Immunological challenges and infections common to Bangladesh due to poor sanitation and health services, could also be affecting steroid levels.

Steroid transfer and flow rate of saliva may also influence assay results. According to Ekins (1982), the rate of steroid transfer from blood to tissues with a high clearance rate, like saliva, is dependent on both unbound and bound concentrations of hormone. The amount of progesterone transferred across the membrane of the salivary gland is determined, not only by the unbound concentration in plasma, but also by the ability of steroids to move across capillary membranes (Verheugen et al 1984). In

general, salivary levels of hormones may not strictly reflect the unbound portion of hormone and/or bound hormones may be more active in organ function than originally thought. Furthermore, the specificity of transport proteins is so high that a minor change in the structure of a hormone will affect its binding properties as well (Brook and Marshall 1982). In relation to flow rate, saliva collected during high flow rates bind a smaller fraction of progesterone than saliva collected during low flow rates (Evans 1986). Perhaps the migrant Bangladeshi women have a higher flow rate than the other two groups. Adult migrant women are likely to have been betel chewers over the long term, as discussed above, and it is possible that because of this their flow rate could have been altered over time.

4.2 Ratios of Saliva to Serum Progesterone and Oestradiol

According to studies using serum samples, the percent of free hormone in blood should be between 1- 2% (Westphal et al 1977, Rosenthal et al 1969). However after reviewing studies comparing saliva (free) hormone to serum (total) , this ratio ranges from 0.62% to 10.17%. But most studies showed a ratio around 1% during the mid-luteal phase, therfore becoming the reference value. My findings found ratios considerably less than 1% except among the migrant Bangladeshi (1.1%). It is interesting that this group fits the expected ratio, even though it had the weakest salivary and blood correlations. The ratio for saliva to serum oestradiol should also be between 1-2% (Donaldson et al 1984, Walker et al 1982). My study found much high values between 10-19%. This could be due to the low serum or high salivary oestradiol concentrations, but most likely it is due to assay problems.

To really determine the actual ratio of unbound to bound steroid hormone, free hormone in serum should be isolated and compared to total hormone concentrations in

blood. Furthermore, it is still unclear whether the ratio of free to total steroid is consistent throughout the menstrual cycle because Luisi et al (1981) showed up to a 10-fold increase in ratios during mid-luteal phase. Although it would be complicated to sample blood from women several times over a cycle, this should be done in order to evaluate whether ratios do indeed change across the cycle. Since samples in this study were not isolated to only the mid-luteal phase, they may indeed be reflecting the changing ratio over the cycle.

4.3 Inter-populational Comparisons of Hormone Concentrations and Ratios

As this study is part of a larger research project, the hormone levels should theoretically match the inter-populational differences that have been demonstrated by Núñez de la Mora et al (2007a, 2008). Specifically, the British women should display the highest levels, while Bangladeshi sedentees and migrants should be lower yet similar to each other. Our findings do not follow such trends. To summarise:

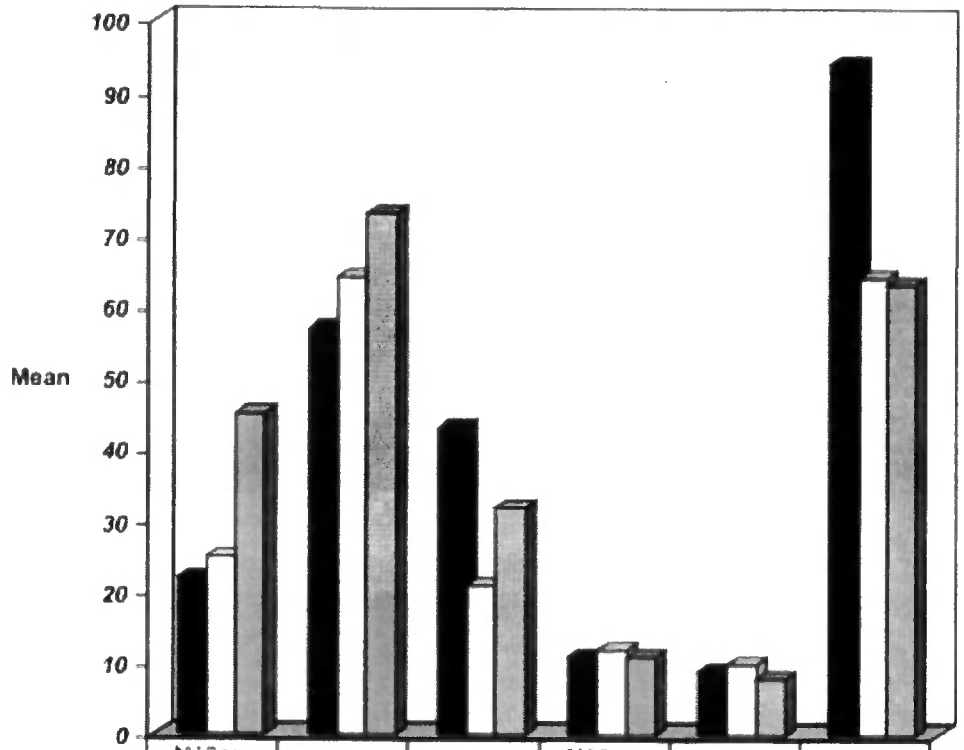
1. Salivary progesterone mean values are similar to those reported by Núñez de la Mora and follow the expected pattern, yet there is no statistical difference between these groups in the current study.

2. Serum progesterone mean values did not match the salivary pattern results demonstrated by the Núñez de la Mora paper because sedentees showied higher values than the British women, and, in the current study, there is no statistically significant difference between groups.

3. The current study's salivary oestradiol levels did not match the earlier results because migrants showed the highest and British women the lowest mean values, and there was no statistically significant difference between these levels.

4. The serum oestradiol mean values showed statistically significant differences between groups. Levels of serum oestradiol were higher among sedentees than both migrant and British groups

FIGURE 4. COMPARISON OF STUDIES' RESULTS: NÚÑEZ DE LA MORA ET AL (2007A, 2008) AND CURRENT STUDY



	Núñez Salivary P pg/mL	Salivary P pg/mL	Serum P ng/mL	Núñez Salivary E2 pg/mL	Salivary E2 pg/mL	Serum E2 ng/mL
Sedentee	22	57	43	11	9	94
Migrant	25	64	21	12	10	64
British	45	73	32	11	8	63

4.4 Study Confounders

The lack of consistency in these results may be due to specific confounders that I was unable to control for. These confounders include: a small sample size, age disparity across groups, variation in participant characteristics and samples taken from different phases of the menstrual cycle

4.4.1 Sample size : Endocrinological studies examining the accuracy of salivary assays included sample sizes ranging between 15-112 samples from 2-29 participants (Walker et al 1979, Luisi et al 1982, Shah and Swift 1982, Read et al 1984, Tallon et al 1984, Zorn et al 1984, Bourqe et al 1986, Lu et al 1997). However these results did not compare groups nor were these studies looking for inter-populational differences. .Caution thus needs to be exercised when drawing conclusions about any group differences within the current study.

4.4.2 Age disparity: Age differences (known to affect hormone levels) could not be controlled for by MANOVA analysis due to sample sizes being less than 30 for each group. There is a significant difference in age among the three groups of women who were sampled, the sedentees being nine years younger than the two other groups. Following Ellison and Lipson's (1992) work, it is apparent that there is a consistent trajectory of hormone levels in relation to age across populations. Women show the highest levels between the ages of 25 and 34; therefore the lower hormone levels of the older women in both migrant Bangladeshi and British women could be affecting the group comparisons. However specific correlations of matched samples should not be affected at the individual level.

4.4.3 Variation across groups in relation to participant characteristics: Differences in education levels, with migrant women having the least amount of schooling, reflect socio-economic status. Differences in BMI, with migrant women having the largest, reflect differences in general health. None of the sedentee women was parous unlike the migrants or British who averaged 3.2 and 1 pregnancies respectively. Even though all women reported regular cycles and had no gynaecological

complaint, there is a possibility that more of the sedentee women have unreported infertility problems. If they are younger women and are not trying to become pregnant then they would have no reason to suspect fertility complications.

4.4.4 Sample variation according to cycle phase : Sample collection occurring at different phases of the cycle when either progesterone or oestradiol might be expected to be higher could sway differences between groups. Difficulties with collecting samples during the luteal phase mirrored those reported in a previous study among Bangladeshi women. While testing the effect of betel nut on salivary steroid analysis during a pilot study with similar sample sizes, Núñez de la Mora (2007b) found 45-55% of samples to be in the follicular phase despite efforts to target only the luteal phase. In the current study, 41% and 77% of women self-reported to be in the mid-luteal and luteal phases respectively. But 95% of all samples were used because of detectable levels of hormone. Since samples were not isolated to the luteal phase, the variability of cycle days became a confounder for the study.

If more women from one group were sampled during the luteal phase -- the Bangladeshi sedentees for example -- then progesterone would be higher compared to the migrant women whose samples were mostly collected during the follicular phase. This would account for the sedentees having the highest serum oestradiol levels, but it does not explain why salivary oestradiol is not equally high in this group. With women varying in so many variables, it is essential to control for confounders; however, the sample sizes do not allow for this kind of multivariate analysis.

4.5 Sampling Methods

The nature of the study—being cross-cultural and requiring blood sampling—elicited some sampling issues when conducting field research. These issues are not easily controlled for by statistical analyses; rather they prompt further consideration and creative approaches to fieldwork within cross-cultural settings. The following section describes certain sampling methods that may have contributed to the inconsistency in the results.

4.5.1 Blood versus Saliva: from the Participants' point of view:

There were cultural constraints when recruiting participants to provide blood samples. Blood is a taboo for many people. In Muslim cultures, blood can represent impurity. In Western cultures, blood can represent risk of infection. For many people, the act of giving blood produces fear. One cannot disregard the cultural connotations of blood when asking participants to provide blood for research. Many women chose not to participate, not wanting to participate in the blood draw. Many Bangladeshi migrant women were willing to participate only if the researchers travelled to the participant's home. Securing a certified phlebotomist to work outside of the clinical setting and to make home visits delayed sample collection, limited time in the field, and reduced the desired sample size. As mentioned by both Luisi et al (1984) and Ellison 1988, blood sampling requires skilled personnel. This requirement does not easily translate into field settings such as Bangladesh or East London.

Women stated their reactions to providing blood and saliva samples after sample collection took place. It is important to note that these opinions came from participants who had already agreed to provide a blood sample. About 45% of women expressed negative reactions towards giving blood referring to feelings of fear and pain. Thirty

percent of women did not like giving saliva samples, stating it was awkward, embarrassing and caused nausea. British women had the fewest problems with blood sampling (40%) while Bangladeshi sedentees expressed the highest problem with blood sampling at 50%. This could be due to the fact that the sedentee women were less likely to have given blood samples prior to this study because of their young age and non-parity. Overall it seems that women would be more likely to provide saliva samples when the awkward/embarrassing connotations were removed. This is easily done when collected privately at home in the participant's own time. However, the ease of saliva collection is a separate issue to that of accuracy; additional blood collection is necessary to validate saliva sampling.

4.5.2 Screening Procedure: For all groups, it is not verified that samples came from ovulatory cycles. For this study's purposes, women were defined as having regular cycles lasting between 25-32 days. These screening criteria are similar to Chatterton et al's (2006) definitions of normal (26-32 days) but are broader than guidelines set out by clinical studies that include women with cycles between 26-30 days (Walker et al 1979, 1982). Allowing for more variability in cycle length may also allow for different concentrations.

There were cultural constraints when asking Bangladeshi women questions regarding their menstrual health. The majority of the sedentees were young single women. Culturally, it is assumed that an unmarried woman is not engaging in sexual behaviour; therefore, questions about the use of contraception were eliminated when interviewing any single Bangladeshi woman. In the beginning of data collection, an interviewer asked one woman who was 23 about her use of contraception. She shook her

head and said, "No, I can't answer that. I am not married." It is also possible that an unmarried Bangladeshi woman does not keep a detailed record of her menstrual history. If there is no chance or desire for pregnancy, then regular menstruation is not an issue of high concern.

4.5.3 Inaccurate Determination of Cycle day: Inaccurate reporting of cycle days affected the successful targeting of mid-luteal phase samples. Matched samples were collected from each woman using on an estimated forward cycle day 21 and, therefore, (hopefully) day -7 of the cycle. With only one sample collected from each woman, data alignment based on cycle day as suggested by Ellison (1988) was not possible. Instead the data reflect ratios from women based on samples collected on a variety of cycle days, and thus are not fully comparable.

The migrant Bangladeshi women had the lowest success rate of correctly estimating their next menstrual cycles for the study's purposes. Instead, the women may have remembered their LMP as "number of days ago" as opposed to documenting the dates on a personal calendar. The majority of British participants referred to their personal calendars when prompted to give their last menstrual period and estimated next menstrual period. Thus, marking their menstrual history on the questionnaire's calendars was an easily transferable exercise for British women. The familiarity of the exercise could account for this group displaying the most accurate rates in all menstrual dating categories.

On the contrary, cultural conceptions of menstruation influenced Bangladeshi women when reporting requested menstrual cycle dates. The Bangla translation for menstruation literally means 'monthly illness'. Therefore most Bangladeshi women, when prompted to mark the questionnaire calendar, recorded their last menstrual period

(LMPcal) and estimated the next menstrual period (ENMP) according to calendar months. For example if a woman's last menstrual period was March 8, she marked her estimated next menstrual period on April 8 despite indicating that her cycle length was 27 days (corresponding instead to April 3).

There is also possible bias when the reporting verified next menstrual period. Some women evidently remembered the date they had estimated during the interview and reported it as the verified next menstrual period (VNMP) possibly for fear of giving the 'wrong answer' to the investigator. Despite a bilingual research assistant collecting data about last menstrual period and estimated next menstrual period from Bangladeshi women, an English-speaking researcher conducted follow-up. Therefore, a language barrier may have also skewed the verification of next menstrual period information.

4.6 Assay Procedure Issues

There were problems in relation to assay procedure and variability during this study. The salivary progesterone concentrations seem to be 10-fold lower than expected. I explored a few possibilities to explain such low values. A Bio Rad protein assay was used on the salivary samples to determine whether the samples were unusually dilute. The protein concentration from a saliva pool taken from many donors over a few weeks was not higher than the protein concentration in the study's samples. Therefore dilute samples cannot account for low progesterone levels. In addition, the preservative used in the collection vials could possibly have interfered. Yet before the samples were assayed, merthiolate was tested and did not interfere with assay results. Furthermore substances that interfere with binding to antiserum give values that are too high, not too low. Preservative interference is thus unlikely.

The quality control pool for the assay is also very low in value. Therefore, it is possible that an incorrect preparation of the assay standard affected the standard curve. However, the concentration of progesterone in the standards was tested via UV absorption and the standard was found to be correct. Still the quality control pool is 5-7 times lower than expected. An additional quality control pool was collected only from women in the luteal phase. This value was considerably higher than the other quality control pool; so while it does not explain the low values in the study, it confirms the low values in the quality control. It remains unclear why values for salivary progesterone are 10-fold lower than expected for such assays. At the moment, abnormal percent ratio values for progesterone and oestradiol cannot be attributed to inter-populational differences since confounders and assay problems cannot be ruled out as influential factors.

Once sample collection is standardised to remove sampling errors, assay procedures should be selected on the basis of sensitivity, specificity, precision and accuracy. The assays conducted in the current study are weak in all these quality control areas. Precision is the ability of the assay to yield consistent results with sub-samples from the same specimen. Precision may exist without a "gold" standard as it represents the scatter of the data and not the exact reported result (accuracy) (BioSciences Research Associates, Inc. http://www.cbrlabs-inc.com/assay-validation.html, 'Assay Validation' accessed June 10, 2008). Intra-assay and inter-assay coefficients are two measures of assay precision. The salivary progesterone assays were conducted 3 times yielding different results, but interassay coefficients were not calculated. Instead, the first two assays were dismissed due to large suspicion of procedure error and the last two assays used I^{25} instead of H^3 as the tracer. Therefore precision was only based on intrassay

coefficients. Most studies use both inter—and intrassay measures to validate their results making this study less comparable to previous studies.

Accuracy is the agreement between the assay result and the excepted referenece value (FDA International Conference on Harmonization 1995). Accuracy requires a "gold" standard but when there is no gold standard, comparison to established reference labs may substitute as was the case with the Tenovus Group studies (BioSciences Research Associates, Inc. http://www.cbrlabs-inc.com/assay-validation.html, 'Assay Validation' accessed June 10, 2008). When interpreting results, it is important to recognise the intrinsic variabilty of results; absolute values will rarely be exactly the same. However, in the current study there may be assay issues and study design confounders affecting the blood samples making these results far from the gold standard against which to compare the saliva.samples. Moreover, directly comparing free hormone in saliva with total hormone in blood does not reveal compelling correlations even if they are significant. Therefore validity can only be given to assay results by contextualising them within known "population means" (Law 1996). However, validation becomes problematic when assaying hormone profiles in new populations, for example Bangladeshi women. Therefore being able to compare the current study's assay results with the results previously reported by Nunez de la Mora (2008, 2007a) is important for determining assay accuracy. However given the problems with sample collection and assay procedure, these studies are not truly comparable.

4.7 Remaining Questions

This study set out to explore some questions brought up by discrepancies between salivary and serum concentrations of progesterone between Bolivian and US women. With matched samples collected between Bangladeshi and British women, the initial

questions regarding whether hormones in saliva or blood best detect ovarian function remained unanswered. We are still asking whether significant salivary and serum correlations are similar across populations. If there are inter-populational differences in steroid correlations, do these differences reflect differences in ovarian function?

4.7.1 Future Implications: The current study points to possible implications for future studies investigating reproductive steroids. It is possible that hormone concentrations found in saliva samples of certain populations are not reflecting the free hormone values. At the same time, total hormone concentrations in blood are not measuring the hormone that is bioavailable to the ovaries either. However, blood has become the gold standard for most medical research, making comparisons with certain studies based on saliva difficult. Instead of dismissing saliva as a reliable diagnostic fluid, the relationship between hormones in blood and saliva must be further investigated and consistently reported as both a correlation and ratio.

Given the problems of the current study, I defer from drawing major conclusions about all research based on data from salivary hormones. Endocrinologists and biological anthropologists alike strongly advocate using salivary sampling due to its convenience and adaptability to field conditions. Blood sampling would be almost impossible in certain field sites without refrigeration. Yet significant hormonal correlations between salivary and serum sample have yet to be verified for anthropological populations. Therefore, I suggest that future anthropological studies utilising salivary sampling should first conduct a pilot study (later described in the following section) investigating the relationship between salivary and serum steroids specific to the population at hand. Simultaneously, I see an opportunity for endocrinological studies to revisit what biological information is actually collected via

blood sampling. What is the definite percent of unbound to bound steroid hormone in blood? Does this vary from individual to individual and also does it vary across the menstrual cycle? Is the bound faction more bioactive than currently thought? What do single blood samples really reveal about ovarian function compared to longitudinal sampling?

4.8 Suggestions for Further Study

A future study specifically investigation blood and saliva hormone correlation should focus on increasing the sample size, establishing a defined age range matched across groups, targeting only the luteal phase and maintaining assay sensitivity, precision, and specificity. To make group comparisons among the Bangladeshi sedentees, migrants, and British women of European descent, the sample size should ideally be 255. The total required sample size comprising 85 per group was determined using an a priori power analysis for ANOVA (using G*Power) with a specified significance value (a= 0.05), power (1-ß =0.95), and a conventional "medium" effect size (Cohen's "f" = 0.25) (Erdfelder et al 1996). Women should be between the ages of 20 and 30 to produce a sample in one age range. In addition, unbound serum hormone must be isolated; and, other components present in blood and saliva should be measured including cortisol, cholesterol, and binding globulins. To answer this question, matched samples from multiple populations need to be assayed, isolating free hormone in serum and then directly comparing it with the assumed unbound hormone existing in saliva.

To better target luteal phase samples, longitudinal tracking of menstrual cycles before sample collection is recommended. For example, participants could keep a menstrual log for one-month recording days of menstruation. Once logs are analysed, women with regular cycles can be scheduled for sampling during their next mid-luteal

phase. More frequent collection of matched samples, even as little as twice a month would also target samples during the luteal phase. But if the period of time allotted to sample collection is still quite limited, the recruitment procedure could restrict sampling to women who menstruated during the week prior to being asked to recall their last menstrual period. This would reduce the time effect on recall error and allow the researcher to concentrate on women reaching their luteal phase within the following three weeks. In addition, refining follow-up procedures could control for self-reporting error. Observed menstrual bloodstains have been used in other studies (Panter-Brick, personal correspondence) to confirm the date of the next menstrual cycle. Although this is an invasive and potentially embarrassing method for women, such techniques could be more reliable than self-reporting.

Overall, this project calls for the validation of salivary sampling for populations whose levels of reproductive hormones have been previously examined. It is still assumed that the correlation of steroids in blood and saliva samples found in European and US women apply to all women. This will remain an assumption until additional studies can validate correlations across populations.

References

Adlercreutz H, Gorbach SL, Goldin BR, Woods MN, Dwyer JT, Hämäläinen E. (1995) Estrogen metabolism and excretion in Oriental and Caucasian women. Journal of the National Cancer Institute 20; 86(14): 1076-82.

Apter D, Reinila M, Vikho R. (1989). Some endocrine characteristics of early menarche, a risk factor for breast-cancer, are preserved into adulthood. International Journal of Cancer 44(5): 783-787.

Banerjee S, Levitz M, Rosenberg CR (1985) On the stability of salivary progesterone under various conditions of storage. Steroids 46: 967-974.

Bentley GR, Aunger R, Harrigan AM, Jenike M, Bailey RC, Ellison PT (1999). Women's strategies to alleviate nutritional stress in a rural African society. Social Science & Medicine 48(2): 149-162.

Bentley GR and Harrigan AM (1990). Ovarian cycle length and days of menstruation of Lese horticulturalists. American Journal of Physical Anthropology 81(2): 193-194.

BioSciences Research Associates, Inc. http://www.cbrlabs-inc.com/assay-validation.html, 'Assay Validation' accessed June 10, 2008

Bourque J, Sulon J, Demey-Ponsart E, Sodoyez JC, Gaspard U (1986). A simple, direct radioimmunoassay for salivary progesterone determination during the menstrual cycle. Clinical Chemistry 32(6): 948-951.

Brook CGD and Marshall NJ (2001). Essential endocrinology. Oxford, Blackwell Science.

Butt W (1982): Discussion. In Read GF, Riad-Fahmy D, Walker RF, Griffiths K, editors. Immunoaasays of steroids in saliva: proceedings of the ninth Tenovus workshop. Cardiff: Alpha Omega Publishing Ltd:d. pp. 141-142.

Corrie JET (1982). Immunoassays for steroid-hormones using radioiodinated tracers. British Veterinary Journal 138(5): 439-442

Chatterton RT, Mateo ET, Lu D, Ling F (2006). Interpopulational differences in the concentrations and ratios of salivary and serum progesterone. Fertility and Sterility 86(3): 723-725.

Choe JK, Khan-Dawood FS, Dawood MY. (1983) American Journal of Obstetrics and Gynecology 147(5):557-62.

Donaldson A, Jeffcoate SL, Sufi SB (1984). Assays of Oestradiol and progesterone in Saliva in the assessment of ovarian function. In: Ferguson DB. Frontiers of Oral Physiology: Steroid Hormones in Saliva. London: Karger, Basel 5:80-86.

Dunger DB, Ahmed ML, Ong KK. (2005) Effects of obesity on growth and puberty. Practice and Research Clinical Endocrinology and Metabolism 19: 375-390.

Ekins RP (1960) The estimation of thyroxine in human plasma by an electrophoretic technique. Journal of Clinica Chimic Acta 5(4): 453-459.

Ekins RP (1982) The role of binding proteins in hormone transport. In: Read GF, Riad-Fahmy D, Walker RF, Griffiths K, editors. Immunoassays of steroids in saliva: proceedings of the ninth Tenovus workshop. Cardiff: Alpha Omega Publishing Ltd. pp. 64-68.

Ellison PT (1988) Human salivary steroids: Methodological considerations and applications in physical anthropology. Yearbook of Physical Anthropology 31: 115-42.

Ellison PT (1990). Human ovarian-function and reproductive ecology- new hypotheses. American Anthropologist 92(4): 933-952.

Ellison PT (1993). Measurements of Salivary Progesterone. Annals of the New York Academy of Sciences 694: 161-176.

Ellison PT (1996). Developmental influences on adult ovarian hormonal function. American Journal of Human Biology 8(6): 725-734.

Erdfelder, E., Faul, F., & Buchner, A. (1996). GPOWER: A general power analysis program. Behavior Research Methods, Instruments, & Computers, 28, 1-11.

Ellison PT (2005) Evolutionary Perspective on the Fetal Origins Hypothesis. American Journal of Human Biology 17:113-118.

Evans JJ (1986). Progesterone in saliva does not parallel unbound progesterone in plasma. Clinical Chemistry 32(3): 542-544.

FDA International Conference on Harmonization (1995). Guideline on Validation of Analytical Procedures: Definitions and Terminology; Availability. Docket No. 94D-0016: March .

Ferguson DB (1984) Steroid Hormones in Saliva (Frontiers of Oral Physiology) New York, Karger.

Fotherby K (1979) Progesterone II: Clinical aspects. In: Gray AC. Hormones in blood. Vol 3. Academic press: 439.

Gann PH, Chatterton RT, Gapstur SM, Liu K, Garside D, Giovanazzi S, Thedford K, Van Horn L. (2003) The effects of a low-fat/high-fiber diet on sex hormone levels and menstrual cycling in premenopausal women: A 12-month randomized trial (the diet and hormone study). Cancer 98(9):1870 – 1879.

Goldin BR, Adlercreutz H, Gorbach SL, Warram JH, Dwyer JT, Swenson L, Woods MN. (1982). Estrogen excretion patterns and plasma levels in vegetarian and omnivorous women. New England Jourbal of Medicine 16;307(25):1542-7.

Goldsworthy GJ, Robinson J, Mordue W (1981). Endocrinology. Glasgow, Blackie.

James VHT and Jeffcoate SL (1974) Radioimmunoassay and saturation analysis. Steroids. British Medicine Bulletin 30: 50-54.

Jasienska G and Ellison PT (1998). Physical work causes suppression of ovarian function in women. Proceedings of the Royal Society of London Series B-Biological Sciences 265(1408): 1847-1851.

Jasienska G and Ellison PT (2004). Energetic factors and seasonal changes in ovarian function in women from rural Poland. American Journal of Human Biology 16(5): 563-580.

Karsch FJ (1984) The Hypothalamus and anterior pituitary gland In : Austin and Short editors. Reproduction in mammals Vol 3: Hormonal control of reproduction. Ed 2. Cambridge University Press. pp. 1-20.

Katz FH and Shannon IR (1969) Parotid fluid cortisol and cortisone. Journal of Clinical Investigation 48(5): 848–855.

Lipson SF and Ellison PT (1989) Development of protocols for the application of salivary steroid analysis to field conditions. American Journal of Human Biology 1(3): 249-255.

Lipson SF and Ellison PT (1992). Normative study of age varaition in salivary progesterone profiles. Journal of Biosocial Science 24(2): 233-244.

Lu YC, Chatterton RT, Vogelsong KM, May LK (1997). Direct radioimmunoassay of progesterone in saliva. Journal of Immunoassay 18(2): 149-163.

Luisi M, Franchi F, Kicovic PM, Silvestri D, Cossu G, Catarsi AL, Barletta D, Gasperi M (1981). Radioimmunoassay for progesterone in human-saliva during the menstrual cycle. Journal of Steroid Biochemistry and Molecular Biology 14(10): 1069-1073.

Luisi M and Franchi F (1984) Salivary steroid measurements. An alternative approach to plasma assays in assessing endocrine function. Frontiers of Oral Physiology 5:124-154.

Luisi M, Franchi F, Kjcovic PM, Barletta D, Buber V (1982). Variation of salivary progesterone throughout the menstrual cycle. Immunoassays of steroids in saliva: proceedings of the ninth Tenovus workshop, Cardiff, Cardiff: Alpha Omega Publishing Limited.

Murphy BEP (1964). Evaluation of a new method for determination of serum thyroxine Clinical Endocrinology and Metabolism. 24:785

Núñez de la Mora A, Bentley GR, Chouhury OA, Napolitano DA, Chatterton RT (2008) The impact of developmental conditions on adult salivary estradiol levels: Why this differs from progesterone? American Journal of Human Biology 20(1):2-14.

Núñez de la Mora A, Chatterton RT, Choudhury OA, Napolitano DA, Bentley GR (2007a). Childhood conditions influence adult progesterone levels. Plos Medicine 4(5): 813-821.

Núñez de la Mora A, Chatterton RT, Mateo ET, Jesmin F, GR Bentley. (2007b). Effect of chewing betel nut on measurements of salivary progesterone and estradiol. American Journal of Physical Anthropology 132(2): 311-315.

Panter-Brick C (2008) Email correspondence in March 2008 from Catherine Panter-Brick. Univeristy of Durham.

Panter-Brick C and Ellison PT (1994). Seasonality of workloads and ovarian-function in Nepali women. Human Reproductive Ecology 709: 234-235.

Panter- Brick C, Lotstein DS, Ellison PT. (1993). Seasonality of reproductive function and weight-loss in rural Nepali women. Human Reproduction 8(5): 684-690.

Prior JC (1985) Luteal phase defects and anovulation: adaptive alterations occurring with conditioning exercise. Seminars in Reproductive Endocrinology 3(1):27-33.

Read GF, Riad-Fahmy D, Walker RF, Griffiths K, editors (1982). Immunoassays of steroids in saliva; . proceedings of the ninth Tenovus workshop, Cardiff, Cardiff: Alpha Omega Publishing Limited.

Read GF, Wilson DW, Hughes IA, Griffiths K (1984). The use of salivary progesterone assays in the assesment of ovarian-function in postmenarcheal girls. Journal of Endocrinology 102(2): 265-268.

Riad-Fahmy D, Read GF, Walker RF (1983). Salivary steroid assays for assessing variation in endocrine activity Journal of steroid biochemistry and molecular biology 19(1): 265-272.

Riad-Fahmy D, Read GF, Walker RF, Walker SM, Griffiths K. (1987). Determination of ovarian-steroid hormone levels in saliva- an overview. Journal of Reproductive Medicine 32(4): 254-272.

Rosenthal HE, Slaunwhite WR, Sandberg AA (1969). Journal of Clinical Endocrinology and Metabolism, 29, 352.

Shah R, Swift AD (1982) Salivary progesterone throughout the menstrual cycle and pregnancy. In: Read GF, Riad-Fahmy D, Walker RF, Griffiths K, editors. Immunoaasays of steroids in saliva: proceedings of the ninth Tenovus workshop. Cardiff: Alpha Omega Publishing Ltd. pp. 134-47.

Sharmeen T (2007). Personal correspondence in March 2007 from Taniya Sharmeen, Anthropology Phd Candidate and native Bangla Speaker, University College London.

Stanczyk FZ (2004). Extraction/Chromatographic Testosterone RIA Can Be Used as the "Gold Standard" for Determining the Reliability of Direct Testosterone Immunoassay Measurements. Clinical. Chemistry. 50: 2219 - 2220.

Stanczyk FZ, Gentzschein E, Ary BA, Kojima T, Ziogas A, Lobo RA (1997) Urinary progesterone and pregnanediol. Use for monitoring progesterone treatment. Journal of Reproductive Medicine 42(4): 216-22.

Sufi SB, Donaldson A, Gandy SC, Jeffcoate JL, Chearskul S, Goh, H, Hazra D, Romero C, Wang HZ (1985). Multicenter evaluation of assays for estradiol and progesterone in saliva. Clinical Chemistry 31(1): 101-103.

Tallon DF, Gosling JP, Buckley PM, Dooley MM, CleereWF, O'Dwyer EM, Fottrell PF (1984). "Direct solid-phase enzyme immunoassay of progesterone in saliva. Clinical Chemistry 30(9): 1507-1511.

Tulchinsky D and Okada DM (1975). Hormones in human pregnancy. IV. plasma progesterone American Journal of Obstetrics and Gynecology. 121, 293.

Van der Molen HJ, Corpéchot C (1968) Isolation and identification of progesterone from urine of nonpregnant women. Journal of Clinical Endocrinology and Metabolism 28(9):1361-6.

Verheugen C, Pardridge WM, Judd HL, Chaudhuri G (1984). Differential permeability of uterine and liver vascular beds to estrogens and estrogen conjugates. Journal of Clinical Endocrinology and Metabolism 59: 1128-32.

Vihko R and Apter D (1984). Endocrine characteristics of adolescent menstrual cycles-impact of early menarche. Journal of Steroid Biochemistry and Molecular Biology 20(1): 231-236.

Vihko R and Apter D (1989). Endogenous steroids in the patho-physiology of breast cancer. Critical Reviews in Oncology/Hematology 9(1): 1-16.

Vining RF and McGinley RA (1986) Hormones in saliva. CRC Critical Review Clinical Laboratory Science 23:95-146.

Vitzthum VJ, GR Bentley, H Spielvogel, E Caceres, K Heidelberg, K Crone, R Chatterton (1998) Salivary progesterone levels at conception and during gestation in rural Bolivian women. FASEB Journal 12(5): 4211.

Vitzthum VJ, Bentley GR, Spielvogel H, Caceres E, Thornburg J, Jones L, Shore S, Hodges KR, Chatterton RT (2002). Salivary progesterone levels and rate of ovulation are significantly lower in poorer than in better-off urban-dwelling Bolivian women. Human Reproduction 17(7): 1906-1913.

Vitzthum VJ, Von Dornum M, Ellison PT (2005)Effect of coca-leaf chewing on salivary progesterone assays. American Journal of Physical Anthropology 92(4):539 - 544

Walker RF, Read GF, Riad-Fahmy D(1979). Radioimmunoassay of progesterone in saliva: application to the assessment of ovarian function. Clinical Chemistry 25(12): 2030-2033.

Walker RF, Walker S, Riad-Fahmy D (1982). Salivary progesterone as an index of luteal function. In: Read GF, Riad-Fahmy D, Walker RF, Griffiths K, editors. Immunoassays of steroids in saliva: proceedings of the ninth Tenovus workshop, Cardiff, Alpha Omega Publishing Ltd. pp. 115-26

Walker SM, Mustafa A, Walker RF, Riad-Fahmy D (1981). The role of salivary progesterone in studies of infertile women. British journal of obstetrics and gynaecology 88(10): 1009-1015.

Walker SM, Walker RF, Riad-Fahmy D (1984) Longitudinal Studies of luteal function by salivary progesterone determinations. Hormone Research 20:231-240

Westphal U, Stroupe SD, Cheng SL (1977). Ann. NY Acad. Sci. 286, 10.

Wright AD and Taylor KW (1967) Immuno-assay of Hormones. In: Hormones in Blood. C. H. Gray and A. L. Bacharach, Eds. Second edition. Academic Press, New York, 1967. Vol. 1.

Yalow RS, Berson SA (1960). Immunoassay of endogenous plasma insulin in man. Journal of Clinical Investigation. 39: 157-75.

Yannone ME, Mueller JR, Osborn RH (1969) Protein binding of progesterone in the peripheral plasma during pregnancy and labor. Steroids 13 773-781.

Zorn JR, McDonough PG, Nessman C, Jansens Y, Cedard L (1984). Salivary progesterone as an index of the luteal function. Fertility and Sterility 41(2): 248-53.

Appendix

Participant No._____

Project title: Salivary and plasma progesterone correlation in Bangladeshi women

We are asking a small number of women if they will give one saliva sample shortly before giving a blood sample scheduled at approximately one week prior to their menstrual period.

All data will be taken during one meeting. At first we will ask you to fill out a questionnaire. We will also measure your height and weight, as well as skin around your arms. We will provide a small tube to spit into. We will give you a piece of vegetarian chewing gum to stimulate saliva flow, so the sample is very quick to obtain. Dr. Kurshida Begum will draw a blood sample from your arm.

The purpose of the matched saliva and blood sample is to compare results obtained for the hormones progesterone and oestradiol which we have studied earlier in Bangladeshi women. We will also analyze these samples for levels of cholesterol, cortisol (a stress hormone) and proteins to which hormones can attach in the blood for transport. The reason for looking at these other substances is to attempt to explain any differences that we might find in levels of the hormones in blood and saliva.

Please answer the following questions so that we can determine your eligibility:

Part I: Demographics

Are you from Sylhet?	YES☐	NO☐
Are you between 23 and 39 years of age?	YES☐	NO☐
Do you menstruate every 25 -32 days?	YES☐	NO☐
If YES, how many days do you have between cycles? _____ days		
Are you willing to give one saliva sample?	YES☐	NO☐
Are you willing to give one blood sample?	YES☐	NO☐

Part II: Health

Do you have a chronic disease including:		
Diabetes	YES☐	NO☐
Thyroid-related problems	YES☐	NO☐
Polycystic ovaries	YES☐	NO☐
Have you taken birth control pills within the last 3 months?	YES☐	NO☐
Have you breast-fed within the last 6 months?	YES☐	NO☐
Have you been diagnosed with any infertility problems?	YES☐	NO☐
Have you ever taken hormonal treatment?	YES☐	NO☐

Participant No._____

Name:		Address:	
Phone:			
Email:			

Please star the best way for us to contact you.

- If you are you willing to participate, please mark on the calendar the date of your last period and when you expected to start your next period. We ask that when we schedule your next appointment, that you do not eat food, drink liquids, or chew beetel nut for at least 1 hour beforehand. Each woman that participates will receive 500 taka as compensation.

MARCH 2007

Sun	Mon	Tue	Wed	Thu	Fri	Sat
				1	2	3
4	5	6	7	8	9	10
11	12	13	14	15	16	17
18	19	20	21	22	23	24
25	26	27	28	29	30	31

APRIL 2007

Sun	Mon	Tue	Wed	Thu	Fri	Sat
1	2	3	4	5	6	7
8	9	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28
29	30					

MAY 2007

Sun	Mon	Tue	Wed	Thu	Fri	Sat
		1	2	3	4	5
6	7	8	9	10	11	12
13	14	15	16	17	18	19
20	21	22	23	24	25	26
27	28	29	30	31		

JUNE 2007

Sun	Mon	Tue	Wed	Thu	Fri	Sat
					1	2
3	4	5	6	7	8	9
10	11	12	13	14	15	16
17	18	19	20	21	22	23
24	25	26	27	28	29	30

Personal Information

Do you know your exact date of birth? ☐YES ☐NO

If YES, what is it? _____

Where were you born? _____

Where was your mother born? _____

Where was your father born? _____

What is your marital status?

☐Single, unmarried ☐Divorced ☐Separated

☐Married ☐Widowed

How many people live in your household?

Please list your family members and their age:

_____ _____

_____ _____

_____ _____

Socioeconomic information

What type of accommodation does your household occupy?

☐House ☐Flat ☐

☐Other, specify_____

Does your household own or rent accommodation?

☐OWNS ☐RENT

2.4 Do you work? ☐YES ☐NO

If YES, what is your occupation? _____

Education

Do/did you attend school? ☐YES ☐NO

What is the highest class in school/college that you have completed?

Please list any qualifications.

Physical activity

Do you exercise or practise any sport? ☐YES ☐NO

If yes, what type?

How often?

Do you walk continuously for more than 20 min. on a daily basis?

☐YES ☐NO

Reproductive History Information

How old were you when you first started to menstruate? Is this an exact age?

What year were you in?

Have you ever been pregnant? ☐YES ☐NO

If yes, how many times?

5.5 How did it feel to give a blood sample? How did it feel to give a saliva sample?