Taking the Lab to the Field: Monitoring Reproductive Hormones in Population Research

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Fertility and mortality, the central processes studied by demographers, are clearly biological in nature. Thus, it is only natural that the interplay between biology and demography has intrigued such renowned population researchers as Alfred Lotka (1934), Louis Henry (1961), and Mindel Sheps (1964). Despite this long-standing interest in biological principles, the actual incorporation of biological variables in population research has started only recently. The advent of new technology that makes collection of relevant biomarkers from human subjects more feasible is causing a surge in biodemographic studies (Wachter and Bulatao 2003; Rodgers and Kohler 2003), and the added value of a multidisciplinary approach that combines biological and social science methods is reflected in an increased range of research questions analyzed in more comprehensive theoretical frameworks.

In the case of fertility, the analysis of individual biological parameters can help explain seemingly puzzling or incongruent data from different populations or from couples within the same population. One of the best examples of the synergy of biodemographic research is the study of the postpartum return to fecundity. In 1961, Louis Henry published a seminal paper on the natural variation in human fertility and the proximate causes of that variation (Henry 1961). He found that the period of postpartum subfecundity was highly variable among natural fertility populations, but that the current social explanations based on postpartum sexual taboos in traditional societies could not account for that variability. Henry suggested that variation in the resumption of ovulation after childbirth might be the most important source of variation in interbirth intervals. He reckoned that the practice of lactation might be related, by some unknown mechanism, to the resumption of post-

partum ovulation. More recently, the possibility of evaluating between- and within-population variation in postpartum hormonal levels has allowed the formulation of well-defined, testable hypotheses that can explain the large variation in the duration of postpartum anovulation (Lunn et al. 1984; McNeilly 1993; Valeggia and Ellison 2004; Holman et al. 2006).

The benefits of incorporating biomarkers in population studies are further illustrated by examining how an understanding of the variation in reproductive hormone levels contributes to understanding demographic phenomena. The variation in reproductive hormone levels can be very high among populations, within populations, and even within the same woman (Valeggia and Ellison 2004; Ellison 1990; Ellison et al. 2002; Ellison et al. 1993). This variation, far from being considered as noise, should become the focus of our research attention. This note provides an overview of methods and techniques for estimating hormonal levels in large-scale population studies and considers their advantages and disadvantages and the ethical issues that biomarker collection may raise. Herein, "large-scale" means the collection and analysis of thousands of samples from the field.

Collection of hormonal biomarkers in field research

The adequate incorporation of biological data into demographic research requires the collection of large numbers of samples. Almost unthinkable a couple of decades ago, recent advances in technology have made the collection of thousands of samples more manageable both logistically and financially (depending on the type of assay chosen, the cost per sample ranges, on average, between US$3.00 and $10.00). I describe below three of the most frequently used techniques: dried blood spots, urine samples, and saliva samples. For each of the three techniques, I describe which hormones can be monitored, the advantages of the technique, and the methods of sample collection, preservation, and shipment. (A brief account of the functioning of reproductive hormones is given in the Appendix.) Table 1 provides a summary of the methods discussed.

Dried blood spots

Dried blood spot assays are a highly sensitive and accurate means for measuring various hormones. Nearly all studies that have compared whole blood with dried blood have shown high correlations for most of the tested biomarkers (Shirtcliff et al. 2001; Worthman and Stallings 1994, 1997).

Dried blood spots, widely used in clinical settings, are best known for their use in neonatal screening for phenylketonuria, congenital hypothyroidism, and hyperplasia (Carreiro-Lewandowsky 2002). This technique is also receiving attention as a tool in clinical care and in large-scale epidemiologi-

TABLE 1 Comparison of methods for monitoring reproductive hormones in human population-scale research

Method	Collection and storage	Shipment	Hormones measured	Advantages	Disadvantages	Publications
Dried blood spots	On filter paper; ideally refrigerated, but can stand room temperature.	No refrigeration needed, mailed without special packaging (except desiccant) within the US.*	testosterone, estradiol, progesterone, FSH, LH, prolactin, cortisol, androstenedione, DHEA-S	No freezing necessary. Easy to store and ship. Broader range of hormones that can be analyzed.	Finger pricking may be uncomfortable.	Worthman and Stallings 1994, 1997; Shirtcliff et al. 2000
Fluid urine samples	Collection in urine cups and divided in small vials. Need freezing as soon as it is possible.	Stored and shipped frozen with special packaging to prevent thawing and leaking.	testosterone, metabolites of estrone and progesterone, hCG, LH, cortisol	Minimally invasive, participants habituated to collect urine samples for medical reasons.	Samples must be stored and shipped frozen.	O'Connor et al. 2003
Dried urine on filter paper	On filter paper; ideally refrigerated, but can stand room temperature.	No refrigeration needed, mailed without special packaging (except desiccant) within the US.*	testosterone, metabolites of estrone and progesterone, hCG, LH, cortisol	Same as above, plus no freezing necessary, easy shipment. No trained personnel needed for collection.	Needs extraction for assaying.	Shideler et al. 1995; Knott 2005; Derr et al. 2006
Saliva samples	Collection in polystyrene plastic tubes. If biocide is added, samples can be stored at room temperature. If available, freezing is recommended.	No refrigeration needed, mailed with special packaging to prevent leaking.*	estrone, estradiol, estriol, progesterone, testosterone, cortisol	Minimally invasive, sample collection does not need privacy or trained personnel.	Collection vials are generally bulky, caution should be taken to ship brittle tubes with abundant cushioning to prevent leaking.	Ellison 1988; Lipson and Ellison 1989, 1992, 1996

* For international shipping, see local regulations.

cal research for the detection of HIV and hepatitis C antibodies (Lukacs et al. 2005; Sherman et al. 2005), hepatitis B antigens (Mendy et al. 2005), iron deficiency (Shell-Duncan and McDade 2004), thyroid-stimulating hormone (Hofman et al. 2004), and C-reactive protein (Shell-Duncan and McDade 2004; McDade et al. 2004, 2005).

Biological anthropologists and human biologists have been successfully using dried blood spots for collecting reproductive hormones data since the mid-1990s (Worthman and Stallings 1994, 1997; Campbell 1994; Shirtcliff et al. 2000).

1. Hormones measured: Blood spot techniques can be used for determination of testosterone (T), estradiol (E2), progesterone (P), follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), and sex hormone binding globulin (SHBG—the primary sex hormone–carrying protein). In addition, they can be used to reliably measure adrenal hormones that impinge upon reproductive function, such as cortisol (C), androstenedione (A), and dehydroepiandrosterone-sulfate (DHEA-S).

2. Sample collection: The materials needed for blood spot collection are minimal. Blood drops resulting from finger pricking are placed on properly labeled filter paper.[1] After the skin is wiped with an alcohol swab, the area is pricked with a disposable needle (lancet) fitted into a self-retractable lancing device.[2] When blood flow begins, the first droplet is discarded and the subsequent droplets are collected on the filter paper.[3] The sample is left to air-dry at room temperature away from heat, sunlight, and, in field conditions, insects and "curious fingers." In humid environments, it is strongly recommended to use a fan to accelerate the process.

3. Sample preservation: After the filter papers are completely dry (usually after four hours in regular ambient conditions), they are placed in sturdy cardboard boxes with a desiccant pack (e.g., silica gel). Ideally, samples should be frozen immediately (–20°C) until shipment to the laboratory. In the field, samples can be stored at refrigerator temperature (–4°C) for approximately eight weeks, at room temperature (22°C) for two to eight weeks, and at tropical temperatures (37°C) for about one week (Worthman and Stallings 1997).

4. Sample shipment: If the samples are to be transported within the United States,[4] dried spots can be mailed via the regular postal system without special mailing cartons (Centers for Disease Control and Prevention Guidelines for the Shipment of Dried Blood Spot Specimens «www.cdc.gov/od/ohs/biosfty/driblood.htm»). Unless there is evidence that a dried blood spot sample contains an infectious agent, there is no need to package the samples in any special way or to place a biohazard label on the envelope.

5. Advantages: The dried blood spot procedure has several advantages over whole blood collection by venipuncture: it does not require specially trained personnel; it uses a small volume of blood; it does not require immediate centrifuging and freezing; and, at least in the United States, it can

be safely mailed via the regular postal system without special packaging. All of these characteristics reduce the cost of sample collection. Compared to saliva samples, dried blood spots have the sizable advantage of being stored in a solid base, thereby avoiding the logistical problems of bulky, brittle, and leaky vials. In addition, they allow for the measurement of circulating levels of protein hormones such as PRL, LH, and FSH, binding proteins such as SHBG, and conjugated steroids that cannot be measured in saliva (see *Saliva samples* below).

Urine samples

Urine samples are ideally suited for addressing research questions that require an integrated measure of reproductive hormone output. This is of special interest to population researchers who are not generally concerned with the daily variation of hormones, but with intra- and inter-population variance.

1. Hormones measured: Several metabolites of estradiol (E2) are excreted in urine, such as free estrone (E1), estrone sulfates, estrone conjugates (E1Cs), and estrone-3-glucuronide (E1G). Of those, E1Cs and E1G are the most frequently used in studies of ovarian function, whereas PdG (pregnanediol-3-glucuronide), the main urinary metabolite of progesterone, has been extensively used as a measure of luteal function and fecundability. Urinary concentrations of human chorionic gonadotropin (hCG) have been used reliably in population studies of early pregnancy and early pregnancy loss (Lasley et al. 1995; Lohstroh et al. 2005). Gonadotropins (LH and FSH), present in urine, have been shown to be reliable biomarkers for ovulation in large-scale studies (Li et al. 2002; Weiss et al. 2004). Cortisol, the so-called stress hormone, can also be measured using urine samples. In addition to its role in the management of physiological challenges, cortisol has been shown to be associated with gonadal hormone levels (Lukas et al. 2005; Nepomnaschy et al. 2004) and can be linked to reproductive mechanisms.

2. Sample collection: The use of first morning void is highly recommended because it integrates the excretion of metabolites overnight and makes samples more standardized. Since this can be a severe limitation for large-scale studies, it should be noted that urine samples for measurement of ovarian hormones can be taken at any time of the day.[5]

Commercially available specimen collection cups are handed to the participants, who are asked to provide a urine sample.[6] In tropical settings, urine samples should be transported from the point of collection to the processing station in coolers with ice packs, particularly if the collection takes a few hours.

3. Sample preservation: Urine samples can be preserved as a fluid in a vial or dried on filter paper. Preservation of samples in fluid form should be the option of choice to avoid extraction steps before analysis.[7] The availability of

freezers, liquid nitrogen, and dry ice, however, is very limited in some field settings. In these situations, urine samples can be reliably preserved using a filter paper method similar to the one used for dried blood spot collection.[8]

4. Sample shipment: In the United States, packing and labeling of samples and containers must follow the Centers for Disease Control and Prevention regulation 42 CFR72 («www.cdc.gov/od/ohs/ biosfty/shipregs.htm»), US Department of Transportation (DOT) Rules and Regulations («http://hazmat.dot.gov»), and the US Postal Service (USPS) Regulations («www.usps.com»).[9] If samples are to be mailed internationally by air, shipment packaging must comply with the regulations of the International Air Transportation Association (IATA, «www.iata.org/whatwedo/cargo/dangerous_goods/index.htm»).

As in the case of dried blood spots (see above), urine samples dried on filter paper can be transported via normal postal systems without special mailing cartons.

5. Advantages: The collection of urine samples has several logistical advantages over serum and blood spot samples. The procedure is painless and less invasive than blood collection, facilitating the compliance of study participants and collection of frequent samples over long periods of time. Even in remote locations, most populations have been made aware of the relationship between the collection of urine samples and health-related issues, which makes explanation of research objectives and the informed consent process considerably easier. Urine sampling may also be a more readily accepted method for the collection of biological specimens from infants, children, and the elderly. Unlike serum samples, urine samples can be collected and stored by untrained personnel or even study participants. Finally, compared to the handling of blood, urine samples carry a smaller risk of infectious disease transmission.

Saliva samples

Saliva samples have been used in large-scale studies of human ovarian function and fecundity since the late 1980s (Ellison 1988; Lipson and Ellison 1989, 1992, 1996).

1. Reproductive hormones measured: Saliva samples can be used to measure estrone (E1), estradiol (E2), estriol (E3), progesterone, and testosterone (Ellison et al. 1989; Ellison 1993; Ellison and Panter-Brick 1996; Jasienska and Thune 2001; Bribiescas 2005). Salivary cortisol (Gozansky et al. 2005) can also be reliably measured and related to reproductive biology variables (Groschl et al. 2000).

2. Sample collection: The collection of saliva samples in adults is relatively simple. Study participants are asked to rinse their mouth thoroughly with tap water and then salivate into polystyrene plastic tubes.[10]

Sample collection protocols need to be modified if the research design includes infants and children. For the collection of saliva from infants and young children, researchers have used disposable micropipettes (Valeggia

and Ellis, unpublished manuscript), cotton-based swabs or dental rolls that absorb saliva (Schmidt 1997; Herrington et al. 2004; Talge et al. 2005), and modified medical pacifiers (Groschl et al. 2003).

Field studies have also shown that cultural traditions may have an impact on the measurement of salivary reproductive hormones. For example, in Andean populations, coca leaf chewing may produce false salivary progesterone values that mimic luteal phase values (Vitzthum et al. 1993). Findings like this emphasize the need for proper pilot work and protocol testing before beginning.

3. Sample preservation: Samples of steroid hormones can be safely stored at room temperature if a biocide is added to prevent bacterial growth and contamination (Ellison 1988). If a freezer is available in the field, it is recommended to store the samples at –20°C. Once in the laboratory, samples should be kept frozen.

4. Sample shipment: Saliva samples can be safely shipped without refrigeration. Tubes must be placed in resealable, leak-proof bags and packed with sufficient cushioning and absorbent material to prevent breaking and leakage.

According to United States regulations, saliva samples (like urine samples) fall within the diagnostic specimen categories and should follow the Department of Transportation and United States Postal Service regulations (see section on sample shipment in *Urine samples*). For sample collection outside the United States, investigators should consult local regulations to ensure safety norms are followed.

5. Advantages: Collection of saliva samples is the method of choice for many large-scale research studies. Of the methods reviewed here, it is the least invasive: it is painless (cf. finger pricking for blood spot collection) and most participants do not need privacy for collecting the sample (cf. urine collection). This allows for the collection of repeated sampling over a long period of time (e.g., in longitudinal studies).

Saliva samples have been successfully used in studies involving participants of a wide range of ages, including pre-term infants (Herrington et al. 2004), children (Ahnert et al. 2004; Azurmendi et al. 2005), and the elderly (Steptoe et al. 2005; Wright et al. 2005). In studies involving adult participants, samples can be entrusted to them for home collection and stored until a convenient pick-up time. In addition, steroid reproductive hormones are very stable at room temperature, which greatly facilitates the collection of samples in remote areas where access to freezers and refrigerators is rare.

Ethical considerations in collecting biological samples

The collection of biological samples requires consideration of its concomitant ethical issues. I will briefly mention two related ethical concerns: the "respon-

dent burden" and omission of the potential health benefits to the population under study (Boerma et al. 2001). The respondent burden refers to the fact that the introduction of biomarkers may increase the percentage of nonresponse and may compromise the participation in subsequent follow-up studies, as an additional burden is imposed on the study participants (Weinstein and Willis 2001). This problem should be anticipated by carrying out a pilot study in a subsample of the selected population and evaluating the rate of response to the collection procedures. The percentage of nonresponse can also be reduced if potential participants see a clear health benefit to providing a sample. Particularly in studies conducted in rural or remote areas, the research team should include trained agents who can provide basic health education and referrals to the public health system. Providing prompt feedback of results to the community can also facilitate future data collection procedures. Many times, biomarkers are collected in such a way that some preliminary results that carry applied value can be obtained relatively fast. For example, the visual inspection of a urine sample could allow the field assistant to identify potential health problems (bloody, very dark, or cloudy urine may indicate infections or kidney disease). Furthermore, if field assistants identify health issues that should be dealt with *in situ*, the research team is required to refer the participant and/or family members to the available public health system.

For studies conducted in developing countries, another ethical issue, one arising from the imbalance in technology, is both evident and sensitive. The collection of biological samples using these minimally invasive techniques can be quite cost-effective, particularly in countries where labor—in the form of field assistance—is relatively inexpensive. However, the costs of assaying those samples in the country of collection are often high. This means that the results are obtained thousand of miles away from the population under study, which raises an obvious ethical concern of omission of potential health benefits to the population originally under study. Well-designed research protocols should include the transfer of information, particularly if results directly relevant for alleviating public health problems are involved (Boerma et al. 2001; Hurtado and Salzano 2004). Public health officials and community leaders should have uncomplicated access to those study results that can potentially assist in improving the welfare of the population. Our research on the reproductive ecology of indigenous communities in the province of Formosa, Argentina (Valeggia and Ellison 1998, 2001, 2003a, 2003b, and 2004) is having a demonstrable impact on the communities we study. Our research protocols have included different ways of disseminating our results, depending on the degree of literacy of the population under study. For example, we have prepared progress and final reports written in Spanish for public health officials. A version of these reports, modified to be easily understood by local community leaders, is then presented at a community meeting. We have also used local radio broadcasts in both Spanish and the indigenous language to

reach remote and illiterate participants. Study participants appreciate learning about the results of the research conducted in their communities and often provide valuable comments that can make future studies intellectually sounder and logistically easier.

Illustrative contributions of biological data

Following are examples of biodemographic questions that have been successfully answered and modeled using reproductive hormones as biomarkers.

Why do some women experience greater levels of pregnancy loss than others? In a population-scale study of women in rural Bangladesh, Daryl Holman (1996) collected urine samples from more than 400 women and measured levels of hCG. The method is highly sensitive and allows the detection of pregnancies before the end of the second week after fertilization (Holman 1996). Using these data, the study modeled the effect of maternal age on fecundability and pregnancy loss—two important components in the analysis of interbirth intervals. Previous interpretations of the decline of fecundability with age pointed at social causes (e.g., decline in coital frequency) or reproductive aging (e.g., a decline in ovarian function). In contrast, the ability to detect very early pregnancy losses in this study allowed the authors to suggest that most of the age-related decline in apparent fecundability could be explained by an age-dependent increase in pregnancy loss (Holman and Wood 2001). Along the same lines, Nepomnaschy et al. (2006) found that, in a population of rural Mayan women in Guatemala, urinary cortisol—a biomarker for stress—was associated with risk of miscarriage within the first three weeks after conception. The link between stress (either physical or social) and fecundity can help explain the high rates of infertility observed in some industrialized populations (Wasser and Place 2001; Cameron 2003).

How can differences in socio-ecological variables explain the variation in fertility levels in different populations? The study of hormone levels and their association with fecundity and health would illuminate the way we model the human response to different environments and its consequences. An example of this contribution is a study of birth seasonality among the Toba Indians of northern Argentina conducted by our research team (Ellison et al. 2005). The distribution of births in this indigenous population was highly seasonal between 1980 and 2005. In the 1980s, most births occurred at the beginning of the spring, whereas birth seasonality was less marked in the following decades. During the period examined there was a significant change in the access that the Toba had to market foods. Beginning in the 1990s, hunting and gathering was supplanted by year-round male employment and government welfare, which provides more or less continuous access to manufactured foods. Our analyses of reproductive hormones (salivary estrogen and progesterone) showed that this new access to market foods had attenuated the effects of

seasonality of resources, which in turn affected the reproductive patterns and the demography of the population (Valeggia et al., unpublished manuscript). The association between socioeconomic variables and reproductive hormone levels has been shown in other populations as well. In Bolivia, for example, progesterone levels and rate of ovulation are significantly lower in poorer than in better-off urban-dwelling women (Vitzthum et al. 2002). The ability to establish these kinds of links between social and biological variables opens the possibility for better, more robust explanatory models (Valeggia and Ellison 2003a; Weiss 1990).

What is the demographic relevance of age-related changes in levels of reproductive hormones? The study of aging patterns is perhaps the area of research that has made the greatest advances in the use of biomarkers as useful tools for understanding underlying population mechanisms (Crimmins and Seeman 2001; Wachter and Finch 1997; Olshansky et al. 2005) as indicated by the number of proposals in behavioral and social research that include the use of biomarkers submitted to the US National Institute on Aging.[11] Starting in 1997 (Wachter and Finch 1997), the study of the biodemography of longevity has gained momentum and has stimulated a productive dialogue between social scientists and human biologists (Finch et al. 2001). Changes in reproductive hormones have been examined in studies of aging. For example, gender differences in longevity have been linked to the actions of estrogen in oxidative processes (Viña et al. 2005). Ovarian and testicular hormone levels have also been used as biomarkers for cancer risks (Eaton et al. 1994; Jasienska et al. 2000). It has been known for many years that long-term exposure to ovarian steroid hormones (estrogen and progesterone) increases a woman's risk of developing breast cancer (Jasienska et al. 2000; Jasienska and Thune 2001; ESHRE Capri Workshop Group 2004). In men, age-related declines in testosterone have been associated with changes in male body composition, bone mineral density, and a variety of health risks, including cardiovascular disease, type II diabetes, and osteoporosis (Travison et al. 2006). In a carefully controlled, cross-population study, Ellison et al. (2002) found that age patterns of testosterone decline vary between populations primarily as a result of variation in the peak levels attained in young adulthood. Young men in Boston, Massachusetts, for example, showed higher levels of testosterone than same-age men in rural Nepal and Paraguay. On the other hand, testosterone levels were similar at older ages, indicating a steeper decline for men in the sample from industrialized countries. This faster drop, the authors suggested, might result in more rapid age-related changes in male body composition, bone mineral density, and related health risks, including prostate cancer (Ellison et al. 2002). A recent study of a population of Bostonian men found that age, health, and lifestyle contributed significantly to the accelerated decline of testosterone levels and associated health risks (Travison et al. 2006). Interestingly, some aspects of lifestyle could be managed to decelerate the decline

in testosterone levels. In addition to the obvious health implications of these findings, an understanding of the links between changes in the hormonal milieu and morbidity in different populations can introduce better-suited variables in demographic models of aging.

New techniques that improve the quality of laboratory assays and reduce their cost are rapidly developing. Furthermore, the development of lab-on-a-chip (LOC) technologies promises to be of great potential for large-scale population research. These techniques allow laboratory operations to be carried out *in situ* using miniaturized LOC devices (Daw and Finkelstein 2006). Although still experimental and expensive, chip-based technologies have been used to test for several salivary biomarkers of potential use in biodemographic research (Yager et al. 2006; Christodoulides et al. 2007; Herr et al. 2007).

This is a promising time for biodemographic studies. The need for, and interest in, interdisciplinary approaches is patent in many population research centers in which biodemography facilities are being formed and strongly supported. The theoretical frameworks within which social and biological science investigators work will become more compatible and the interaction between them will be more productive. As a result of that impetus, the next decade will witness an expansion of the research questions and associated working models. Taking the laboratory to the field will undoubtedly contribute to that end.

Appendix

Hormonal processes in human reproductive physiology: The basics

The reproductive cycle in human females is governed by the functioning of the major reproductive hormonal axis: the hypothalamus-pituitary-ovarian (HPO) axis. Male reproductive endocrinology is regulated mainly by the hypothalamus-pituitary-testicular (HPT) axis. Below is a brief description of the HPO and HPT axes and their endocrine products as they relate to some of the proximate determinants of fertility (Wood 1994). The descriptions emphasize mainly the function of those hormones that are mentioned above as useful biomarkers in demographic studies. (For greater detail, see Knobil and Neil 2005.)

The ovarian cycle has usually been described as biphasic: a follicular phase, during which follicles grow in the ovary, is followed by a luteal phase, during which the reproductive system prepares for conception and implantation. Ovulation (the release of a single mature egg from the follicle) marks the transition from one phase to the other, and it occurs about the mid-portion of the cycle. Just before the beginning of each cycle, the pituitary gland, stimulated by GnRH (gonadotropin-releasing hormone), starts releasing FSH and LH. These hormones stimulate the growth of ovarian follicles. As follicles grow, they begin secreting estradiol, which feeds back

to the pituitary and the hypothalamus to inhibit the release of additional FSH and LH. The concentration of estradiol increases almost exponentially during the follicular phase. Conversely, the levels of FSH and LH remain low during most of the follicular phase. However, approximately one day before ovulation, there is a sharp surge of LH. This peak in LH concentration is the trigger for ovulation. LH and FSH levels drop back to baseline at the time of ovulation. After ovulation, the follicle cells remaining in the ovary develop into a specialized gland called the corpus luteum, which secretes progesterone in large quantities and estradiol to a lesser extent. The postovulatory increase in progesterone defines the luteal phase. If there is no conception, the corpus luteum disintegrates and progesterone and estradiol levels drop, which signals the beginning of a new cycle. If conception occurs, the corpus luteum remains active and progesterone and estradiol levels increase dramatically as does the concentration of hCG secreted by the conceptus.

Male reproductive physiology is not organized in recognizable cycles. The hypothalamus releases pulses of GnRH, which signals the pituitary to release LH and FSH. Increasing levels of LH stimulate the production and release of testosterone in the testes, while FSH (aided by testosterone) promotes the production of sperm. As is the case in females, feedback control mechanisms play an important role in this system. Testosterone inhibits the release of pituitary and hypothalamic hormones.

In women, each reproductive phase or status has its own hormonal "signatures" or markers that can be monitored using the minimally invasive techniques described in this article. Fecund cycles are characterized by regular, measurable changes in ovarian hormones. Thus, monitoring estradiol and progesterone levels during the postpartum period can help identify two important fertility components: the return to postpartum fecundity and the waiting time to conception. In contrast, prepubertal and postmenopausal levels of ovarian hormones are noncyclic and very low. Therefore, the beginning and the end of women's reproductive life can be established precisely at a population level by estimating estradiol and progesterone levels in blood, urine, or saliva. As another example, an increase in progesterone levels during the luteal phase indicates that ovulation has occurred. Researchers interested in finer-grained analysis of the risk of conception may find it useful to incorporate an estimation of the ratio of ovulatory versus nonovulatory cycles, which could result from monitoring progesterone levels in nonpregnant/nonlactating women.

Notes

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1 Samples are typically collected on specimen collection filter papers (Schleicher and Schuell #903) that can be ordered in different formats (e.g., blank cards or pre-printed cards with half-inch circles) to meet the needs of sample recording («www.whatman.com/products»).

2 Although pricking manually with a lancet is also a possibility, the lancing device makes pricking more controlled and less painful. Given the widespread use of lancing devices for self-testing of glucose levels in diabetics, there is now a wide variety of lancets and lancet devices. Some of the most commonly used include Beckton-Dickinson®(«www.bddiabetes.com»), PenletPlus® («www.lifescan.com»), Tenderfoot® («http://www.itcmed.com/products_6.html»), and Surgilance® safety lancets («www.surgilance.com»).

3 Care must be taken to hold the finger or earlobe slightly above the filter paper to avoid any contact between the skin and the filter paper.

4 In the case of field or community studies, samples should be mailed or shipped to the laboratory where the analyses will be performed. If samples are collected in a country different from the country where they will be analyzed, shipment regulations should be checked for both countries. For example, in the United States, shipment regulations have been published as a national standard by the National Committee for Clinical Laboratory Standards (1992). Researchers working in other countries should consult local regulations for the export and shipment of biological samples.

5 Depending on the hormone, there is a lag between the secretion of the hormone and the excretion of its metabolite in urine. In gonadal hormones the lag is approximately 24 hours (O'Connor et al. 2003). This delay needs to be considered when, for example, the day of ovulation or miscarriage is estimated.

6 For the measurement of reproductive hormone metabolites, it is not necessary to ask the participant to discard the first stream of urine.

7 *Preservation of urine samples in fluid form:* Using disposable plastic pipettes or pipette tips, urine samples are divided in several properly labeled polypropylene vials. Two or three duplicates of 1 ml each are sufficient for most measurements and for replication studies. Immediate freezing (at or below –20°C) of the samples until shipment or analysis is recommended. Storage at room temperature (22–25°C) leads to a loss of 2–4 percent per day for estrogen and progesterone metabolites (O'Connor et al. 2003). Urine samples should not be stored at room temperature for more than two days. On the other hand, gonadal steroid hormone metabolites (E1C and PdG) can usually withstand several freeze-thaw cycles without significant loss. When freezing facilities are available, samples should be kept frozen until shipment.

8 *Preservation of urine samples on filter paper:* This preservation technique requires minimal materials and equipment. Using gloves to prevent contamination, the filter paper strip (Schleicher and Schuell #16110) is cut in approximately 5 cm pieces and stored in air-tight containers with silica gel desiccant bags until use. After properly labeling the piece of filter with pencil, the paper is grasped with a pair of tweezers and dipped in the cup containing the urine sample until thoroughly soaked (about 5 minutes). If urine touches the tweezers' tip, it must be rinsed with water and detergent before the next use. Soaked pieces of filter paper are then left to air-dry, away from heat and sunlight. In humid environments, it is advisable to use a fan to accelerate the process. The filter paper strips should not be stored until thoroughly dried to prevent the growth of fungi and other microorganisms. A well-dried filter paper should feel to the touch as dry as an unused piece of filter paper. Once the filter papers are dried, they can be stored in cardboard boxes with silica gel at room temperature or in a refrigerator.

9 The mailing of fluid urine samples usually falls within the diagnostic specimen category. For transportation to and within the United States, DOT and USPS regulations impose a volume limit of 500 ml per package and require a Diagnostic Specimen label to be placed on the outer packing. The USPS and other express carriers require the use of absorbent material when shipping biological materials. The absorbent material is placed with the vials inside the leak-resistant plastic bag. Several companies produce special containers for transportation of biospecimens (Rockett et al. 2004), and many air transportation companies post detailed explanations on how to pack specimens (e.g., «www.abxair.com/hazmat/Index.htm»).

10 It generally takes participants between 2 and 15 minutes to collect the saliva sample

if unstimulated. In cases of dry mouth the use of salivation stimulants is recommended to increase saliva flow and facilitate collection. A number of studies have reported the use of products containing citric acid—lemon juice (Lipson and Ellison 1989), drink-mix crystals (Talge et al. 2005), wax, and sugared and sugarless gum (Lipson and Ellison 1989). Whichever stimulant is chosen, it should be evaluated for potential cross-reactivity with the hormones to be studied (Ellison 1988).

11 Online information at «www.nia.nih.gov».

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