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Panagiota D. Tryphonopoulos, BN, RN, PhD¹,
Nicole Letourneau, PhD, RN², and Rima Azar, PhD³

Abstract

Salivary cortisol is becoming more commonly utilized as a biologic marker of stress in observational studies and intervention research. However, its use with infants (12 months of age or younger) is less widespread and poses some special challenges to researchers. In order to decide on the most suitable collection procedure for salivary cortisol in infants, a number of criteria should be considered. This article will aid investigators interested in integrating salivary cortisol measurement into their research studies by presenting (1) an overview of the patterns of cortisol secretion in infancy including the development of diurnal rhythm and response to stress; (2) a comparison of the most commonly used approaches for collecting salivary cortisol samples in infants including cotton rope, syringe aspiration technique, filter paper, hydrocellulose micro sponge, and the Salimetrics children's swab; (3) a discussion of the factors contributing to heightened cortisol variability in infancy and how these can be limited; (4) analytical issues associated with cortisol measurement; and (5) examples of criteria to consider when choosing a saliva sampling method and lab for conducting assays.

Keywords

cortisol, stress measurement, saliva collection, infants

Salivary cortisol has emerged as a practical, noninvasive biologic marker of stress. Researchers have used cortisol to measure responses to stressful stimuli and to determine the effectiveness of interventions aimed at reducing stress (Hanrahan, McCarthy, Kleiber, Lutgendorf, & Tsalikian, 2006; Kidd et al., 2009). Although cortisol testing has been used extensively to assess adrenocortical activity in adults and children, its use with infants (12 months of age or younger) is less widespread and poses some special challenges to researchers. Infant basal cortisol levels can be highly variable and reactive to numerous factors, including developmental age, environment, and biological factors (de Weerth, Zijl, & Buitelaar, 2003), all of which must be considered prior to incorporating this variable into research with infants. The purpose of this article is to provide investigators with an overview of information required to reliably utilize salivary cortisol in infant studies.

Cortisol Secretion

Cortisol is the final product of the hypothalamic–pituitary–adrenocortical (HPA) axis. Virtually every cell in the human body is affected by cortisol, and it has a variety of important functions such as aiding in energy release, immune activity, mental activity, growth, and development, and reproductive function (Finn & England, 1997; Kirschbaum & Hellhammer, 1989). In young children and infants, typical elevations in early morning cortisol levels may increase interest in exploration and

promote acquisition and consolidation in learning (Larson, White, Cochran, Donzella, & Gunnar, 1998). Cortisol plays a critical role in stress responses (Kirschbaum & Hellhammer, 1989), and its levels are sensitive to both physical and emotional stimuli. Many studies have used salivary cortisol as a measure of stress or stress response when examining various aspects of infant development; however, descriptions of normal development of basal cortisol levels in large study populations of infants less than 12 months of age are limited (Tollenaar, Jansen, Beijers, Riksen-Walraven, & de Weerth, 2010).

Basal Cortisol Levels in Infancy

Few references of the normal ranges of basal salivary cortisol for infants and young children have been established, and commercial manufacturers of salivary steroid assays do not

¹ Interdisciplinary Studies, University of New Brunswick, Fredericton NB, Canada

² Norlien/ACHF Research Chair in Parent–Infant Mental Health, Faculty of Nursing, University of Calgary, Calgary, Alberta, Canada

³ CIHR/RPP New Investigator, Psychobiology of Stress & Health Lab, Psychology Department, Mount Allison University, Sackville, New Brunswick, Canada

Corresponding Author:

Panagiota D. Tryphonopoulos, BN, RN, PhD, Interdisciplinary Studies, University of New Brunswick, PO Box 4400, Fredericton NB, Canada, E3B 5A3.

Email: penny.tryph@unb.ca

typically provide sufficient reference data for their products (Gröschl, Rauh, & Dörr, 2003). de Weerth and van Geert (2002) noted, however, that not only did basal cortisol levels decrease during the first year of life but also 5- to 8-month-olds exhibited the highest degrees of intraindividual variability in basal cortisol levels. This observation is of particular significance if a researcher is considering whether to use one-time sampling or sampling over multiple days.

Normative Ranges of Cortisol

Tollenaar and colleagues (2010) sought to establish normative values for infant basal cortisol levels and to further examine the development of intraindividual variability in cortisol levels during the first year of life ($N = 300$). They observed a decrease in mean midmorning basal cortisol levels over the course of the study as well as a peak in intraindividual variability at 5 months of age. Basal cortisol levels within the 90% range (i.e., between the 5th and 95th percentiles) ranged between 4.4 and 25 nmol/L and remained fairly stable throughout the first year of life (Tollenaar et al., 2010).

Development of Cortisol Circadian Rhythm in Infancy

Cortisol is secreted by the adrenal cortex via pulsations that follow a 24-hr circadian profile. In adults, cortisol levels are highest in the early morning hours (peaking around 30 min after waking), followed by a sharp decrease during the mid-morning; then a more gradual decline occurs throughout the remainder of the day, with the lowest levels present around midnight (Edwards, Evans, Hucklebridge, & Clow, 2001; Kirschbaum & Hellhammer, 1989). In utero, fetal circadian rhythm is entrained via maternal stimuli (e.g., maternal temperature and food intake, placenta-crossing hormones such as melatonin). While the exact mechanism of action for postnatal synchronization of circadian rhythm is unknown, this process is chiefly coordinated by the suprachiasmatic nucleus (SCN) of the hypothalamus and re-entrained to the day–night cycle (Serón-Ferré et al., 2012). Following birth, infants exhibit both diurnal (24-hr) and ultradian (90–120 min) patterns of salivary and urinary cortisol (Bettendorf et al., 1998; Valenzuela, Hoffman, Hess, & Serón-Ferré, 1998; Zadik et al., 1999). Iwata and colleagues (2013) reported that while diurnal circadian rhythm was present in newborn infants during the first 5 days of life, the acrophase of the newborn's cortisol rhythm was principally defined by time of birth (as opposed to adult cortisol rhythm, wherein acrophase occurs in the early morning). In a longitudinal study of newborns, Sippell, Becker, Versmold, Bidlingmaier, and Knorr (1978) observed cortisol patterns consisting of two peaks (as opposed to the single morning peak typical in adults) 12 hr apart, which were not correlated with the day–night cycle.

While cortisol circadian rhythms continue to develop throughout infancy and toddlerhood (Watumura, Donzella, Kertes, & Gunnar, 2004), fully matured configurations, characterized by lower cortisol levels midafternoon than midmorning,

are not reliably obtained until approximately 4 years of age (Gunnar & Donzella, 2002). Researchers differ on the point at which infants acquire cortisol circadian rhythm. These contradictory views can be attributed to a number of factors that have impacted studies on the topic such as differing working definitions of circadian rhythm, inconsistent sampling times, infant exposure to antenatal steroids, and variation in data collection procedures and methods of data analysis (de Weerth et al., 2003). Opinions regarding the age of onset of diurnal cortisol patterns include 2–20 weeks (Antonini, Jorge, & Moreira, 2000), 2–3 months (Mantagos, Moustogainnis, & Vagenakis, 1998; Price, Close, & Fielding, 1983), 2–5 months (de Weerth et al., 2003), and up to 9 months (Kiess et al., 1995). However, it is most likely that a diurnal cortisol pattern is reliably present by at least 6 months (Davis & Granger, 2009; Ramsay & Lewis, 1995). Given that circadian maturation of premature infants occurs at a similar postnatal age as is typical of full-term infants, it is likely that there is a parallel between the emergence of diurnal cortisol rhythm and the onset of a circadian sleep rhythm. These results support the hypothesis that length of exposure to environmental time cues (i.e., light versus dark) has a greater impact than neurological maturity on the ontogenetic maturation of the circadian cycle in pre- and full-term infants (Antonini et al., 2000).

HPA Axis and Stress Reactivity

The human stress response involves the interaction of two systems: the rapidly activated norepinephrine–sympathetic adrenomedullary (NESAM) system associated with the “fight or flight” response (Gunnar & Cheatham, 2003) and the more slowly activated HPA axis (Hanrahan et al., 2006). The activation of the HPA axis by stressors initiates a cascade of hormone secretion that results in the release of cortisol from the adrenal glands. The physiological effects of cortisol are numerous, including the enhancement of an organism's ability to adapt to stressful conditions (Essex, Klien, Cho, & Kalin, 2002).

Acute Stress Reactivity. Levels of stress can range from positive or tolerable (e.g., temporary stress responses) to toxic (Gunnar, Talge, & Herrera, 2009). In the normative function of the HPA system, some stress can be a normal and necessary part of development, especially when it occurs within the context of stable and supportive relationships. For example, “positive” stress in infants and children (e.g., playing with an unfamiliar toy or peer, being introduced to a new food) is associated with surges in cortisol that increase heart rate, blood sugar, and brain functioning, which, in turn, promote infants' learning capabilities and facilitate bonding with caregivers (Gunnar, 1996, 2009). Even at birth, humans are capable of discriminating between types of stressors and responding accordingly to perceived stress (Gunnar & Cheatham, 2003; Gunnar, Herrera, & Hostinar, 2009). At as early as a few days old, infants are capable of showing small but measurable elevations in cortisol in response to mild stressors such as weighing/measuring or being subjected to physical examinations (Gunnar, 1992).

Stressors involving painful stimuli (e.g., circumcisions) elicit even higher elevations of cortisol. Cortisol levels peak approximately 20–30 min after an infant encounters an acute stressor and return to prestressor baseline levels 120–150 min postevent (Gunnar, 1992; Gunnar, Malone, Vance, & Fisch, 1985; Ramsay & Lewis, 1995).

Infants are most reactive to minor stressors in their first year of life and may show larger responses to stressors at this period than they will as they age (Gunnar & Cheatham, 2003). Several studies show that a decreased response in HPA axis functioning (and subsequent dampening of cortisol reactivity) develops over an infant's first year of life. For example, using the well-baby exam/inoculation stressor protocol as a pretest/posttest indicator of stress reactivity, Lewis and Ramsay (1995) reported that infants aged 2 and 4 months showed an increase from baseline cortisol levels in conjunction with behavioral indications of distress (e.g., crying and fussing); however, at 6 months of age, posttest cortisol levels did not rise above the baseline readings, despite infants' visible distress. Davis and Granger (2009) obtained similar results, reporting that stress-related cortisol increases were present at 6 and 12 months but not at 2 and 24 months. These observations point to a developmental shift in adrenocortical functioning between 6 and 12 months, which is a compelling evidence for the stabilization of the HPA axis and the reliable appearance of adult-like cortisol circadian rhythm by 6 months of age (Davis & Granger, 2009; Ramsay & Lewis, 1995).

Chronic Stressors. Exposure to chronic stressors, such as those associated with early life adversities (e.g., neglect, abuse, exposure to violence, parental mental illness, parental substance abuse, and low parental nurturance/sensitivity), results in frequent overactivation of the HPA axis. Chronic stressors result in prolonged elevations in cortisol levels, which can be maladaptive and have deleterious effects on physiologic, emotional, and behavioral processes (Essex et al., 2002; Gunnar & Donzella, 2002), in contrast to the shorter periods of cortisol elevation associated with acute stressors. Studies show that prolonged exposure to elevated cortisol can lead to increased insulin resistance and obesity, reduced cognition, attention deficits, impaired memory, diminished immune responses, and disturbances in emotional regulation (Essex et al., 2002). In contrast to the above findings, however, some studies in children exposed to chronic stressors, such as severe deprivation (e.g., orphanages or institutional care), neglect, or abuse, have reported lower basal levels of cortisol or hypocortisolism (which is equally maladaptive to children's development), in response to the prolonged periods of hyperactivity of the HPA axis (Fries, Hesse, Hellhammer, & Hellhammer, 2005; Gunnar, Morison, Chisholm, & Schuder, 2001; Lupien, McEwen, Gunnar, & Heim, 2009).

There is both potential for remarkable growth and a great deal of vulnerability to harm the rapidly developing infant brain. Perhaps the most troubling consequences of frequent overactivation of the HPA system are the deleterious effects on the developing infant brain, including decreases in brain

volume, inhibition of neurogenesis, disruption of neuronal plasticity, and abnormal synaptic connectivity. During the sensitive periods of enhanced plasticity, the infant brain is particularly vulnerable to the long-term effects of stress hormones, and overactivation may result in divergence from the typical pathways and organization of the young brain (Gunnar, Herrera, & Hostinar, 2009). This divergence may result in irrevocable long-term deficits to the particular regions of the brain developing during the time of toxic stress exposure (Gunnar, Herrera, et al., 2009).

Strategies for Collecting Salivary Cortisol in Infants

Advantages of Salivary Cortisol Testing

Saliva collection offers several advantages over collection of other diagnostic fluids: it is minimally invasive, inexpensive, painless, and uncomplicated, characteristics that are of particular importance when collecting samples from the very young (Salimetrics, 2008). Due to the ease with which it can be collected, salivary cortisol is appropriate for use in studies that require multiple samples to be taken over the course of the day (e.g., for assessing diurnal rhythm). Provided that collection protocols (e.g., time of sample) are clear, saliva can be self-collected or collected by a family member, allowing a degree of ecological validity that enables stress reactivity to be monitored in everyday situations (D. Granger, personal communication, April 7, 2010; Salimetrics, 2009). Finally, because blood collection, as opposed to saliva collection, may be stress inducing (particularly in young children), using saliva as a testing medium rather than serum eliminates the risk of measuring the reaction to the collection process itself (Kirschbaum & Hellhammer, 2007; Salimetrics, 2008).

The accuracy of salivary cortisol testing has been established, and serum and salivary cortisol levels have been shown to be well correlated ($r = .83-.94$; Chang, Anderson, & Wood, 1995; Francis et al., 1987; Gozansky, Lynn, Laudenslager, & Kohrt, 2005; Kurihari et al., 1996; Riad-Fahmy, Read, Walker, & Griffiths, 1982). Binding proteins present in serum may complicate measurement of active cortisol levels (Gozansky et al., 2005). Cortisol is thought to be biologically active only when it is not bound to corticosteroid-binding globulin (CBG). Only the unbound fraction of cortisol (12% in the lower range and 89% in the upper range) is available to diffuse into the saliva (Gozansky et al., 2005; Hellhammer, Wüsta, & Kudielka, 2009). For this reason, salivary cortisol levels are consistently lower than serum levels; however, the low level of cortisol measured in saliva is a direct measure of the biologically active, free fraction in serum (Gozansky et al., 2005).

Collection Methods and Devices

Although salivary cortisol testing has been used widely in developmental and behavioral research, collection in very young children (i.e., infants aged 12 months or younger) poses

a special challenge. Researchers must contend with factors such as the high possibility of insufficient specimen volume (Herrington, Olomu, & Geller, 2004) and the potential for choking when a collection device is placed in an infant's mouth (Salimetrics, 2009). The most commonly used approaches for collecting salivary cortisol samples in infants are the braided cotton rope, syringe aspiration technique, filter paper, hydrocellulose micro sponge (Sorbetto), and the Salimetrics children's swab (SCS). See Table 1 for a side-by-side comparison of collection devices.

Cotton Rope. Cotton, particularly braided cotton rope, was one of the first materials researchers used for saliva collection (Berry, Blair, Willoughby, & Granger, 2012; Granger, Stansbury, & Henker, 1994; Gunnar, Fisch, Korsvik, & Donhowe, 1989; Gunnar, Mangelsdorf, Larson, & Hertsgaard, 1989). Typically, a small section of rope is placed in an infant's mouth to absorb saliva for 2 min or more, while the caregiver/researcher firmly holds the other end to prevent choking. Once the rope is saturated with saliva, a needleless syringe is used to extract the sample from the rope, which is then expressed into a collection vial (Gunnar, Fisch, et al., 1989; Salimetrics, 2009). Alternatively, the saturated portion of cotton can be placed into a saliva storage tube for centrifuging. The cotton must be thoroughly saturated to obtain accurate cortisol results (Salimetrics, 2009). Given that cotton absorbs liquids efficiently, a problem occurs when salivary volume is small relative to the capacity of the absorbent material. The fluid can be so diffusely distributed throughout the fiber network that no amount of centrifugation or pressure will recover a volume sufficient for testing (Harmon, Hibel, Romyantseva, & Granger, 2007). Although use of the cotton rope has the advantage of being simple and efficient, the rope is reported to have an unpleasant taste that may be unpalatable to young children, thus affecting compliance. Moreover, many studies suggest that the molecular structure of the cotton rope interferes with the immunoassay and may alter cortisol values (Gunnar, Fisch, et al., 1989; Salimetrics, 2009; Shirtcliff, Granger, Schwartz, & Curran, 2001). Due to these drawbacks, cotton has begun to fall out of favor as a collection device, leading to the need to explore alternative approaches to saliva collection (Harmon et al., 2007).

Feeding Tube. Another approach to saliva collection involves placing a small plastic feeding tube or suction catheter into the infant's mouth and aspirating the sample with a syringe (Harrison, Johnston, Spence, Gillies, & Nagy, 2005). Harrison and colleagues briefly described this method: Samples are obtained by inserting a 3-cc syringe attached to a shortened size 8-Fr feeding tube into the mouth, inside the cheek and under the tongue of participants, to obtain sample volume. Unfortunately, only 35% ($n = 49$) of infants in their study produced samples that were sufficient for analysis. Another disadvantage of the aspiration method is that it can be rather intrusive and places the infant at risk for damage to the delicate mucous membranes, often resulting in bleeding and the attendant risk of

contamination of the saliva samples (Neu, Goldstein, Gao, & Laudenslager, 2007).

Filter Paper. Neu and colleagues (2007) recently established the validity of a new approach for collecting samples in very young children, involving the use of filter paper strips to absorb saliva. The authors described the procedure that they used in their study of preterm infants: Specially cut (2.4×9 cm) Whatman Grade No. 42 filter papers were folded in half lengthwise and placed on the infants' tongues until the lower portion was completely saturated with saliva (paper appears translucent when it is adequately wet). Sampling times ranged from 30 s to 2 min (an average of 59 μ L of saliva was absorbed in 20 s), with the investigators reporting that the infants readily sucked on the paper strips without any signs of distress. Once saliva samples were collected, the filter paper was set, wet end facing down, for 3–4 hr until completely dried. Laudenslager, Calderone, Philips, Natvig, and Carlson (2013) described a modified approach for collecting diurnal saliva samples, whereby strips of paper were assembled in a specially constructed booklet containing four filters for use over the course of a single day of collection. To prevent sample cross-contamination between adjacent filters, waxed weigh paper (Whatman Grade B2 parchment paper) cut slightly larger than the filters was used to separate individual papers, and the entire booklet was secured with a staple. Upon completion of sampling, the entire booklet was stored in a perforated (allowing adequate airflow for filter paper drying) plastic medicine bottle.

Filters can be stored for up to 6 months at room temperature until assayed and can even be sent through the regular mail without the need for refrigeration, giving this method one obvious advantage over collection devices (e.g., Sorbetto, SCS) that require cold storage (Granger, Cicchetti, et al., 2007; Neu et al., 2007). Filter paper is particularly useful in situations where collection of adequate saliva volume is likely to be a challenge (e.g., neonates). Collection time is relatively brief, minimally invasive, and results in little or no infant arousal during sampling, which makes filter paper especially suitable when obtaining multiple samples (Granger, Cicchetti, et al., 2007; Neu et al., 2007). Whether or not the molecular structure of filter paper alters immunoassay results was not evident from the literature reviewed.

The filter paper method is not without limitations. For example, data collection may be especially difficult when saliva samples are viscous or stringy or contain particulate matter or blood contamination. Also, it is challenging to accurately estimate the volume of saliva absorbed when the paper is placed in the infant's mouth (Granger, Cicchetti, et al., 2007).

Hydrocellulose Microsponge. The hydrocellulose microsponge (Sorbetto) is a small (0.7×1.8 mm), tasteless, arrowhead-shaped absorbent device attached to a short plastic applicator shaft (approximately 0.4×5.2 mm) that serves as a handle (Granger, Kivlighan, et al., 2007; Salimetrics, 2009). It is especially recommended for collecting saliva samples from infants (Capriolo et al., 2013; de Weerth, Jansen, Vos, Maitimu, &

Table 1. Comparison of Salivary Cortisol Collection Devices Most Commonly Used in Infants.

Characteristic	Cotton Rope	Feeding Tube	Filter Paper	Hydrocellulose Microsponge (Sorbettes)	Salimetrics Children's Swab (SCS)
Description	Braided cotton rope, 12 × 153 mm	3 cc Syringe attached to size 8-Fr feeding tube	Whatman Grade No. 42 paper, 2.4 cm × 9 cm	Arrowhead-shaped sponge, 0.7 × 1.8 mm, attached to a short plastic applicator shaft, 0.4 × 5.2 mm	Cylindrical inert polymer, 8 × 125 mm
Directions for use	Insert one end of the cotton braided rope into the infant's mouth and hold onto the other end Leave in the mouth for approximately 2 min; can be intermittently removed and reinserted Remove the rope, cutoff wet end and place into storage tube, recap securely.	Insert syringe attached to shortened feeding tube into infant's mouth Collect saliva from the infant's mouth, inside the cheek and under the tongue Aspirate syringe into collection vial	Fold paper in half lengthwise and place on infant's tongue until lower portion saturated (30 s–2 min) To dry, lay paper flat or place in perforated container	Place Sorbettes under infant's tongue for 15–30 s at a time and reintroduce as needed until saturated (60–90 s) Place Sorbettes into conical tube cap with tips facing the cap end of the tube (positioning critical for recovering absorbed saliva during centrifugation) Slide storage tube over shaft and secure cap	Place SCS into infant's mouth under tongue for 1–2 min Place SCS into storage tube and recap tightly
Ease of use	Moderate	Moderate	Simple	Simple	Simple
Materials commercially available	http://www.salimetrics.com/store/all/cotton-rope	Purchase from medical suppliers	http://www.whatman.com/CelluloseFilters.aspx	http://www.salimetrics.com/	http://www.salimetrics.com/store/collection-supplies/salimetrics-childrens-swab
Ideal uses	Best in older infants with excess saliva production	Best in infants with excess saliva production	Can be used when saliva volume diminished	Can be used when saliva volume diminished	Appropriate for infants of all ages
Advantages (+)	(-) Molecular structure of rope may interfere with immunoassay	(-) May not yield sample volume sufficient for analysis	Appropriate for infants of all ages, particularly neonates	Appropriate for infants of all ages, particularly neonates	(+) Long length eliminates choking hazard
Disadvantages (-)	(-) May not yield sample volume sufficient for analysis	(-) Risk for damage to the mucous membranes	(+) Can be stored without refrigeration/freezing (-) Ineffective sample absorption when saliva is viscous	(+) Small enough to be introduced into infant's mouth without detection (-) Small size increases the risk of choking	(+) Palatable texture (-) May cause temporary dryness of mucosal membranes

Lentjes, 2007; Donzella, Talge, Smith, & Gunnar, 2008; Harmon et al., 2007; Matsukura et al., 2012). This device was designed for use with very small volumes of liquid. Up to 50–200 μL of fluid can be recovered from each Sorbette, and two to three Sorbettes can be used simultaneously to ensure adequate sample volume. For sample collection, the Sorbette is placed under an infant's tongue for 15- to 30-s intervals until the arrowhead is saturated (usually 60–90 s total). In contrast with cotton rope, the untrained eye can easily determine when enough saliva has been collected because the sponge portion becomes swollen and shiny (de Weerth et al., 2007). Once sufficient saliva volume is obtained, the Sorbettes are then placed, arrowhead tip facing down, into the conical cap of the accompanying swab storage tube (Salimetrics, 2009).

A major advantage of the Sorbette is that it is small enough for collecting samples from sleeping infants' mouths without waking them (Granger, Kivlighan, et al., 2007). Granger and colleagues noted that infants were often more willing to allow the Sorbette to be introduced into their mouths than a cotton rope. Using the Sorbette method, researchers have been successful in quickly securing an adequate sample volume, even in instances where saliva is limited. However, the Sorbette's small size can cause choking if appropriate precautions are not taken (Donzella et al., 2008; Granger, Kivlighan, et al., 2007; Salimetrics, 2009). Teething infants can also bite off and swallow or choke on the arrowhead-tip sponge (de Weerth et al., 2007; D. Granger, personal communication, April 7, 2010).

Salimetrics Oral Swab. The Salimetrics oral swab (SOS) is considered the gold-standard device for saliva collection in adult populations. It is not recommended for infants and children under the age of 6, however, because its small size (30 mm \times 10 mm) and cylindrical shape render it a choking hazard (Salimetrics, 2009). To address this issue, Salimetrics introduced the SCS, specifically designed for use with infants and young children (Salimetrics, 2010). The SCS is similar in composition to the oral swab but is longer and smaller in diameter (8 \times 125 mm), thus eliminating the choking hazard. The swab's extra length allows a caregiver or investigator to secure one end of the swab while placing the other end in the child's mouth underneath the tongue (for approximately 1–2 min). The swab can withstand chewing, and its taste and texture are palatable to children. The SCS can typically absorb 200–1000 μL of saliva. Once the swab has absorbed an adequate amount of saliva, the saturated portion can be cut free and placed in a storage tube.

One disadvantage of the SCS is that the required 1 to 2-min collection period may be too long for infants to tolerate, which may lead to noncompliance (Harmon et al., 2007). The SCS also may cause temporary dryness of mucosal membranes, resulting in some mild discomfort (Salimetrics, 2009). Finally, since the SCS was only recently released, studies describing and validating its use in young children and infants are not yet available.

Summary. There is no universally ideal strategy for infant salivary cortisol collection. The method that is most appropriate

for a given research project will depend on a number of criteria, including age of infant, setting (i.e., lab vs. home collection), ease of use, frequency of sample collection, cost of materials, and efficiency in yielding sufficient sample volume. For example, both the filter paper and hydrocellulose micro sponge methods are particularly well suited for use with very young infants who may produce smaller volumes of saliva. Using Laudenslager and colleagues' (2013) booklet method, filter papers are also useful for collecting multiple specimens over a single day with relative ease. The SCS is minimally invasive and easy to use (a contributing factor for participant compliance) and absorbs adequate saliva volumes; thus, this device is practical for use with older infants in a home setting.

Although the advantages associated with salivary cortisol testing are numerous, it remains an effortful undertaking (though much less onerous than the collection of other diagnostic fluids); thus, decisions regarding sample collection strategies should not be made casually. Researchers seeking to assess salivary cortisol in infants should consider all of the aforementioned criteria prior to deciding on the most suitable collection method.

Factors Affecting Cortisol Analysis and Results

There are many factors contributing to heightened cortisol variability in infancy that must be taken into consideration when designing research involving the measurement of salivary cortisol (de Weerth & van Geert, 2002; Hanrahan et al., 2006). Synchronizers (i.e., behavioral and environmental factors) such as sleep, food, or stressful stimuli may cause alterations in circadian expression and, thus, alter cortisol levels as well (de Weerth & van Geert, 2002). For example, Spangler (1991) found that cortisol levels in infants aged 11 weeks to 7 months were higher when sleep had occurred in the hours preceding sample collection (longer sleep episodes were associated with an increase in adrenocortical activity). Food and liquids may cause variability in salivary cortisol for a number of reasons. First, contamination of the saliva sample with food or drink may interfere with the cortisol assay (Salimetrics, 2009; Schwartz, Granger, Susman, Gunnar, & Laird, 1998). Second, ingesting solid food causes a postprandial surge in cortisol (Hertsgaard, Gunnar, Larson, Brodersen, & Lehman, 1992). Third, breast milk or formula may contain various hormones that can interfere with results (Magnano, Diamond, & Gardner, 1989). Cortisol levels can also be influenced by a broad spectrum of diagnoses (e.g., Addison disease; King & Hegadoren, 2002) and medications (e.g., preterm neonates may be treated with steroids to promote pulmonary function; Bettendorf et al., 1998). Maternal medication used while breast-feeding may also influence infant cortisol levels (Hibel, Granger, Kivlighan, Blair, & the Family Life Project Investigators, 2006).

Another factor with the potential to impact cortisol analysis is the tendency of infants younger than 3 months of age to generate very little saliva. Consequently, gathering sufficient testing volumes poses a special challenge when working with this

population (Herrington et al., 2004). Although modern immunoassays are designed for use with very small quantities of saliva, insufficient fluid volume for performing assays occurs all too often and results in missing data and compromised study findings (Granger, Kivlighan, et al., 2007). There is debate over whether or not oral stimulants should be used to promote sufficient sample volumes in young children. In their "Saliva Collection and Handling Advice" protocol, Salimetrics (2009) recommends against the use of oral stimulants when collecting saliva samples due to the risk of causing assay interference and the alteration of cortisol levels. Yet, the company also notes that, in cases in which stimulants are absolutely necessary (i.e., sample collection would be impossible without them), they must be used in a consistent manner throughout the study. Regardless, in instances when there is the risk of insufficient saliva volume, it would be prudent to use a collection device (such as the Sorbette or filter paper) that effectively absorbs small specimen volumes rather than risk assay interference.

The later age of emergence of cortisol diurnal rhythm, which may not be consistently present until 6 months of age, suggests that cortisol stress reactivity may be a more reliable measure in young infants than diurnal rhythm (de Weerth et al., 2003; Hanrahan et al., 2006). Very young infants show elevations in cortisol levels in response to mild stressors such as examination or inoculation (Gunnar, Broderson, Kruger, & Rigatuso, 1996), while older infants exhibit diminished adrenocortical reactivity to the same procedures (though these procedures may still elicit behavioral responses such as fussing and crying; Davis & Granger, 2009). It is likely that older infants require a more intensive perturbation (such as a social stressor like parental separation) in order to elicit a cortisol response.

A number of controls can be implemented to ensure the maximum reliability of cortisol analysis and limit the impact of factors that contribute to heightened cortisol variability in infancy. It is critical to establish sample collection protocols (e.g., using consistent materials and sampling methods; Hanrahan et al., 2006), particularly in instances where specimens are obtained in the home (as is often the case with diurnal sampling). Parents should be provided with written instructions emphasizing the importance of adherence to the protocol and cautioning against food or drink within 30 min prior to sampling. Participants should also be provided with a log or questionnaire to document relevant activities during the time of sampling (e.g., sleep patterns, the presence of any illnesses, medication use, whether the infant is teething or anything else that may be a departure from the child's routine). Precise timing of sample collection is critical to ensure accurate assessment and interpretation of diurnal cortisol profiles; however, a major limitation of ambulatory assessment of cortisol is the lack of control over the timing. To encourage adherence to collection protocols and record exact timing of sample collection, electronic monitoring devices such as the MEMS® 6 Track-Cap (MEMS, AARDEX, Lux, Switzerland) may be employed. These devices consist of a conventional medicine bottle fitted with a special closure that records the time and date of each opening and closing of the container through integrated

microcircuitry. Although this is an indirect measure of adherence because there is no way to verify whether the sample was taken at the time the cap was opened, it has been shown to reliably assess participant adherence with a saliva collection protocol and improves compliance (because subjects know they are being monitored; Hall et al., 2011; Hanson & Chen, 2010). Furthermore, the ability to eliminate or otherwise reclassify samples taken at the wrong times will reduce error variance during statistical analysis.

The reliability of cortisol measurement is also influenced by the selection of an appropriate immunoassay and laboratory service (Hanrahan et al., 2006). Commonly used commercial assays for cortisol measurement include radioimmunoassay (RIA), immunofluorescence assay, and enzyme-linked immunosorbent assays (ELISAs; Jessop & Turner Cobb, 2008). These assays vary in buffer composition, antiserum specificity, and equipment needs, making it difficult to compare the outputs of different assay types (Hanrahan et al., 2006; Jessop & Turner Cobb, 2008). While collection materials are relatively inexpensive, analysis is often the most costly aspect of salivary cortisol measurement. Depending on the number of saliva samples requiring analysis (labs may offer discounts for a large volume of samples), rates for duplicate analyses may range from US\$2.50 to US\$15.00 per sample (Kirschbaum, n.d.). Numerous commercial labs operating internationally offer assay services for salivary cortisol. The labs most frequently cited in behavioral science research include IBL International (www.ibl-hamburg.com), Diagnostic Systems Laboratories, Inc. (www.dsl.com), and Salimetrics, LLC (www.salimetrics.com). When selecting a lab for conducting assays, researchers should consider the following criteria: experience of technical staff, cost, and most crucially, the quality performance measures used by the lab (e.g., establishing quality control using duplicate analyses, random specimens, and control specimens; Hanrahan et al., 2006). Often, cost and assay quality are inversely related because labs with the most rigorous protocols also tend to have extensive experience, deal with higher volumes of samples, and have automated procedures. Labs are required to provide details of their quality assessment programs and proficiency testing upon request (Nicolson, 2007).

In addition, it is highly recommended that researchers consult with the selected lab personnel before initiating sample collection regarding recommendations for proper collection techniques, storage, and shipping of samples. Some labs conduct workshops to train investigators seeking to integrate salivary measurement into their research studies. For example, Salimetrics, LLC, offers a 2-day "Spit Camp" that covers the fundamentals of salivary biomarkers, sample collection procedures, and immunoassay techniques and offers a hands-on lab component (see <http://www.salimetrics.com/spit-camp/> for details).

Once researchers have identified an assay and laboratory, it is important that they clarify the units in which the cortisol results should be reported and establish a normal range of values in order to identify outliers (Hanrahan et al., 2006). Cortisol is typically reported as micrograms per deciliter ($\mu\text{g}/\text{dl}$), but it is not uncommon for labs to use units as diverse as micrograms

per liter ($\mu\text{g/l}$), nanograms per milliliter (ng/ml), nanograms per deciliter (ng/dl), milligrams per deciliter (mg/dl), and nanomoles per liter (nmol/l) SI (standard international units; Jessop & Turner Cobb, 2008).

Analytical Issues

Cortisol data tend to be positively skewed (Cruz, 2007; Gunnar, 1996). Studies involving small sample sizes (which are typical in stress research involving infants) are particularly susceptible to threats to normality created by extreme observations. Investigators thus must conduct screening to identify potential outliers and data transformation for increasing interpretability and for correcting skewed distributions (Cruz, 2007). Examples of transformations for dealing with positively skewed cortisol data include the square root function transformation (SQRT) and logarithmic transformations (Tabachnick & Fidell, 2001).

Cortisol levels are typically analyzed in one of three ways: (1) diurnal with 3–5 measurement points throughout a 24-hr period (e.g., at waking, 30 min later, midmorning, midafternoon, and bedtime); (2) cortisol awakening response (CAR) with two measurement times (i.e., at waking and 30 min later), although this response may not be reliably observed in younger infants until a mature circadian rhythm is established; and (3) reactivity/recovery from a stressor (e.g., immunization or laboratory procedure) with three measurement times (i.e., pre-stressor, poststressor, and delayed poststressor). With only two data points, CAR analysis is relatively straightforward, and slope or change scores can be calculated (Clow, Hucklebridge, Stalder, Evans, & Thorn, 2010). However, with techniques calling for more than two data points (i.e., stress reactivity/recovery and diurnal), more complex analytic methods are required (Fekedulegn et al., 2007; Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003).

In research involving repeated collection of cortisol samples from the same participant over time (e.g., examining the development of cortisol rhythm in infancy), the amount of data gathered often represents a problem for statistical analysis; thus, approaches for summarizing information are required (Fekedulegn et al., 2007; Pruessner et al., 2003). Area under the curve (AUC) analysis can be used to incorporate multiple time points to estimate the circadian changes in cortisol and to facilitate the manageability of data without sacrificing the information contained in repeated measurements without necessitating adjustment of the significance level (comparisons between groups being reduced). Pruessner and colleagues present two formulas for calculating AUC. The first, AUC with respect to increase (AUCI), is calculated with reference to the baseline (first) measurement (as opposed to 0 on the x–y axes) and best reflects the magnitude of the increase in cortisol over the day. The second, AUC with respect to ground (AUCG), reflects the total AUC of all measurements and best reflects total cortisol output over the day (Fekedulegn et al., 2007; Pruessner et al., 2003). The usefulness of AUC calculation in repeated cortisol measurements is thus twofold—to measure the magnitude of the cortisol response and to measure the pattern of response over time

(Fekedulegn et al., 2007). A major advantage of AUC is that it can be applied even when time intervals between repeated measurements are not identical (which is often the case with self-collection of cortisol samples; Pruessner et al., 2003).

Conclusion

Salivary cortisol has emerged as a simple and effective biologic marker of stress; however, cortisol levels in infants are subject to a high degree of variability due to environmental differences and the rapid neuroendocrine and developmental changes that are associated with this stage in life. Consequently, it may be difficult to establish a gold standard of salivary cortisol measurement with regard to collection device, cortisol pattern being assessed, timing of samples, and normal ranges of cortisol. Rather, using consistent collection techniques and laboratory analysis procedures, ensuring a comprehensive understanding of individual factors relating to the behavior of cortisol levels and considering how infants' developmental age may influence cortisol rhythm will contribute to improved accuracy of cortisol testing and help advance the understanding of infants' responses to stressful events.

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