Advancing the Social Neuroscience of Human-Animal Interaction: The Role of Salivary Bioscience

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Introduction

While the field of human-animal interactions (HAI) has been recognized for decades, there have been recent calls to increase the number of sound scientific studies, including the integration of valid and repeatable biobehavioral measures of both short and long-term effects (Esposito, McCune, Griffin & Maholmes, 2011). The advent of new technologies and techniques has increased the opportunities for developmental, health and therapeutic investigations in varied human populations, as well as the study of the welfare implications of HAI on animals. As investigators move to include these techniques in their research, it is important that they understand both the potential applications and limitations of each measure. In recent decades, the opportunity to explore biosocial relationships in humans and animals has been facilitated by the ability to measure inter- and intra-individual differences in the activity of biological systems such as the hypothalamic-pituitary-adrenal (HPA) axis, hypothalamic-pituitary-gonadal (HPG) axis, and autonomic nervous system (ANS) non-invasively in oral fluids (saliva). In fact, technical innovations in the field of salivary bioscience reveal that information may be obtained from oral fluid specimens about the activity of a broad array of physiological systems, pathogen and chemical exposures, and genetic variability relevant to basic biological function, health, and disease. The attention saliva has received as a research biospecimen is largely due to the perceptions of sample collection as quick, uncomplicated, cost-efficient, minimally invasive, and

acceptable to children and parents, as well as to animals and their handlers. The purpose of this chapter is to describe best practices, and provide a roadmap to enable investigators interested in the social neuroscience of HAI to integrate the tools of salivary bioscience into their conceptual and measurement models. While we focus on a variety of analytes in oral fluid, readers interested in vasopressin and oxytocin are referred to other chapters in this volume on the topic (see Chapter ## by Carter and Porges and ## by Beetz and Bales).

Theoretical and Conceptual Issues

The study of hormones and behavior is the scientific foundation for most research employing salivary analytes in the social, behavioral, and health sciences. Modern thought assumes that biological changes influence behavior and vice versa, enabling individuals to respond flexibly and fluidly to changes in the environment. Gottlieb (1992) elaborates and suggests that certain physiological processes are activated only when components of the "behavioral surface" are unable to accommodate the challenge. In his model, the first line of "adaptation" and the most flexible and fluid mechanisms available to an individual to adjust to changes in the environment involve coping resources, change in behavior, restructuring cognitions and perceptions of the event, or all of the above. Thus, physiological systems sensitive to context (i.e., psychobiology of stress) are only activated when the adjustment cannot be handled by the behavioral surface. In theory, the activation of these physiological subsystems adjusts over repeated encounters with the same situation or circumstances. Habituation is essential to maintain homeostasis and the integrity of the organism, whereas repeated or chronic activation of the HPA or ANS has deleterious consequences via effects on multiple body systems (McEwen, 1998). The relation of activity and regulation of the biological systems to behavior is assumed to be dependent on the social context. To paraphrase Sapolsky (2005), hormones do not cause behavior; they increase

the probability that existing behavioral tendencies will be expressed given the right circumstances. Related to this, it has been suggested that developmental mechanisms calibrate activation thresholds and response magnitudes within environmentally responsive biological systems to match ecological conditions encountered in life, rendering certain individuals intrinsically more or less biologically sensitive and susceptible to context (e.g., Ellis, Boyce, Belsky, Bakermans-Kranenburg, & Ijzendoorn, 2011).

These theoretical issues suggest that examining the associations and dissociations between concurrent activity of biological systems in relation to behavior, health and social relationships is critical. Also, advancing our understanding how social and contextual forces influence the coordination of these systems may provide insight into how individual differences in physiological reactivity and regulation contribute to social, behavioral, cognitive processes and well-being. Viewed from this set of assumptions and perspectives, HAIs represent a specialized context in which biobehavioral relationships may be modified or changed. Since Friedman's landmark study of pet ownership and cardiac patient survival (Friedman, Katcher, Lynch & Thomas, 1980), much HAI research has focused on physiological measures (e.g., stress hormone levels, sympathetic nervous system activation, and cardiovascular reactivity) to examine the effects of animals on their human companions. While HAIs refer to the interaction and effects of each species on the other, fewer investigators have examined their bi-directional effects. Salivary analytes have the potential to advance measurement models that aim to examine these complex reciprocal and context-dependent influences both in humans and in animals.

Human Salivary Bioscience

To integrate biology, behavior, and context into theoretical and analytical models, biospecimens must be collected (1) repeatedly from the same individuals, (2) without causing burden or stress to the human or animal, and (3) in a variety of settings. Historically, the initial wave of studies utilizing salivary analytes (those prior to the late 1990s) often ignored key facts about oral biology and the nature of saliva as a biospecimen. This may have compromised the value of the information gained. Biobehavioral research on HAI can benefit from that unfortunate history, by reviewing and understanding some of the fundamentals of oral biology related to the special characteristics of oral fluid.

Oral Fluid. The "saliva" specimen is actually a composite of oral fluids secreted from many different glands (Veerman, Van Den Keijbus, Vissink, & Nieuw Amerongen, 1996). The major source glands are located in the upper posterior area of the oral cavity (*parotid gland* area), lower area of the mouth between the cheek and jaw (*submandibular gland* area), and under the tongue (*sublingual gland* area). A small fraction of oral fluid (crevicular fluid) also comes from serum leakage in the cleft area between each tooth and its surrounding gums, or via leakage from serum due to mucosal injury or inflammation.

Each secretory gland produces a fluid that differs in volume, composition, and constituents (Veerman et al., 1996), thus each source gland's contribution to the pool of oral fluid varies. For instance, *mucins* make saliva viscous, elastic, and sticky to protect tooth enamel against wear and to encapsulate microorganisms. These glycoproteins are not present in oral fluid secreted by the parotid gland. Under resting conditions—when there is minimal fluid contribution from the parotid gland and the levels of mucins in saliva are high and consequential—specimens will be more viscous (Nieuw Amerongen, Bolscher, & Veerman, 1995).

Oral fluid is water-like in composition and has a pH (acidity) range between 6 and 9. Foods and substances placed in the mouth are capable of changing salivary acidity very quickly because the fluid has minimal buffering capacity. Immunoassays are a method of choice for assaying many salivary analytes. The antibody-antigen binding step during an immunoassay is compromised when the specimen is highly acidic ($pH < 3$) or basic ($pH > 9$). This unique characteristic of saliva interacts with procedures used to collect it and can compromise measurement accuracy.

Many of the salivary analytes employed in biobehavioral studies (e.g. steroid hormones) are serum constituents transported into saliva either by *filtration* through the tight spaces between acini (duct cells in the salivary glands) or *diffusion* through acinar membranes (Vining, McGinley, & Symons, 1963). Some of the analytes found in oral fluids (e.g. enzymes, mucins, cystatins, histatins) are synthesized, stored, and released from the granules within the secretory cells of the saliva glands. Still others are components of humoral immunity (antibodies, complement) or signaling molecules (cytokines, chemokines, growth factors) secreted by cells of the mucosal immune system. Furthermore, saliva collected using procedures common in biobehavioral studies contains sufficient cellular material to obtain a high quantity and quality DNA. An understanding of whether an analyte is transported into oral fluid by filtration or passive diffusion, secreted from salivary glands, or released or derived from cells locally in the oral mucosa, is essential to interpreting the meaning of individual differences in that measure.

The secretion of oral fluids is influenced by several factors: the day-night cycle, chewing movement of the mandibles, taste and smell, iatrogenic effects of medications that cause xerostoma (dry mouth) and medical interventions (radiation), and conditions (e.g., Sjorgen's syndrome) that affect salivary gland function (Atkinson, Travis, Pillemer, Bermudez, Wolff, &

Fox, 1990). Salivary glands are directly innervated by ANS nerves (e.g., Garrett, 1987), and activation of the sympathetic and parasympathetic components of the ANS response to stress decrease or increase saliva flow rates, respectively. The levels of salivary analytes produced in the mouth, like alpha-amylase (sAA) and secretory IgA, and the levels of those that migrate into saliva from blood by filtration through the junctions between duct cells in the salivary gland (e.g., dehydroepiandrosterone-sulfate [DHEA-S] and other conjugated steroids) are influenced by the rate of saliva secretion (e.g., Kugler, Hess, & Haake, 1992). For these saliva analytes, a correction must be made by multiplying the measured concentration or activity of the analyte (e.g., U/mL, pg/mL, ug/dL) by the flow rate (mL/min) to express the measure as *output as a function of time* (e.g., U/min, pg/min, ug/min; for example .50 ug/dL x .5 mL/min = 1 ug/min), or at a minimum, flow rate (mL/min) should be used as a covariate in the statistical analyses.

The U.S. Centers for Disease Control note that unless visibly contaminated with blood, oral fluid is not a class II biohazard. This statement has contributed to the perception that saliva is *safer* to work with than blood. In reality, other than obviously not needing needles during the collection steps, this may be something of a misperception. Even under normative-healthy conditions, more than 250 species of bacteria are present in oral fluids (Paster et al., 2001). During upper respiratory infections, oral fluids are highly likely to contain agents of disease. Oral fluid specimens should be handled like all class II biohazards with *universal precautions* in both research and diagnostic applications.

Sample Collection. In the past, saliva collection devices have involved cotton-based absorbent materials. Cotton placed in the mouth for 2–3 minutes is rapidly saturated by oral fluids, which are then expressed into collection vials by centrifugation or compression. Most of the time, this is convenient, simple, and time-efficient. However, when the absorbent capacity is

large and sample volume small, the specimen absorbed can be diffusely distributed in the cotton fibers, making sample recovery problematic, with possibly higher rates of missing data and artificially low cortisol estimates. This absorption process has the potential to interfere with the immunoassay of several salivary analytes.

Early studies employed serum assays modified for use with saliva by, among other things, requiring large saliva test volumes (200–400 ul). To collect sufficient test volumes, saliva flow was often stimulated via chewing (gums, dental wax) or tasting (sugar crystals, powdered drink mixes, citric acid drops) substances. When not used minimally and/or consistently, some of these methods are capable of changing immunoassay performance (e.g., Schwartz et al., 1998). Indirectly, stimulants also influence measurement of the levels of salivary analytes dependent on saliva flow rate (SIgA; DHEA-S; Neuropeptide Y, NPY; Vasoactive Intestinal Peptide, VIP). We advise avoidance of these techniques unless pilot studies show that their application does not adversely affect measurement validity of the salivary analytes of interest.

Given this quick review of the sources of oral fluids, it is not surprising that studies show the placement of oral swabs in the mouth has the potential to introduce variation in the measured levels or activity of some salivary analytes. Depending on where in the mouth an absorbent device is placed, a different fluid type may be collected, and if not controlled, may contribute to measurement error across sampling occasions within and between subjects. Caution must be exercised to minimize this threat to measurement validity by standardizing instructions and monitoring compliance.

Collecting *whole saliva* by passive drool can minimize these threats to validity (Granger et al., 2007). Briefly, participants are asked to imagine that they are chewing their favorite food,

slowly move their jaws in a chewing motion, and allow the oral fluid to pool in their mouth without swallowing. Next, they gently force the specimen through a short device (e.g., SalivaBio LLC, Carlsbad, CA) into a vial. There are several advantages of this procedure: (1) a large sample volume (.5-1.5 mLs) can be collected within a short collection timeframe (3–5 mins.); (2) target collection volume can be confirmed by visual inspection in the field; (3) the fluid collected is a pooled specimen mixture of the output from all salivary glands; (4) it does not introduce interference related to stimulating or absorbing saliva; and (5) samples can be aliqoutted and archived for future assays.

Most techniques that have been studied have unique benefits as well as shortcomings that prevent universal application. When possible, saliva collection methods should always be piloted in the field to ensure that they do not contribute to measurement error, in relation to the exact assay protocols to be employed.

Blood Leakage into Oral Fluid. To meaningfully index *systemic* biological activity, quantitative estimates of an analyte (e.g., hormone) in saliva may need to be highly correlated with the levels measured in serum. The magnitude of this serum-saliva association depends, in part, on consistency in the processes used to transport circulating molecules into oral fluids. When the integrity of diffusion or filtration is compromised, the level of the serological marker in saliva will be affected. Most serum constituents are present in serum in much higher levels (10–100 fold) than in saliva.

Blood and blood products can leak into oral fluids via burns, abrasions, or cuts to the cheek, tongue, or gums. Blood in oral fluid is more prevalent among individuals who suffer from poor oral health (i.e., open sores, periodontal disease, gingivitis), endure certain infectious diseases, or engage in behavior known to influence oral health negatively.

Spiking whole blood into saliva reveals that samples visibly contaminated with blood will present varying degrees of yellow-brownish hue. A simple 5-point Blood Contamination in Saliva Scale (BCSS; Kivlighan, et al., 2004) offers the following response options: (1) “saliva appears clear, no visible color”; (2) “saliva has a hint of color, a little brown or yellow tint is barely visible”; (3) “saliva has a clearly visible yellow or brown tint”; (4) “yellow or brown coloring is more than just a tint, color is obvious but not very deep”; and (5) “saliva is very colored, deep, rich, dark yellow or brown is very apparent” (pp.41-42). Under healthy conditions, BCSS ratings (N = 42) averaged 1.33; after microinjury caused by vigorous tooth brushing, ratings averaged 2.42.

In the context of research on HAI: (1) participants should be screened for events in their recent history that could cause blood leakage into saliva by asking questions related to oral health (i.e., “Do your gums bleed when you floss or brush your teeth?”), shedding teeth, or open sores or injury to the oral cavity; (2) sampling saliva within 45 minutes of microinjury to the oral cavity (e.g., brushing teeth) should be avoided (Kilvighan et al., 2004); and (3) samples should be systematically inspected at the collection point and, if visibly contaminated with blood, excluded from analyses.

Particulate Matter and Interfering Substances. The integrity of oral fluid samples can also be influenced by items placed in the mouth. Food residue in the oral cavity after drinking or eating may change salivary pH or composition (viscosity), and/or contain substances (e.g., bovine hormones, active ingredients in medications, enzymes) that cross-react in immune- or kinetic- reaction assays. We recommend a simple solution: research participants should not consume food or drink within the 20 minutes prior to sample donation. If anything has been eaten within this time window, participants should rinse their mouth with water prior to

providing a specimen. Importantly, however, they must wait at least 10 minutes after drinking before a specimen is collected to avoid diluting it with water and artificially lowering concentration/volume (ug/dL, ng/mL, pg/mL) or activity/volume (U/mL) estimates of salivary analytes. Access to food and drink should be carefully planned and scheduled when study designs involve repeated sample collections over long time periods.

Sample Handling, Transport, and Storage. Typically, once specimens are collected, they should be kept cold or frozen to prevent degradation of some salivary analytes and restrict the activity of proteolytic enzymes and growth of bacteria. How samples are handled, stored, and transported has the potential to influence sample integrity and measurement validity. Our recommendation is conservative: after collection, saliva samples should be kept frozen (at least – 20 ºC), or at a minimum kept cold (on ice or refrigerated) until they can be frozen that day, and repeated freeze-thaw cycles should be avoided. Also note that some salivary analytes (e.g., neuropeptides) may require specimens to be treated with inhibitors (such as EDTA or aprotinin), or flash frozen on the spot to minimize rapid degradation.

Medications. We recommend that the name, dosage, and schedule of all prescription and OTC medications taken within the last 48 hours be recorded in the field. This information should be used (covaried, controlled) to statistically rule out the possibility that medication use is driving the primary salivary analyte-outcome relationships of interest.

Animal Salivary Bioscience

Oral fluid has been collected and analyzed for a variety of compounds in many species. While the principles of salivary bioscience in other animals are similar to those of humans, there are species-specific considerations that should be taken into account when planning and carrying

out saliva collection in animals. As oral fluid varies in production, composition and function, investigators should familiarize themselves with the species with which they are dealing.

In herbivores, such as the horse, saliva plays a particularly important role in combining with food to create a bolus and provide lubrication for swallowing. Like humans, horses have parotid, mandibular and sublingual salivary glands. Smaller glands in the labial, buccal and lingual regions also provide moisture for the area in which they are found (Schummer, Nickel, & Sack, 1979). Horses produce large quantities of saliva (10-12 L/day), which, like that of humans and canines, is a hypotonic solution. Unlike dogs and humans, the parotid reflex of horses is not conditioned to external stimuli, that is, salivary secretion does not begin until food is actually prehended and the animal begins to eat (Alexander & Hickson, 1969).

In addition to the parotid, mandibular and sublingual salivary glands, dogs and cats have a zygomatic salivary gland located in the upper jaw behind the eye. The pH of dog saliva varies between 7.34 and 7.8, while that of cat saliva averages around 7.5 (National Research Council, 2006). Canine saliva also has some buffering capabilities to protect individuals against ingestion of more acidic substances. Dogs and cats (as well as marine mammals, dolphins, sea lions) lack the salivary alpha-amylase enzyme that is found in very low concentration in the equine. Unlike many other species, an important function of canine saliva is evaporative cooling; the flow rate of saliva increases greatly in response to increased environmental and body temperature (Swenson & Reece, 1994).

Sample Collection. A number of investigators have successfully collected and analyzed oral fluids in companion animal species involved in animal assisted therapy and activities, including horses and dogs (Dreschel & Granger, 2005, 2009; Peeters, Sulon, Beckers, Ledoux & Vandenheede, 2011). While it is possible to collect saliva from domestic cats (Siegford,

Walshaw, Brunner, Zanella, 2003), it is difficult to obtain adequate sample volume to process. Collection devices must be used to collect oral fluid from animals, as no technique for collecting passive drool in common companion animals has been developed. A primary consideration in oral fluid collection from animals is safety to the researcher; because saliva collection requires close proximity of the handler's fingers to the animal's mouth, it should not be attempted on aggressive, fearful or anxious dogs. Many of the same limitations (poor recovery of fluid from cotton fibers, interference of cotton with salivary analytes, etc.) reported in humans also apply to animals, so it is recommended that an inert material, such as methylcellulose, which has not been shown to interfere with immunoassay of cortisol, be used for sample collection (Dreschel & Granger, 2009). Salivary stimulants such as citric acid have been used by some researchers, but the danger of interference with salivary analytes exists in animals as in humans (Dreschel & Granger, 2009; Schwartz et al., 1998); as with human sampling, we recommend avoiding these unless pilot studies show that they do not adversely affect the measurement validity of the compounds of interest. Likewise, "flavoring" of the collection device has been shown to interfere with salivary cortisol analysis (Dreschel & Granger, 2009).

The technique we have found to be most useful in collecting saliva from dogs is as follows: (1) one handler holds the dog steady to avoid it walking away, while the other firmly holds a 5" saliva collection swab in one hand and lifts the subject's lip with the other hand; (2) place the swab into the mouth through the space between the upper and lower canine teeth and the premolars, which often causes the dog to open his or her mouth; (3) hold the muzzle loosely shut so that the dog can chew on the swab, stimulating saliva flow; (4) hold the swab firmly and move it around to sample from several areas of the mouth. It may take 2-3 minutes for saturation of the swab. The swab can also be used to wipe saliva from the areas between the gingiva and

cheeks of the dog. Training using positive reinforcement can facilitate oral fluid collection procedures. After saturation, the swab can be compressed through a 5-10cc syringe to extract the saliva, or placed in a storage tube. Handling post-collection is similar to that of human samples. This technique has been successful for veterinarians, veterinary technicians, researchers, and dog owners (Dreschel, 2007; Dreschel and Granger, 2005, 2009).

Blood Leakage, Particulate Matter, and Interfering Substances. As with humans, the contamination of oral fluid with blood and other substances can be a limitation with animal sample collection. In domestic canines, the presence of dental disease and gingivitis will increase the risk of blood contamination and should be avoided or at least noted. Additionally, dogs often retain food between their teeth, or eat or chew on other substances (grass, dirt, sticks, rawhide bones) that could interfere with salivary analytes, so dogs should not eat for 30 minutes before sample collection. While it is recommended that researchers reward the dog immediately after sample collection, if a follow up sample will be collected, the reward should be a small treat that will not require much chewing. Likewise, because dogs retain fluid in their mouth after drinking, there is a risk of sample dilution if the sample is taken too soon after the dog drinks.

Medications and neutering status. Many dogs take monthly heartworm and flea preventitives. Corticosteroids and other anti-inflammatory drugs are also commonly prescribed. While the influence of these on salivary analytes has not been well documented, any medications taken should be recorded. In addition, it is important to record age, gender, and neutering status of animals used in HAI research; the role of spaying and castration on salivary hormone levels has not been researched.

Research Designs, Sampling Schemes, and Analytical Strategy

In this section, we describe the logic behind some common research designs and saliva sampling schemes and describe several analytical strategies for these designs.

Basal Levels. A "basal level" is the level of activity of an analyte that represents the "stable state" of the host during a resting period. One approach to assessing basal levels has been to sample early in the morning, before the events of the day are able to contribute variation. Levels of salivary analytes may be influenced by inherent moment-to-moment, diurnal and/or monthly variation in their production/release, rate of their metabolism/degradation, and sensitivity to environmental influences, and whether they are measured quantitatively or qualitatively. Given these issues, a single time-point measure of salivary analytes (other than invariant genetic polymorphisms), except under very unique circumstances, is *unlikely* to yield meaningful results for basal levels. The minimally invasive nature of oral fluid collection enhances the reliability of basal estimates of salivary analytes by sampling at the same time of day across a number of sampling days, then aggregating across days. Theoretically, the more inherent variation in the analyte, the more days of sampling would be required.

Stress-reactivity and Regulation to Acute Events. The vast majority of studies have used research designs that test time-dependent changes in salivary analytes (i.e., cortisol, sAA) following exposure to a discrete event. The number of samples collected depends on the specific analyte, questions being addressed, tolerance for sampling burden by participants, and logistical and practical issues. The optimal design for the measurement of salivary cortisol and sAA reactivity and regulation involves a pre-pre-[task]-post-post-post-post sampling scheme with samples collected on arrival to the lab (after consent), immediately before the task (after a period of relaxation), then again immediately, 5, 20 and 40 minutes post-challenge.

Although some developmental studies have yielded consistent mean-level differences in salivary analytes before and after exposure to a stressful or novel event, there are generally wide ranging inter-individual differences in stress-related reactivity. Some individuals will exhibit unexpected patterns of change, including no change, as well as continuous increases or decreases in analyte levels at least during the time period in which the analyte was sampled.

Person-oriented Approach for Identifying Reactivity and Recovery. Studies employing pre-post task saliva sampling designs have also explored individual differences in reactivity or recovery. Early studies often classified youth as cortisol reactors or non-reactors based on a 10–15% difference between pre- and post-task levels (e.g., Susman, Dorn, Inoff-Germain, Nottelmann, & Chrousos, 1997). The logic was that a difference of this size was 2 to 3 times larger than the intra-assay coefficient of variation (CV; 3–5%). The intra-assay CV reflects the error inherent in the assay by comparing results from the same samples assayed twice, with an intra-assay CV equal to 0 meaning perfect "reliability". For cortisol, we added the criterion of an absolute difference of at least .02 ug/dL, as this value is 2–3 times higher than the lower limit of our salivary cortisol assay's sensitivity (i.e., the smallest value distinguishable from zero is .007 ug/dL). The next step typically involved either multivariate logistic regression or discriminant function analyses to predict reactor status (e.g., Granger, Weisz, McCracken, Ikeda, &Douglas, 1996). The limitation of this simple approach is that all reactors are grouped together, even though some may only show a minor increase and others may show substantial change. This approach becomes complex when multiple post-stress samples are collected and the focus is on individual differences in the trajectory of reactivity and recovery over time. For these designs, latent growth modeling approaches, such as growth mixture modeling (GMM), may be employed to identify homogenous subpopulations within a larger heterogeneous population and for the

identification of meaningful groups of individuals with specific growth trajectories. Given the wide-ranging individual differences in physiological responses to stress, continued efforts to employ this advanced type of individual-oriented approach seem valuable.

Patterns of Reactivity and Recovery across Multiple Occasions. Consistent patterns of HPA or ANS activation across time or situations (e.g., high or low reactivity across conditions) may be especially informative when investigating individual differences in risk or resilience. Before much progress can be made on this front, a consensus is needed regarding the best manner by which to group individuals into these different patterns of reactivity and recovery. Growth mixture modeling could be a useful tactic to identify these patterns and profiles.

Diurnal Rhythm. An important component of variability within individuals in salivary analyte levels is the diurnal rhythm of production (e.g., Gunnar & Vasquez, 2001). In humans, most salivary hormone levels (e.g., cortisol) are high in the morning, decline before noon, and then decline more slowly in the afternoon and evening hours (Nelson, 2005). By contrast, levels of sAA show the reversed pattern with low levels in the morning and higher levels in the afternoon (Nater, Rohleder, Scholtz, Ehlert, & Kirschbaum, 2007). The non-linear nature of these patterns requires multiple sampling time points to create adequate statistical models. A typical sampling design for salivary cortisol and sAA involves sampling immediately upon waking, 30-minutes post waking, midday (around noon), in the late afternoon, and immediately prior to bed (Hellhammer, et al., 2007; Nater et al., 2007).

Horses have also been shown to have a diurnal secretion of cortisol, with levels higher in the morning (0600h) and lower in the evening (1800h) (Van der Kolk, Nachreiner, Schott, Refsal, & Zanella, 2001). Interestingly, while episodic secretion of cortisol has been shown in

both cats and dogs, a diurnal rhythm to cortisol secretion has not been identified (Kemppainen & Peterson, 1996, Koyoma, Omata, & Saito, 2003).

Measurement of Momentary Biobehavioral Associations in Everyday Contexts. Documenting everyday events and emotions that help explain changes in analyte levels or activity across the time period of interest may strengthen causal inference when these assessments are paired with samples across multiple days. Recent advances in information technology (computerized handheld devices, such as PDAs) have made these self-assessments of momentary emotions and events possible during the course of individuals' everyday lives (e.g., Stone, et al., 2003). Research designs typically involve diary-sample pairings several times per day and across multiple days. In studies focusing on cortisol, saliva samples are collected approximately 20 minutes after each diary entry.

While much HAI research has concentrated on therapeutic interventions, more recent work has focused on the general effects of living with and working with animals on daily human lives (e.g. Allen et al., 2002). The role of animal influence on specific populations continues to be examined (e.g. Aydin, et al., 2011; Viau et al., 2010). Using non-invasive physiological measures such as salivary analytes in any population can add to the richness of these data. In addition, the role of human influence on the welfare of animals in shelters (e.g. Bergamasco et al., 2010), competition (Jones and Josephs, 2006), therapy work (Glenk, et al, 2014) and military, police and search and rescue working situations (Haverbeke, Diederich, Depiereux & Giffroy, 2008; Horvath, Doka, & Miklosi, 2008) has been examined using canine salivary cortisol. Because of the relative ease of canine saliva collection, handlers, pet owners, and researchers are able to collect samples at home and during training and working sessions. This research will

help to identify and ameliorate stress or other negative outcomes that could be associated with the inclusion of animals in therapeutic and working situations.

Associative Relations of Salivary Analytes between Dyads. In several studies, salivary analyte levels (e.g., cortisol, testosterone, and sAA) are associated in dating couples (Powers, Pietromonaco, Gunlicks, & Sayer, 2006), newlywed couples (Cohan, Booth, & Granger, 2003), siblings (Schreiber et al., 2006) and parent-child dyads (Sethre-Hofstad, Stansbury, & Rice, 2002). Constructs related to these patterns of symmetry have varied substantially across studies. Studies have found dyadic physiological symmetry to be associated with negative correlates such as exposure to domestic violence and harsh parenting practices (Hibel, Granger, Blair, & Cox, 2009), maternal depression (Laurent, Ablow, & Measelle, 2011), marital dissatisfaction (Saxbe & Repetti, 2010), and shared negative affect (Papp, Pendry, & Adam, 2009). Yet other studies relate dyadic physiological symmetry to positive correlates such as friendship strength (Goldstein, Field, & Healy, 1989), and maternal sensitivity (van Bakel, & Riksen-Walraven, 2008), making it difficult to form decisive conclusions about the implications of this coordination. One area of discrepancy in the examination of physiological symmetry is the type of methods employed in analyses.

The associative dyadic relationship between animals and humans is an underdeveloped area of research in HAI. Most research to date has focused on the effects of animals on humans (particularly in animal assisted therapy and activities), or the influence of humans on animals (in the context of animal welfare), but few studies have looked at the association of each species in biobehavioral terms and the dyadic relationships of this bi-directional influence. Odendaal and Meintjes (2003) presented some of the first research examining this dyadic relationship using neurophysiological correlates of positive human-dog interactions based on blood sampling and

arterial blood pressure measurement. Handlin et al. (2011) also examined the physiological response to interaction between dogs and their owners using blood sampling. The use of salivary analytes in such studies allows for research beyond the laboratory into other areas of HAI. Jones and Josephs (2006) examined the change in salivary cortisol in agility dogs, relative to their handlers' basal levels and changes in testosterone in winning and losing teams. Lit, Boehm, Marzke, Schweitzer, & Oberbauer (2010) compared human salivary cortisol and testosterone to dogs' pulse and body temperature during search and rescue certification tests.

Analytes in Saliva of Interest to Research on HAI

To date, the range of salivary analytes that have been integrated into studies of HAI has been restricted relative to the possibilities. Many may not know that the National Institute for Cranofacial and Dental Research (NIDCR) initiated a multi-site program project charged with characterizing the salivary proteome in humans. The list includes more than 1,000 analytes (Hu, Loo, & Wong, 2007). Salivary analytes vary in terms of how they can be interpreted, which influences their *value* to HAI research.

Some analytes are present in saliva because oral fluid represents an ultra-filtrate of serum constituents. This group of analytes has high value because their levels in saliva are highly correlated with and reflect levels in general circulation. These measures enable investigators to make inferences about systemic physiological states. Adrenal and gonadal hormones are exemplars of this category of salivary markers (see Table 1). Cortisol or corticosterone (in relevant species) is the most common analyte measured in animal saliva as a physiological marker of stress.

Insert Table 1 about here

The majority of analytes in oral fluid are produced locally in the oral cavity and secreted from salivary glands; their levels may reflect features of and variations in oral biology rather than systemic physiology. Many salivary immune and inflammatory markers such as neopterin, beta-2-microglobulin, cytokines (see Table 1) fall into this category. Secretory IgA in domestic canines is a potentially useful marker of stress (Kikawa, Uchida, Nakade, & Taguchi, 2003). Individual differences may represent systemic immune function or status, but a more likely major contributor is local inflammatory processes related to oral health and disease.

A subset of analytes is produced locally by salivary glands but the levels vary predictably with systemic physiological activation. The activation of the autonomic nervous system (ANS) affects the release of catecholamines from nerve endings, and these compounds' action on adrenergic receptors influences the activity of the salivary glands. Salivary alpha-amylase (sAA) is considered a *surrogate marker* of ANS activation, with the majority of findings linking it to sympathetic activation via beta-adrenergic pathways in humans. Cats and dogs do not produce salivary amylase, so this is of no value in these species (Dreschel, 2007). Salivary measures of neuropeptide Y and vasoactive intestinal peptide may also serve as surrogate markers of ANS.

Antibodies to specific antigens are also measurable in oral fluids. Antibodies to human immunodeficiency virus (HIV) and Hepatitis C (HCV) are the exemplars in this category of salivary analytes, and Table 1 offers several additional examples. The presence of an antibody in oral fluids reflects immunological history of pathogen/microbe exposure, and depending on the specific antibody measured may represent local and/or systemic immune activity or current or prior exposure. A variety of pharmaceuticals, abused substances, and environmental contaminants can be quantitatively monitored in oral fluids. Cotinine, a metabolite of nicotine, is

routinely measured in oral fluid to estimate primary and secondary exposure to nicotine. Urinary cotinine has been previously measured in dogs and cats to measure the effects of environmental tobacco smoke (Bertone-Johnson et al., 2008; McNiel et al., 2007).

Within the recent past, technical advances confirm that high quantity and quality DNA can be extracted from whole saliva. Genetic polymorphisms can be determined from the same specimens already in use, or planned for use, to assess individual differences in salivary analytes and biomarkers. Our preliminary studies suggest that global and specific methylation assays are technically feasible using DNA extracted from cells in oral fluid, raising the possibility that saliva-based measurements may contribute to study epigenetic phenomena in research related to HAI.

Conclusions

Sampling oral fluid is minimally invasive, collection is simple and discrete, and specimens can be collected repeatedly without interrupting the flow of social interaction. As the number of substances that can be reliably measured increases, oral fluid may become a biospecimen of choice for studies of HAI. These technical advances enable the construction and evaluation of measurement models related to how individual differences in several integrated biological systems are related to behavior, cognition, psychopathology, and health, and how these differences moderate the effect of HAI on subsequent psychosocial and behavioral adjustment in humans and animals. With careful attention to the special issues noted here, salivary bioscience has the potential to profoundly impact our understanding of the social neuroscience of HAI.

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Table 1. *Salivary Analytes of Potential Interest to HAI*

Endocrine	
Aldosterone	Estradiol, Estriol, Esterone
Androstenedione	Progesterone, 17-OH Progesterone
Cortisol	Testosterone
Dihydroepiandrosterone, and -sulfate	Melatonin
Immune/Inflammation	
Secretory Immunoglobulin A (SIgA)	Beta-2-microgolublin (B_2M)
Neopterin	Cytokines, Chemokines
Soluble Tumor Necrosis Factor Receptors	C-Reactive Protein (CRP)
Autonomic Nervous System	
Alpha-Amylase (sAA)	Neuropeptide Y (NPY)
Vasoactive Intestinal Peptide (VIP)	
Nucleic Acids	
Human Genomic	mRNA
Mitochondrial	Microbial
Bacterial	Viral
Antibodies Specific for Antigens	

Measles	Hepatitis A,B,C,E	HIV
Mumps	Herpes Simplex	CMV
Rubella	Epstein Barr	