Methods Series (invited only)

Beyond Catecholamines: Measuring Autonomic Responses to Psychosocial Context

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ABSTRACT: Despite longstanding interest among human biologists in autonomic responses to socioecological context or culture change, the adoption of autonomic measures has been limited by methodological challenges. Catecholamine secretion is the most direct measure, but not all study designs are amenable to urinary sampling, and blood pressure lacks specificity to the parasympathetic or sympathetic division of the autonomic nervous system. This article reviews three alternative approaches for measuring autonomic responses: salivary α-amylase as a nonspecific autonomic marker, respiratory sinus arrhythmia as a specific parasympathetic marker, and the pre-ejection period as a specific sympathetic marker. Study design considerations are discussed in detail, including ambulatory sampling protocols that permit the evaluation of autonomic responses to everyday life. Researchers interested in how culture and social experience "get under the skin," as well as those concerned with the evolution of social engagement, can benefit from these well-validated biomarkers that are nevertheless relatively novel in human biology. Am. J. Hum. Biol. 00:000–000, 2015.

The autonomic nervous system (ANS), which can further be subdivided into the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS), is responsible for wide-ranging, largely involuntary regulatory functions. The ANS and the hypothalamic-pituitary-adrenocortical (HPA) axis together comprise the principal mediators of allostasis that permit maintenance of homeostasis in the face of physical and psychosocial stressors. Measured directly through catecholamines (Brown, 2007) or indirectly through blood pressure (James, 2007), between- and within-individual variation in ANS responses across socioecological contexts have been used by anthropologists and human biologists to assess the biological impact of social context, migration and culture change (e.g., Bergey et al., 2011; Brown, 1982; Brown and James, 2000; Harrison et al., 1980; James et al., 1987; James and Brown, 1997; Pearson et al., 1990; Van Berge-Landry et al., 2013). However, deeper integration of ANS measures into human biology research has been limited by two methodological challenges. First, unlike cortisol in saliva, catecholamines are difficult to assess on a moment-to-moment basis; the noninvasive approach requires collecting urine and calculating catecholamine output over time. Second, indirect measures like ambulatory blood pressure lack specificity with respect to the division of the autonomic response being activated.

While it is common to describe the SNS as the "accelerator," the PNS as the "brake," and treat them as reciprocal, a more accurate description would be that they are co-regulated, distinct adaptive systems for allostatic regulation, continually adjusting arousal states across various organ systems in response to changing external and internal conditions (Hastings and Miller, 2014). For example, the role of the PNS in regulating heart rate, facial expressiveness, and other arousal parameters through the myelinated vagus (Xth cranial) nerve appears to have an important place in the evolution of the capacity for social engagement in mammals (Porges et al., 1994; Porges and Furman, 2011). The parasympathetic moderation of arousal counteracts the socially defensive posture evoked by the sympathetic fight-or-flight response, and also conserves energy during low-threat situations (Hastings and Miller, 2014). By contrast, sympathetic arousal can be understood as preparation for active coping strategies, particularly in novel and uncertain contexts that may require a rapid behavioral or physiological response. In this sense, it is essential to fight or flight, although more subtle momentary regulation prepares individuals to respond adaptively to changing social conditions of a mundane variety as well (Kelsey, 2012).

The SNS and PNS often respond differently depending on the type of stressor encountered or social engagement required. They can fluctuate on a second-by-second basis independently of one another, and need not be reciprocal, as co-activation and co-inhibition also have been observed (Benevides and Lane, 2013; El-Sheikh and Erath, 2011; Hastings and Miller, 2014; Obradović and Boyce, 2012). Finally, the effects of central autonomic arousal are not uniform across target organs, so indirect markers of autonomic activity often show weak correlations with direct, system-wide measures like serum catecholamines, and with each other (Brown, 2007; Nagy et al., 2015; Yim et al., 2015). For all these reasons it is highly questionable to posit a general autonomic stress response (Obradović and Boyce, 2012). Instead, careful study of human interactions in socioecological context benefits from considering the two ANS divisions separately where possible, and always with the highest achievable documentation of momentary context.

SALIVARY AUTONOMIC BIOMARKER: α-AMYLASE

Validation as an autonomic biomarker

Salivary α-amylase (sAA) is an enzyme that catalyzes the breakdown of starch and glycogen in the oral cavity,

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but also serves as a marker of autonomic arousal due to the tight autonomic control of salivary protein secretion. Several reviews of the underlying physiology are available (Bosch, 2014; Nater and Rohleder, 2009; Proctor and Carpenter, 2007; Rohleder and Nater, 2009); space is sufficient here only for a brief summary. The salivary glands are directly innervated by both the SNS and PNS, from the upper thoracic spinal cord and brainstem respectively. The major salivary glands (parotid, submandibular, and sublingual) are differentially innervated by the SNS and PNS, and differently regulated at the local cellular level. This permits highly complex, stressor-specific patterns of saliva secretion, involving interactions among ANS divisions and salivary glands. As a broad gloss, the sympathetic division is most influential in protein secretion, and the parasympathetic division in salivary flow. The highest concentration of α-amylase is found in saliva from the parotid gland, but the other glands contribute as well (Bosch, 2014). sAA is the only salivary autonomic biomarker. Although sAA has received surprisingly little attention from human biologists or anthropologists (but see DeCaro, 2008; Lynn et al., 2010; Lynn et al., 2015; Ponzi et al., in press; Vigil et al., 2009, 2010), it is a common marker of the stress response in psychobiology research, especially in experimental designs (Granger et al., 2007; Nater and Rohleder, 2009; Nater et al., 2013b; Rohleder and Nater, 2009). sAA is even more appealing for ambulatory study designs, because research subjects can be coached to collect a series of samples on their own. Paired with observational, experience sampling, or diary data, this allows for the description of physiological responses to transient socioecological conditions and emotional states (cf. Flinn et al., 2012; Pollard and Ice, 2007). Saliva collection methods for cortisol and amylase are compatible, allowing sAA to enhance ecological studies of the adrenocortical response by adding an autonomic biomarker at no additional burden to the participant and minimal cost.

While some authors frame sAA as a specific SNS marker, this may be misleading. Evidence for a sympathetic role in sAA regulation includes increased secretion following norepinephrine infusion (Kuebler et al., 2014), reduced secretion under beta adrenergic blockade (van Stegeren et al., 2006), and (less consistently) correlations between plasma catecholamines and sAA (Chatterton et al., 1996; Ditzen et al., 2014; Nater et al., 2013b; Thoma et al., 2012b). Yet salivary assays measure amylase activity, not secretion; enzyme activity per volume of saliva is also a function of flow rate (Nagy et al., 2015). Adjusting for salivary flow may help to isolate the sympathetic response, and should be a standard element of study design. Even then, SNS/PNS interactions cannot be ruled out. Nagy et al. (2015) repeatedly sampled saliva *during* as well as after a cognitive challenge, and observed that sAA secretion first declined during the task, and rapidly increased afterwards. Through comparison with a parallel parasympathetic marker, the respiratory sinus arrhythmia (RSA) (see below), they argued that the response pattern was most consistent with parasympathetic control. Considering this body of evidence as a whole, it remains advisable to treat sAA as an autonomic biomarker without specifying an ANS division.

Responsiveness to stress and ecological context

Psychobiologists have documented rapid elevations in sAA following a wide array of acute cognitive, physical, emotional and psychosocial challenges in laboratory contexts (Doane and Van Lenten, 2014; Nater and Rohleder, 2009; Nater et al., 2013b; Petrakova et al., 2015; Schumacher et al., 2013; Strahler et al., 2015). Other researchers have evaluated sAA responses to chronic stress or to shifting socioecological contexts in everyday life (Adam et al., 2011; Lynn et al., 2010, 2015; O'Donnell et al., 2015; Schumacher et al., 2013; Thoma et al., 2012a; Vigil et al., 2009; Wolf et al., 2008). There is no consistent evidence that average sAA activity functions well as a summative marker of chronic stress. Instead, the strongest research designs take into account both interindividual and intraindividual variability. For instance, Doane and Van Lenten (2014) instructed adolescents to collect 5 saliva samples per day across 3 days, and complete a diary entry regarding the positive/negative valence and the intensity of emotion each time. High arousal with positive affect was associated with higher momentary sAA levels, whereas negative affect and low arousal were not. By contrast, high arousal with negative affect during the *previous day* was associated with a greater early morning decline in sAA, and a flatter diurnal slope.

Such findings highlight the importance of collecting multiple samples across several days, and employing statistical methods such as multilevel modeling and lagged analysis that can identify both within- and between-individual patterns of association, a methodological consideration shared with the ambulatory study of blood pressure (James, 2007) and cortisol (Hruschka et al., 2005). Reducing multiple samples to an average will fail to capture these complex time-course dynamics and obscures important associations between physiology and context (Hruschka et al., 2005). Beyond approximately 3–4 samples, increasing the number of days is likely more important to detecting meaningful intraindividual variation than increasing the number of samples per day (Out et al., 2013).

Pairing sAA with cortisol, a marker of activity in the HPA axis, has demonstrated possible linkages between psychosocial adaptation among children experiencing chronically high stress and HPA/ANS symmetry or assymetry (Ali and Pruessner, 2012; Berry et al., 2012; Engert et al., 2011; Gordis et al., 2008; Koss et al., 2014; Ponzi et al., in press; Schumacher et al., 2013). However, while cortisol peaks in saliva 20–30 min after the onset of an acute stressor, sAA peaks in under 10 min (Nagy et al., 2015; Strahler et al., 2015). Hence, cortisol and sAA are time-linked but not simultaneous markers of HPA and ANS responses when assayed in the same sample.

Confounds and diurnal rhythm

Aside from flow rate, recent food consumption, active chewing during collection (e.g., chewing on an absorbent swab), and exercise increase sAA activity. Medications that affect autonomic responses, such as adrenergic agonists or antagonists, will interfere with naturalistic sAA responses. Smoking, alcohol, sleep disturbances, and caffeine are also potential confounds (Bosch, 2014; Ditzen et al., 2014; Koibuchi and Suzuki, 2014; Ligtenberg et al., 2015; Nagy et al., 2015; Rohleder and Nater, 2009; Veen et al., 2012). Rohleder and Nater (2009) advise neither drinking anything other than water nor eating for an hour before sample collection, and a water rinse prior to sampling to wash away food components. sAA levels tend to increase

throughout the lifespan while diurnal secretion patterns and reactivity profiles change in shape (Adam et al., 2011; Hill-Soderlund et al., 2015; Nater et al., 2013a; Strahler et al., 2010a; Strahler et al., 2010b; Yim et al., 2015), so controlling for age also is important. sAA displays a diurnal rhythm that is nearly a mirror image of the familiar cortisol pattern. sAA declines rapidly during the first 30 min post awakening, then gradually increases throughout the rest of the day. While time of sample collection must be recorded and controlled, the shape of the diurnal rhythm itself is a variable of potential interest (Nater et al., 2007; Out et al., 2013). With self-collected samples an accurate description of the diurnal rhythm can be difficult, because of unreliable sample collection immediately after awakening. For this reason researchers increasingly rely on technology to monitor compliance, such as motion detection (actigraphy) to detect the time of awakening, and electronic caps on saliva tubes that record exactly when the sample was collected (Doane and Van Lenten, 2014).

Best practices for saliva collection

Saliva collection can be either stimulated or unstimulated. Within each category, saliva can be collected by placing absorbent material in the mouth, or through "drooling/spitting" directly into a collection tube (Rohleder and Nater, 2009). The gold standard for saliva collection is unstimulated drooling/spitting (Bosch, 2014; DeCaro, 2008; Nagy et al., 2015; Rohleder and Nater, 2009). The variable mass of absorbent materials makes it difficult to reliably compute saliva flow rate, and swabs can interfere with full sAA recovery (Bosch, 2014; DeCaro, 2008). It is also preferable to measure protein secretion without stimulating salivary flow through chewing, since stimulation results in reflex secretion of proteins and a change in the proportion of saliva derived from each of the salivary glands. These effects decouple sAA concentration from the psychosocial processes under investigation (Bosch, 2014).

It is nearly always possible with 60 s of saliva flow to collect enough sample for sAA determinations, which easily can be performed with as little as 100 μl, so stimulating saliva collection should not be necessary. However, it is sometimes easier for research participants to use an absorbent swab than to drool or spit into a tube; this is particularly an issue for younger subjects (Rohleder and Nater, 2009). In ambulatory study designs wherein participants are expected to self-collect samples over multiple days, reducing participant burden in this manner may be appealing. Collection using an absorbent swab is still considered unstimulated if the swab is rolled around the mouth without chewing. However, clear instructions are required to ensure consistency. The swab should be rolled around the *entire* mouth to capture saliva from all the glands that secrete amylase. Finally, in the exceptional case where stimulating saliva flow is necessary, acidic compounds should never be used for this purpose since the lowered pH of the saliva may interfere with the assay (Rohleder and Nater, 2009).

Absorbent materials should be synthetic polymers that have been validated for the collection of sAA and shown neither to artificially increase nor decrease sAA recovery relative to passive drool (DeCaro, 2008). The SalivaBio Oral Swabs, Children's Swabs, and Infant Swabs from Salimetrics, LLC (Carlsbad, CA, http://www.salimetrics.com) are validated synthetic absorbent polymers. The synthetic swab in the Salivette Cortisol Code Blue from Sarstedt (Nümbrecht, Germany, https://www.sarstedt.com) may also be suitable, although it is not clear this device has specifically been validated for sAA. Many researchers have employed cotton swabs, such as those found in the traditional Sarstedt Salivette. However, cotton is a bioactive compound, and decreases sAA activity in extracted saliva relative to passive drool while also increasing random error variance (Bosch, 2014; DeCaro, 2008). Samples previously collected using cotton may be tested for sAA if sample volumes are high. Yet given superior alternatives, absorption of saliva using cotton is categorically unjustified in future study designs.

To distinguish between amylase activity (or concentration) and secretion, flow rate should be measured whenever possible. This can be achieved by weighing each collection device before and after it is used, on a device-by-device basis because collection tube and/or swab weights are not perfectly uniform. Premeasurement and postmeasurement of the entire device is preferred to weighing only the saliva extracted after the fact, especially for devices that use absorbent swabs. This avoids problems with incomplete recovery of sample volume confounding saliva flow calculations for small samples. The exact length of time the swab was in the mouth also must be recorded. Volume is estimated using the density of water (1 g/ml), which permits calculating flow as ml/min. Under ideal conditions samples are stable for three weeks at room temperature. However, it is still best to freeze samples at −20°C or below as soon as possible to prevent microbial growth (DeCaro, 2008).

Quantifying sAA activity in saliva

A widely employed kinetic reaction assay for sAA activity, which is directly proportional to protein concentration, is available from Salimetrics (catalog #1-1902). Saliva diluted 1:10 in an assay buffer is incubated at 37°C with 2-chloro-p-nitrophenol linked with maltotriose, which serves as a chromogenic substrate for α-amylase. Color development over 2 min at 405 nm is proportional to amylase activity, reported as U/ml. No standard curve is required. The equipment required to undertake the assay in-house includes an incubator and plate rotator, or preferably a microplate incubator/rotator, to warm the substrate evenly; a microplate reader capable of measuring absorbance at 405 nm, preferably with a programmable kinetic read mode; a single channel pipettor capable of delivering 8 μl; and a multichannel pipettor capable of delivering 320 μl. In preparation for the assay the saliva must be thawed and spun in a centrifuge at 1500 × g to remove solids. Multiple academic labs and at least one commercial lab (Salimetrics) are equipped to undertake this assay at low cost, so it is feasible for researchers without in-house assay capabilities. If flow rate was measured, secretion (U/min) can be estimated as activity (U/ml) × flow rate (ml/min). As discussed earlier, unadjusted amylase activity is still an autonomic biomarker, but likely a less specific one (Bosch, 2014).

Biosensors that provide immediate "point-of-care" sAA readings may be a viable alternative to the collection and analysis methods described above (Robles et al., 2013; Rohleder and Nater, 2009; Yamaguchi et al., 2006). In this case, samples should be collected using unstimulated drool and the biosensor applied to the sample from the

tube rather than through direct collection onto the sensor from the mouth (Robles et al., 2013). This ensures whole saliva collection, and also makes samples available for other assays or quality control checks. For field research where access to a biomarkers laboratory is a financial or logistical barrier, biosensors are likely to see increased adoption as they become securely validated and widely commercially available.

PARASYMPATHETIC AND SYMPATHETIC ACTIVITY ASSESSED THROUGH CARDIOGRAPHY: RESPIRATORY SINUS ARRHYTHMIA AND THE PRE-EJECTION PERIOD

Validation as specific parasympathetic and sympathetic markers

A wide array of cardiovascular parameters is subject to autonomic control. The most commonly measured, blood pressure and heart rate, are regulated in exceptionally complex ways through interactions of parasympathetic and sympathetic activity and are not very specific (Hastings and Miller, 2014). Yet it is possible to identify and distinguish PNS and SNS responses using electrocardiography (ECG) and impedance cardiography (ICG).

Cardiac parasympathetic activity is indexed by the RSA, a normal fluctuation of heart rhythm tied to respiration; in fact, this is the *only* well-accepted noninvasive marker of PNS activity across all organ systems. Sympathetic activity is indexed by the pre-ejection period, which is the delay between the onset of ventricular polarization and left ventricular ejection also called electrical systole (Hastings and Miller, 2014; Obradović and Boyce, 2012). The specificity of RSA and PEP at rest and in response to stressors have been confirmed through autonomic blockade using the muscarinic antagonist atropine and the beta-1 antagonist metoprolol; through beta adrenergic stimulation; and in the case of PEP, through correlation with catecholamines (Berntson et al., 1994; Cacioppo et al., 1994a; Kelsey, 2012). Although RSA displays sympathetic influence under extreme conditions such as respiratory distress and very low heart rate (Grossman and Taylor, 2007), this is unlikely to be a concern in typical human biology research.

In mammals, heart rate increases during inspiration and decreases during expiration. This effect may have adaptive significance as a mechanism to improve gas exchange during inspiration by increasing blood perfusion of the lungs (Taylor et al., 2014). The specific neural mechanisms are complex and beyond the scope of this review (although see Berntson et al., 2007; Berntson et al., 1993; Grossman and Taylor, 2007; Porges, 2007; Porges et al., 1994; Taylor et al., 2014), but parasympathetic signals through the vagus are *inhibited* during inspiration, which results in *withdrawal* of the parasympathetic "brake" from the sinoatrial node and acceleration of the heart. The degree of parasympathetic activity (sometimes referred to as "vagal tone" when measured at rest) thus may be quantified as heart rate variability (HRV) within the frequency band of respiration. By convention this is the high frequency (HF) band, so RSA is a *marker* of parasympathetic activation that is *measured* by quantifying HF-HRV.

PEP is a momentary marker of activity in the SNS, reflecting contractility of the left ventricle. Increased contractility, which shortens the PEP, is a feature of beta adrenergic acceleration in heart rate and the increase in cardiac output in response to a challenge or threat (Seery, 2011). PEP is quantified using ICG, wherein a constant current is applied between electrodes positioned at the top and bottom of the thorax, and two receiving electrodes that also bracket the thorax are used to detect voltage change. The voltage change is proportional to the impedance of the thorax, which fluctuates systematically during systole (Cybulski et al., 2012).

RSA and PEP response to socioecological context

Rather than the magnitude of the RSA or PEP response per se being associated with psychosocial functioning or wellbeing, adaptive flexibility and context sensitivity in the autonomic response—even in the course of a single encounter—best support prosocial behavior on one hand, and rapid active coping responses on the other (El-Sheikh and Erath, 2011; Hastings and Miller, 2014). Moreover, the association between autonomic reactivity and persistent ecological stress appears to be U-shaped in children, perhaps because greater biological sensitivity to context is most beneficial to those inhabiting highly favorable *or* highly risky environments (Boyce and Ellis, 2005; Obradović et al., 2010).

A series of ambulatory studies that track momentary fluctuations in RSA and PEP during real-life conditions serve as compelling models for human biology and anthropology researchers (Wilhelm et al., 2012). For instance, among white-collar males, Vrijkotte et al. (2004) found that excessive work involvement was associated with lower PEP throughout the day, lower PEP variability and a smaller day/night PEP difference. Reijman et al. (2014) showed distinctive patterns of PEP change during infant crying between maltreating and nonmaltreating mothers. Ambulatory RSA has been linked to marital conflict (Gates et al., 2015), and qualities of mother-infant interaction (Giuliano et al., 2015), while both ambulatory RSA and PEP have been linked to flight anxiety during air travel (Busscher et al., 2013).

Data collection through ambulatory monitoring

Experimental reactivity protocols remain a viable option for human biologists, and it is possible to combine ecological sampling of daily experience with experimental manipulations to determine how responses in controlled conditions vary across general social settings (DeCaro and Worthman, 2008). There also are compelling theoretical frameworks that use variation in autonomic responsiveness under standardized laboratory conditions as a window into the evolution of social behavior and the stress response (Boyce and Ellis, 2005; Porges, 2007; Porges and Furman, 2011; Porges et al., 1994).

However, ambulatory monitoring of RSA and PEP is well-developed in cardiology and psychophysiology, and ripe for adoption by human biologists interested in biological regulation during everyday life. Several comprehensive reviews are available (Cybulski, 2011; De Geus and Van Doornen, 1996; Houtveen and de Geus, 2009; Wilhelm and Grossman, 2010; Wilhelm et al., 2012). Contemporary ambulatory monitors may easily be carried in a shirt or pants pocket, or can be clipped to the waist or arm. For instance, the Mobile Impedance Cardiograph from MindWare Technologies. (Gahanna, OH, http://www.mindwaretech.com/) measures 117 × 79 × 47 mm, weighs 370 g including the battery pack and leads, and can collect data continuously for at least 18 h on a charge with local

storage of data. While it is possible to purchase ambulatory ECG-only monitors for RSA, all impedance cardiographs for PEP integrate ECG as well. Many ambulatory systems incorporate or can be configured with additional sensors, such as triaxial accelerometers, light sensors, and electrodermal response (skin conductance) amplifiers. Other popular mobile monitoring systems include the Mobita from BIOPAC Systems (Goleta, CA, http://www.biopac.com), and the VU-AMS Ambulatory Monitoring System from Vrije Universiteit (Amsterdam, The Netherlands, http://www.vu-ams.nl).

In ambulatory monitoring of RSA and PEP, the researcher must consider whether to continuously collect data, and if so, over what period of time. Another alternative is to trigger data collection periodically through an experience sampling protocol, or in response to specific predetermined events (Wilhelm et al., 2012). Ambulatory data are of little use without knowledge of activities and social settings, and so context must be documented separately. The most salient contextual elements, and the frequency with which they need to be recorded, will depend on study design. Diaries and experience sampling are useful for subjective experience, whereas sensors may be used to obtain objective data, as when integrated accelerometers allow for an estimate of physical activity and posture (Wilhelm et al., 2012).

The collection of ECG data suitable for RSA requires only three leads (positive, negative, and ground), which typically are arranged in the Lead II configuration to maximize the prominence of the R-wave. In Lead II, the positive lead is on the left leg or left lower torso and the negative lead is on the right arm or right upper torso. The ground can be placed on the left arm/left upper torso or right leg/right lower torso. Lead I and Lead III configurations are also possible (Mendes, 2009). Disposable spot ECG monitoring electrodes are suitable, with snap-style connectors preferred over alligator clips. The site of electrode placement should be cleaned with an alcohol wipe and allowed to dry to improve electrical contact. Artifact related to movement of the electrodes and leads can be minimized by placing the electrode directly over bone and in a location with a minimum of hair, taping the electrode and the lead wire to the skin, and positioning the electrodes centrally on the trunk rather than distally on the limbs (Alkon et al., 2003; Mendes, 2009; van Dijk et al., 2013)

For PEP, which is assessed using ICG, an additional four leads are required (Cybulski et al., 2012; Reijman et al., 2014; Sherwood et al., 1990; Spangler and Friedman, 2015). Disposable spot ECG monitoring electrodes are suitable, and the site should be cleaned and electrodes taped. The two electrodes that will apply a constant current are placed posterior, at the base of the neck, approximately at C3/C4, and over the eighth or ninth thoracic vertebra. The two recording electrodes are placed anterior, over the jugular notch and the xiphoid process. Current and recording electrodes should be separated vertically by at least 3 cm (Alkon et al., 2003; De Geus and Van Doornen, 1996; Reijman et al., 2014). An example of such a set-up using the VU-AMS Ambulatory Monitoring System, including ECG and impedance electrode and lead placement with an online video as a supplementary material, is available from van Dijk et al. (2013).

An ECG and ICG sampling rate of 1,000 Hz is suitable for RSA and PEP (Reijman et al., 2014; Spangler and Friedman, 2015). After hookup and prior to the formal start of data collection, the ECG waveform and the first derivative of the impedance curve, dZ/dt from ICG, should be visualized to ensure signal quality. There should be clearly distinguishable ECG R-waves for RSA, clear ECG Q-waves and a distinct ICG B-point for PEP, and a minimum of noise. Proper identification of these landmarks is discussed in the analysis section, below. Most ambulatory equipment includes a screen that continuously displays bioelectric signals during data collection to facilitate quality control. Changing, repositioning, or re-taping electrodes may be required if signal quality is poor. In an ambulatory study, where possible it is beneficial to train research participants to recognize common data quality problems so they can take basic troubleshooting steps or contact investigators. Some ambulatory devices are capable of detecting loss of signal and audibly alerting the research subject (De Geus and Van Doornen, 1996). Despite such precautions, it may be necessary to discard as much as 20% of the raw data from an ambulatory study because of signal quality problems, and entire monitoring periods may need to be repeated if loose electrodes go undetected (De Geus and Van Doornen, 1996).

RSA and PEP confounds

RSA and PEP are subject to confounding by all variables that affect heart rate or autonomic arousal, including the use of stimulants (e.g., nicotine, caffeine) or depressants (e.g., alcohol), food intake, medications that alter autonomic activity, posture, and physical activity. In a laboratory study, such variables are carefully controlled. In an ambulatory design these confounds must be recorded and accounted for using sensors or diaries, eliminated through instructions that preclude specific behaviors during the monitoring period, or dealt with using exclusion criteria for study participants (De Geus and Van Doornen, 1996; Houtveen and de Geus, 2009; Wilhelm et al., 2012). RSA and PEP at rest generally increase across childhood and adolescence, although age-related developmental changes are neither entirely consistent nor linear (Alkon et al., 2003; Benevides and Lane, 2013; Obradović and Boyce, 2012). Hence, it may be more helpful to control for age categorically than as a continuous variable.

Best practices in data analysis

The cleaning of ECG and ICG signals and reduction to RSA and PEP requires specialized software, such as AcqKnowledge from BIOPAC, or Physiology Analysis Software from MindWare, each a proprietary commercial package that can import raw data from other vendors' equipment. For the analysis of HRV only, Kubios software from the Biosignal Analysis and Medical Imaging Group at the University of Eastern Finland is available free of charge (http://kubios.uef.fi/).

Before analysis, the ECG and ICG signals must be broken into epochs of sufficient length to capture HF-HRV. The minimum length varies depending on age but should typically be 30–60 s (Bar-Haim et al., 2000). It is not necessary to analyze all the data that were collected; instead, relevant segments can be sampled purposively or at random. Valid RSA and PEP calculations require manual review of the peak detection results to confirm correct placement of all R-waves within each epoch; misplaced peaks bias RSA and PEP calculations. Automated algorithms included in the packages listed above are effective

for typical and clean ECG waveforms, but movement artifacts, poor electrode and lead contact, and atypical ECGs can cause algorithms to partially fail. All software packages include tools to adjust R-wave placement and/or clean the waveform. Some also include tools to extrapolate undetectable R-waves so as to avoid discontinuities in the data.

Once correct R-wave detection has been confirmed, the remainder of the process for calculating RSA can be fully automated. For each epoch, an interbeat interval time series is determined based on distances between R-waves. The IBI is then detrended and transformed using a Hamming window (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Often a Fourier transformation is used to generate a frequency power spectrum and the area under the curve across the HF band computed, although alternative statistical approaches to quantifying HF-HRV also are available (Alkon et al., 2003; Berntson et al., 1993; Cacioppo et al., 1994b; Mendes, 2009; Spangler and Friedman, 2015). RSA derived from spectral analysis is reported as the natural logarithm of this HF power value; the conventional unit is $\ln(ms^2)$. Because respiration rates are not constant, the correct HF band depends on the age of the subject. From infancy through age 4, a frequency range of 0.24–1.04 Hz is appropriate (Alkon et al., 2003; Bar-Haim et al., 2000); for elementary age children, the frequency range is 0.15–0.80 Hz (Obradović et al., 2010); and among adolescents and adults, 0.15–0.40 Hz (Berntson et al., 1993; Mendes, 2009; Spangler and Friedman, 2015). Some authorities recommend confirming or controlling for respiratory frequency on a subject-by-subject basis rather than using typical frequency bands (Grossman and Taylor, 2007). Respiration rate can be derived from ICG in a mixed RSA/PEP design, or measured using a separate respiratory effort transducer. However, RSA studies often rely on ECG alone, and it is acceptable to use an age-typical respiratory range if individualized data are unavailable (Mendes, 2009; Porges, 2007). Paced-breathing calibration at the start of an RSA recording is an additional way to adjust for individual differences in respiratory rate and volume (Ritz, 2009).

Calculation of PEP requires the correct placement of two landmarks: the Q-wave peak from ECG, and the B-point on the dZ/dt from ICG. In Lead I, II, or III configurations the Q-wave is a downward deflection immediately before the R-wave, and signals the onset of ventricular polarization. In a clean ECG, the Q-wave may be identifiable using automated software tools, but Q is small and easily obscured by noise, so placement must be visually verified (Berntson et al., 2004). The dZ/dt is the first derivative of the impedance curve. A rising dZ/dt thus reflects the change of the impedance slope in a positive direction. The B-point, which is defined as the beginning of the longest upward deflection in the dZ/dt waveform, marks the initiation of left ventricular ejection. While automated algorithms to detect B are available as time-saving devices (Lozano et al., 2007), none are fully effective. Hence, visual confirmation is required and manual adjustment of B-point placement frequently is needed (Mendes, 2009). Inter-rater reliability should be calculated on a subset of test cases both during training and for ongoing quality control. Because coding both Q and B on a beat-by-beat basis is difficult and time consuming, the epoch often is collapsed using a signal processing approach called ensemble averaging. This produces a single combined ECG and dZ/dt waveform that represents a "typical" beat (Mendes, 2009). PEP is reported as the time in milliseconds between Q and B.

Limitations

The limitations of RSA and PEP are similar. They are not systemic markers of parasympathetic or sympathetic activity, for which they are sometimes inappropriately treated as shorthand; rather, they specifically reflect parasympathetic and sympathetic control over the heart (Ritz, 2009). ECG and ICG are subject to movement artifact, which can be challenging in an ambulatory study, or with children. Research participants may not be willing to wear electrodes and leads for extended periods of time. Initial equipment costs are high, although properly cared for the equipment is durable and can be repeatedly reused. Significant skill is required to analyze the data correctly. Finally, ECG and ICG monitoring may be impossible in some field settings, such as when there is no reliable access to electrical power to charge equipment, or where the device cannot be protected against excessive moisture or extreme temperatures. Good electrode contact is also more difficult when the skin is moist from sweat (Mendes, 2009). Since psychophysiology equipment has overwhelmingly been developed for and used by scientists conducting research in North America and Europe (although see Seligman, 2010), manufacturers have limited data regarding the performance of their devices in highly variable environmental conditions.

CONCLUSIONS

sAA, RSA and the pre-ejection period complement catecholamines and blood pressure monitoring, and expand the autonomic toolkit for human biology. Each confers specific advantages that can overcome practical difficulties associated with monitoring autonomic responses across changing socioecological contexts. sAA is the first and so far the only validated salivary autonomic biomarker. It can easily be integrated into sampling protocols designed for cortisol research, and is stable under field-typical conditions (DeCaro, 2008; Nater et al., 2013a; Nater and Rohleder, 2009). It is not an instantaneous or continuous measure of ANS activity, but it allows finer time resolution than urinary epinephrine or norephinephrine. However, sAA is a very indirect way to examine autonomic arousal. Because of the complexity of the regulation of saliva secretion, sAA is not a reliable proxy for catecholamines (Bosch, 2014).

RSA is the only reliable noninvasive marker specific to peripheral parasympathetic activity across any organ system (Hastings and Miller, 2014). ECG is simple to collect and specialized software is available to assist in data reduction. Isolating parasympathetic responses is helpful for research on stress responses, biological sensitivity to socioecological context, and the evolution of social behavior (Boyce and Ellis, 2005; Porges et al., 1994; Porges and Furman, 2011). Furthermore, as parasympathetic and sympathetic responses are not perfectly in sync and neither can be inferred from the other (Obradović and Boyce, 2012), RSA opens a window onto an allostatic system that has largely been overlooked in human biology research to date.

PEP shares its status as a specific marker of sympathetic activation with urinary catecholamines and electrodermal

responses (Brown, 2007; Mendes, 2009). However, given tissue specificity of autonomic effects (Ritz, 2009), there are theoretical benefits to measuring sympathetic and parasympathetic responses in the same organ system, which can only be accomplished by pairing PEP and RSA. Using PEP, momentary responses to changes in context can be evaluated in experimental or ambulatory designs.

None of these measures, per se, illuminate differential well-being. While the autonomic response has widespread effects on human physiology, there is no true baseline. Instead the ANS is continuously responding to characteristics of the social and physical context, including anticipation of upcoming challenges (Obradović and Boyce, 2012; Wilhelm et al., 2012). Even low arousal should be understood as a *response to* low-threat conditions and, perhaps, the need for social engagement, not merely the *absence of* a threat. The dedication of an entire division of the ANS to actively lowering physiological arousal should make this evident.

Moreover, high levels of arousal are consistent with well-being if they are context-appropriate adaptive responses. For these reasons much of the variation of interest to human biologists is likely to be within the range of normal, much as is true for cortisol levels or blood pressure. When investigating these mediators of allostasis, persistent patterns of arousal over time in response to social environment rather than arousal levels at a given moment are what generate allostatic load and consequent health risk (McEwen, 2012). The evaluation of momentary associations between context and physiological responses, however, provides early clues regarding social processes through which allostatic load accumulates, and is essential to discovering how everyday experience "gets under the skin."

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