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Heart rate variability reveals that a decrease in parasympathetic ('rest-and-digest') activity dominates autonomic stress responses in a free-living seabird

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Abstract

The autonomic stress response, often referred to as the 'fight-or-flight' response, is a highly conserved physiological reaction to stress in vertebrates that occurs via a decrease in parasympathetic (PNS) activity, which promotes self-maintenance 'rest and digest' processes, and an increase in sympathetic (SNS) activity, which prepares an animal for danger ('fight-or-flight'). Though the PNS and SNS both innervate most organs, they often control different tissues and functions within those organs (though the pacemaker of the heart is controlled by both). Moreover the PNS and SNS are regulated independently. Yet until now, most studies of autonomic stress responses in non-model species focused only on the SNS response. We used external electrocardiogram loggers to measure heart rate and heart rate variability indexes that reflect PNS and SNS activity in a seabird, the Streaked Shearwater (*Calonectris leucomelas*), during the stress of handling, and during recovery in the nest burrow or during restraint in a cloth bag. We show for the first time in a free-living animal that the autonomic stress response is mediated primarily by a rapid decrease in PNS activity: handling stress induced a large and long-lasting depression of PNS 'rest-and-digest' activity that required two hours to recover. We also found evidence for a substantially smaller and shorter-lasting SNS 'fight-or-flight' response. Confinement in a cloth bag was less stressful for birds than handling, but more stressful than recovering in nest burrows. We show that quantifying autonomic activity from heart rate variability is effective for non-invasively studying stress physiology in free-living animals.

Keywords autonomic nervous system, heart rate, stress response, sympathetic, parasympathetic, capture-and-restraint protocol, fight-or-flight, heart rate, heart rate variability, shearwater

Abbreviations

ECG	electrocardiogram
PNS	parasympathetic nervous system (promotes 'rest-and-digest' processes)
SNS	sympathetic nervous system (promotes 'fight-or-flight' processes)
IBI	inter-(heart)beat interval
'SDNN'	standard deviation of all IBIs (reflects combined SNS and PNS activity)
'rMSSD'	standard deviation of the differences between successive IBIs (reflects PNS activity)
'SDNN:rMSSD'	the ratio between 'SDNN' and 'rMSSD' indexes (reflects the SNS:PNS balance)

1. Introduction

When a vertebrate encounters a threat such as a predator, signals from its sensory organs and body quickly initiate physiological and behavioural response patterns in the upper brain stem and hypothalamus (Jänig, 2006) that help protect it from danger. This includes a rapid change in the activity of the highly conserved autonomic nervous system (Taylor et al., 2014). The autonomic nervous system comprises two neural branches that have broadly opposing physiological effects: the parasympathetic nervous system (PNS) promotes self-maintenance processes such as digestion, repair, energy conservation, and the resting of major organs, whereas the sympathetic nervous system (SNS) prepares an animal for danger by e. g. increasing alertness, blood pressure, and mobilizing energy reserves to support muscular action (Porges, 1992, 1997, 2007; Jänig, 2006; Romero and Wingfield, 2016).

During an autonomic stress response, 'rest-and-digest' PNS activity rapidly decreases (via the 'vagal brake' on the fast-acting myelinated ventral vagal complex; Porges, 1997, 2007; Jänig, 2006, Carravieri et al., 2016). If the threat persists, then 'fight-or-flight' SNS activity increases via neural connections to target organs (Jänig, 2006) and also through the slower acting sympathetic-adrenal-medullary hormonal pathway that stimulates catecholamine release (in birds: epinephrine from the adrenal medulla and norepinephrine from adrenals and sympathetic nerve terminals, Dzialowski and Crossley II, 2015). The two autonomic branches also innervate the heart: when activated, the PNS reduces heart rate and the SNS increases heart rate (Brindle et al. 2014; Kuenzel, 2015). The combined reduction in PNS activity and activation of SNS activity during an autonomic stress response therefore very rapidly increases heart rate. Heart rate has become a commonly used measure of the overall autonomic stress response in studies of animals in captivity (Dickens et al., 2006; Nephew et al., 2003; Wascher et al., 2009) and in the wild (Gabrielsen et al., 1985; Steen et al., 1988; Nimon et al., 1996; Bisson et al. 2009; Bisson et al. 2011; Viblanc et al. 2015, Romero and Wingfield, 2016).

But many studies interpret stress-induced increases in heart rate as a reflection of reciprocal activation of the PNS and SNS, or more often, activation of the SNS alone (perhaps why increased heart rate is often called the 'fight-or-flight' response). This approach may lead to inaccurate characterisations of the autonomic responses and their downstream physiological effects. Although autonomic stress responses can indeed reciprocally activate PNS and SNS branches, they also can independently activate or even co-activate them (Paton et al., 2005, 2006), with substantial variation between individuals in the degree of their reciprocal, independent, or co-activation (Berntson and Cacioppo, 1999). For example, in humans, male erection and ejaculation (Berntson et al., 2003), sky diving (Allison et al., 2012), and hypoxia (Kollai and Koizumi, 1979; Fukuda et al., 1989), are three very different situations that co-activate the PNS and SNS. Co-activation of both branches can under some circumstances can result in a net increase in heart rate, such as during pain in both humans and laboratory animals (reviewed in Paton et al., 2006), or by a net decrease in heart rate such as during diving (reviewed in Paton et al., 2006), stimulation of peripheral chemoreceptors (Kollai and Koizumi, 1979) or ocular trauma or surgery (Paton et al., 2006) making it difficult to quantify PNS and SNS activity from heart rate alone. Yet it is important to quantify the activity of both branches, because many tissues in the

body are only innervated by either the PNS or the SNS (though both branches innervate most organs and in some cases even innervate similar tissues and modulate similar functions within those organs like in the heart, iris, bladder, and some blood vessels; Jänig, 2006).

Analysing heart rate variability provides a non-invasive way of measuring the individual activity of the PNS and SNS. Both autonomic branches modulate heart rhythm, each creating oscillations in inter-heartbeat intervals at different frequencies (Stauss, 2003; van Borell et al., 2007) so the contributions of the PNS and SNS to heart rate variability can be quantified using various indexes that reflect the amplitude of these oscillations (Stauss, 2003; von Borell et al., 2007). Changes in PNS- and SNS-driven components of heart rate variability during an autonomic stress response provide a much more detailed picture of autonomic regulation and its downstream effects than analyses of heart rate alone.

In this study we measure heart rate and use heart rate variability to quantify PNS- and SNS-driven components of the autonomic stress response in the Streaked Shearwater (*Calonectris leucomelas*), a pelagic long-lived seabird that breeds in Japan. Shearwaters are convenient models for behavioural and physiological field research as they breed in large colonies, nest inside excavated burrows that make them easy to find and capture (Ogawa et al., 2015) and are large enough for attaching bio-logging instruments for tracking their movements or physiology (e.g. Müller et al., 2014, 2015; Carravieri et al., 2016). We attached miniaturised, external electrocardiogram (ECG) loggers to breeding adult shearwaters and recorded their heart activity just after exposure to the acute stress of handling and (1) during their subsequent recovery in their nest burrows (which has the advantage of being a familiar non-stressful environment, birds remain mostly motionless in the narrow space, and are easy to re-capture for logger retrieval) or (2) during a period of stress-inducing confinement in an opaque cloth bag, a commonly used standardised test for comparing the magnitude of the stress responses within and between individuals (capture-and-restraint protocol, Wingfield et al., 1982). We also recorded heart rate of adults incubating eggs inside their nest burrows for 24 hours to obtain baseline values of heart rate and heart rate variability indexes.

We compared the PNS and SNS activity from heart rate and heart rate variability indexes across time intervals and tests to investigate the importance of the PNS relative to the SNS in driving the autonomic stress response, and to determine the time required for PNS and SNS activity to stabilise/recover after the acute stress of handling. We hypothesise, based on human and laboratory animal studies that the PNS ('rest-and-digest' branch) plays an important role in the autonomic stress response in a free-living wild animal, though this has until now, to our knowledge, never been tested.

2. Material and methods

2.1 Data collection

Fieldwork was conducted in a large Streaked Shearwater (*Calonectris leucomelas*) breeding colony on Awashima Island (38° 18'N, 139° 13'E, ca. 84,000 birds) in the Sea of Japan, during the chick-rearing season (mid-August to mid-October) in 2014 and 2015 and during the late incubation period between August 13-16

of 2016. Streaked Shearwaters lay one egg inside a narrow ca. 0.5–1 m deep nest cavity excavated into the soil on a coastal slope facing the sea. Parents share reproductive duties during incubation and chick-rearing (Matsumoto et al., 2016).

Adult breeding birds were captured from their nest burrows and equipped with externally attached miniaturised ECG data loggers and returned to their nest burrows (or placed into a cloth bag, see below), to measure the effect of handling stress and the duration of recovery in heart rate, and in PNS and SNS indexes of heart rate variability. We used Little Leonardo ECG loggers (model W400-ECG, 21 x 109 mm cylindrical logger, 1 ms sampling interval, voltage range +/- 5.9 mV, 60 g, 2 GB memory) and Neurologger 2A for ECG (0.625 ms sampling interval, voltage range +/- 3 mV, 20g, 1 GB memory, Evolocus LLC, Fig. A.1; for details see Vyssotski et al., 2009; Anisimov et al., 2014). Three wires extend from the ECG logger, with small safety pins soldered to the ends that function as electrodes. Electrodes were subcutaneously attached to the skin under the feathers of the breast, the wires were wrapped around one side of the bird and the logger was firmly taped to the dorsal feathers (see Yamamoto et al., 2009). This method (as opposed to gluing) has the benefit of not requiring the removal of feathers, results in much faster attachment of the logger (reducing handling time), and has become a standard method for seabirds (e.g. Ropert-Coudert et al., 2006; Yamamoto et al., 2009; Carravieri et al., 2016) and other animals (Ropert-Coudert et al., 2009). Several birds were repeatedly tested (up to 10 times within a season) and even when recaptured within a few days, we detected no skin wounds or irritation from previous ECG logger attachments. Pins and skin were cleaned with alcohol wipes before logger attachment.

We attached ECG loggers to incubating adults for 24 hours (in 2016) to obtain baseline values of heart rate and heart rate variability indexes of resting birds. Nine incubating adults were captured from their nests in the early morning (between 6 am and 8 am), equipped with loggers and returned to their burrows for 24 hours. Though incubation bouts in shearwaters typically last several days, to ensure that no birds escaped with a logger, nest entrances were blocked with a cloth-covered 2L bottle filled with water.

During the chick-rearing period, adults are present in the colony only at night when they return from foraging trips to feed their chicks, so in 2014 and 2015, fieldwork was conducted at night between ca. 8 pm and 4 am. Birds were placed into their burrows for 2–3 hours (see results for sample sizes), with a total handling time, including capture and logger attachment, of 7–12 minutes. Another group of birds was equipped with loggers in the same way, but instead of being returned to the burrow, they were placed into an opaque cloth bag (28 x 35 cm) that was closed with a drawstring, and secured on a level area on the ground for 20–90 min (see results for sample sizes). Unringed birds were given a metal ring, and one end of a 1.5 m long string was tied to the ring and the other end was attached outside of the nest burrow to ensure that the logger-equipped bird did not fly away. The investigator sat motionless in the dark outside the nest and quickly captured any logger-equipped birds that attempted to escape, and removed the logger and string before releasing them. Streaked Shearwaters have short, thick legs for propelling themselves from the water's surface during take-off. If an animal briefly pulls on the string during an escape attempt, this does not cause injury in this species. Data from birds that attempted to escape were not included in the analyses.

At the end of each test, the bird was retrieved, the string and ECG logger was removed, bill length was measured with callipers, and mass was measured to the nearest 5 g.

2.2 ECG data processing

ECG data were analysed using Igor Pro version 6.37 (Wavemetrics, USA) in five-minute intervals, based on the recommendations of von Borell et al. (2007). The R peak in the PQRS complex (the main graphical deflections in an ECG tracing of an individual heartbeat, Fig. 1A) was easily detectable. R peaks were primarily identified using the software Ethographer (Sakamoto et al., 2009), manually identified when necessary, and used to create a data frame of the occurrence time of each heartbeat (in milliseconds).

Using the *RHRV* package (Mendez et al., 2014) in *R* computing software (version 3.2.1), these beat positions were then filtered to eliminate spurious beats that came from misidentified peaks in the ECG wave caused by muscle noise, and were used to calculate inter-beat intervals (IBIs). Heartbeat positions plotted over time produce a tachogram that reveal oscillations in heart rate caused by the autonomic nervous system, which generate the greater part of total heart rate variability (Fig. 1B). High-frequency oscillations (between 0.3 and 2 Hz in this species, or every 0.5-3.3 s, Fig. 1B, 1C) reflect variability in heart rate that is modulated by the PNS and corresponds to respiration - during inhalation heart rate accelerates, during exhalation heart rate slows (respiratory sinus arrhythmia, Stauss, 2003; Taylor et al., 2014; Carravieri et al. 2016). The strength, or amplitude, of these oscillations in heart rate is therefore an index of PNS activity. Low-frequency oscillations (0.04 - 0.3 Hz in this species, or every 3.3-25 s, Fig. 1B, 1C) are modulated by both the SNS and PNS, and the amplitude can be therefore used to quantify combined SNS and PNS activity (von Borell et al., 2007; Malik et al., 1996; Yamamoto et al., 2009; Carravieri et al., 2016).

From the IBI data we calculated a series of indexes that reflect the amplitude of the oscillations generated by PNS and/or SNS activity using *RHRV*. We calculated the standard deviation of the differences between successive IBIs ('rMSSD'), which reflects the amplitude of high frequency oscillations and therefore PNS activity, the standard deviation of all IBIs ('SDNN'), which reflects the amplitude of low frequency oscillations and therefore the combined SNS and PNS activity (hereafter SNS+PNS index), and the ratio between the 'SDNN' and 'rMSSD' ('SDNN:rMSSD') which reflects SNS:PNS balance (Malik et al., 1996; Kjaer and Jørgensen, 2011; von Borell et al., 2007; Shaffer et al., 2014, see Carravieri et al., 2016 for more details about heart rate variability analysis in this species). We also performed fast Fourier transform analyses that produce a power spectrum separating the high frequency and low frequency oscillations and quantified the power (i.e. squared amplitude) of the different oscillations in heart rate (Altimiras, 1999; von Borell et al., 2007; Carravieri et al. 2016). This produced broadly the same results as calculations of indexes based on standard deviations of IBIs (results are reported in supplementary material). Finally, we also used *RHRV* to compute average heart rate (which reflects SNS:PNS balance, but see introduction) over the course of each five-minute interval.

2.3 Statistics

Statistical analyses were performed using *R* (version 3.2.1). All indexes were log-transformed to achieve normality except for heart rate, which was already normally distributed. We performed mixed models using the *lme4* package (Bates et al., 2015) and *lmerTest* (Kuznetsova et al., 2015). Several birds were tested more than once, so we included individual ID as a random factor in all models except for models performed exclusively using data from birds confined in a bag or incubating birds recorded for 24 hours, because those datasets included only one observation from each individual. We did not correct for time of night in our analyses, as we found no strong circadian patterns in 24-hour records from incubating birds. Furthermore, there was little concern that time of night would introduce bias into our analyses as most comparisons were among different indexes within test.

To compare the magnitude by which heart rate and heart rate variability indexes differ between baseline and stress, we performed a series of mixed models on standardised (i.e. z-scored) data and compared the regression coefficients (referred to as *b*) between models. We standardised the data by subtracting the distribution mean from each observation and dividing it by the standard deviation. This creates a new distribution with a mean of 0 and a standard deviation of 1 for each variable. Models contained 'Time' as a continuous fixed predictor (0, 20 and 90 min post-handling), individual ID as a random effect and standardized heart rate or heart rate variability index as the dependent variable. The standardised regression coefficients from the models are hereafter referred to as 'effect sizes'. For ease of interpretation, in Fig. 4 we multiplied the effect sizes by -1 so effect sizes reflect the magnitude of change between baseline and stress, which is the more conventional way of presenting results in stress research (rather than the change over the course of the recovery period in the burrow). 95% confidence intervals (CIs) for effect sizes were calculated by multiplying the standard error (*s. e.*) by 1.96.

3. Results

3.1 Baseline PNS and SNS activity in incubating birds inside their burrows

We recorded ECG of incubating birds inside their burrows for 24 hours, and chose heart rate and heart rate variability indexes calculated 18-hours after ECG logger attachment, to use as a baseline reference to compare with stress-induced values. Eighteen hours post-handling in the 24-hour tests occurred at midnight, which is the midpoint of the usual 8 pm–4 am nightly testing period for the shorter burrow/bag recovery tests reported below, which matches the timing of measurements of the baseline reference values and those from the short tests. We compared indexes calculated at 18 hours to those calculated just after handling (0 hrs), and to those calculated after one and 24 hours in the burrow. All indexes calculated just after handling (0 hrs) differed from those calculated at 18 hours (Table 1, Fig. 2). Indexes calculated after one hour in the burrow and those calculated after 24 hours in the burrow did not differ from those at 18 hours (Table 1, Fig. 2), indicating that baseline values remained stable.

3.2 Duration of recovery in burrow

A subset of birds were placed into their burrows after handling for a period of time lasting up to 180 min, and heart rate and heart rate variability indexes were calculated for several time intervals (0, 20, 60, 90, 120 and 180 min post-handling) to determine how much time is required for indexes to stabilise to resting baseline values. Changes in indexes were analysed between the time intervals specified below. Analyses across the full recovery time (across 0 through 180 min post-handling using unstandardized data) produced results that were qualitatively similar to our findings for the larger dataset of standardised indexes measured over the course of 90 minutes in the burrow (see below): The PNS index 'rMSSD' gradually increased over the course of 180 min (Table 2A, Fig. A.2A, N=24 for 0, 20, 60 and 90 min, N=23 for 120 min, N=12 for 180 min) and the SNS+PNS index 'SDNN' also increased (Table 2A, Fig. A.2B). The 'SDNN:rMSSD' ratio (an SNS:PNS index) decreased (Table 2A, Fig. A.2D), and heart rate also decreased (Table 2A, Fig. A.2C).

Autonomic activity returned to baseline already after 120 min of recovery in the burrow, as none of the indexes changed significantly between 120 min and 180 min post-handling (Table 2B, Fig. A.2). The 'rMSSD' (PNS activity) and 'SDNN' (SNS+PNS activity) were, however, still changing between 90 min and 120 min post-handling (Table 2C, Fig. A.2A, A.2B), but the 'SDNN:rMSSD' ratio and heart rate (SNS:PNS balance, Fig. A.2D, A.2C) did not change (Table 2C). The 'SDNN:rMSSD' ratio already returned to baseline by 60 min post-handling, as it did not change between the 60 and 90 min mark (b=0.00341, *s. e.*=0.00284, T=1.203, P=0.245, Fig. A.2D), though it was still decreasing between the 20 and 60 min mark (b=-0.00416, *s. e.*= 0.00164, T=-2.534, P=0.019, Fig. A.2D). Heart rate returned to baseline by 90 min post-handling, but it was still decreasing between 60 and 90 min post-handling (b=-0.517, *s. e.*=0.101, T=-5.12, P<0.001, Fig. A.2C).

Stabilised values of 'SDNN:rMSSD' ratio at 60 min, heart rate at 90 min, and 'rMSSD' and 'SDNN' at 120 min, did not differ significantly from baseline values of those indexes measured after 18 hours in incubating birds (Table 2D).

3.3 PNS and SNS activity after handling stress and recovery in burrow

We performed a series of models on standardised (i.e. z-scored) indexes to compare the magnitude of the changes in heart rate and heart rate variability indexes over the course of the 90-min recovery period in the burrow (including measures taken at 0 min, 20 min and 90 min post-handling, N=188 tests from 93 different birds, Fig. 3A-D show changes in raw, unstandardised indexes between 0, 20 and 90 minutes). For easier interpretation, Fig. 4 shows the degree of change in heart rate and heart rate variability indexes with the onset of stress (between unstressed state of ca. baseline values at 90 min post-handling compared to stressed state at 0 min post-handling). In other words, the sign and magnitude of the effect size for a particular index in Fig. 4 reflects the direction and degree of change due to handling stress. For example, the large positive effect size in heart rate reflects the strong increase in heart rate during stress, and the negative effect size in 'rMSSD' reflects the decrease in 'rMSSD' (PNS activity) during stress.

When birds were returned to their burrows after handling, 'rMSSD' (PNS activity) increased significantly over the course of 90 min (b=0.0135, *s. e.*=0.000752, T=18.0, P<0.001; Fig. 3A). 'SDNN'

(SNS+PNS activity) also increased significantly (b=0.00688, *s. e.*=0.000913, T=7.54, P<0.001; Fig. 3B). The 'SDNN:rMSSD' ratio (SNS:PNS balance) decreased significantly (b=-0.0112, *s. e.*=0.000884, T=12.643, P<0.001, Fig. 3D). Heart rate, which also can reflect SNS:PNS balance, also decreased strongly (b= -0.0176, *s. e.*=0.000626, T=-28.07, P<0.001, Fig. 3C).

3.4 PNS and SNS activity after handling stress and during confinement in a bag

Birds showed a marked increase in PNS activity during confinement in a bag for 90 minutes, as 'rMSSD' increased over the course of 0, 20 and 90 minutes post-handling (b=0.00516, *s. e.*=0.00138, T=3.732, P<0.001, bag sample sizes: N=60 at 0 min, N=59 at 20 min, N=36 at 90 min; all tests on a different individual; Fig. 3A). The SNS+PNS index 'SDNN' did not change over the course of that time (b=0.000429, *s. e.*=0.00120, T=0.358, P=0.721, Fig. 3B). The SNS:PNS index 'SDNN:rMSSD' ratio decreased over time (b=-0.00470, *s. e.*= 0.00121, T=-3.90, P<0.001, Fig. 3C). Heart rate (which also can reflect SNS:PNS balance) decreased substantially over time (b=-0.688, *s. e.*=0.113, T=-6.07, P<0.001, Fig. 3D).

3.5 Comparing PNS and SNS activity between bag vs. burrow

After handling, 'rMSSD' (PNS activity) was higher in birds returned to their burrows than in birds confined in cloth bags, but only after 90 min had passed (Table 3A, Fig. 3A; bag sample sizes: N=60 at 0 min, N=59 at 20 min, N=36 at 90 min, all tests on a different individual; burrow sample sizes: N=213 at 0 min [112 individuals], N=214 at 20 min [113 individuals], N=194 at 90 min [94 individuals]).

The SNS+PNS index 'SDNN' was higher in birds placed into bags than birds returned to their burrows during the time interval immediately (0 min) after handling, but this difference was no longer present 20 or 90 min after handling (Table 3B, Fig. 3B).

The SNS:PNS index 'SDNN:rMSSD' ratio was consistently higher in birds inside a bag than in those placed inside their burrows throughout the test (Table 3C, Fig. 3C,). Heart rate was also higher in birds inside a bag than those in the burrow 20 and 90 min post-handling, but no difference was detectable immediately after handling (Table 3D, Fig. 3D).

4. Discussion

In this study, handling birds induced a strong autonomic stress response, exhibited by a strong elevation in heart rate to almost 300 beats per minute (bpm) that gradually decreased as the birds recovered in their nest burrows, to stabilise at ca. 160 bpm (ca. 90 min, Figs. 2C, 3C, A.2C), similar to baseline values measured after a long period of rest in undisturbed conditions (Fig. 2C). Heart rate is controlled by the largely reciprocal actions of the two branches of the autonomic nervous system: the sympathetic nervous system (SNS), which activates the 'fight-or-flight' response including increased heart rate; and the parasympathetic nervous system (PNS), which activates 'rest and digest' processes including decreased heart rate (Porges, 1992; Jänig, 2006). As the SNS and PNS also create oscillations in heart rate at different frequencies, we were

able to quantify the relative activity of these two branches by calculating different indexes of heart rate variability, to assess the relative importance of the PNS and SNS in driving autonomic stress responses.

4.1 Changes in PNS activity during the autonomic stress response

Analyses of heart rate variability revealed that the principle autonomic action driving the autonomic stress response in Streaked Shearwaters is a strong drop in 'rest-and-digest' PNS activity: PNS activity was much lower just after the stress of handling ('rMSSD', Figs. 2A, 3A, A.2A) compared to baseline PNS activity. Furthermore, as birds recovered in their burrows, PNS activity dramatically increased, indicating a return of PNS drive toward the high level observed in unstressed conditions (Fig. 2A, 3A, A.2A). The size of the decrease in PNS activity during stress (effect size 'rMMSD' in Fig. 4), was similar to the size of the increase in heart rate during stress (effect size 'HR' in Fig. 4), indicating a dominating role of PNS withdrawal (rather than increasing SNS drive) in causing the strong increase in heart rate during an autonomic stress response.

This falls in line with a recent study on the same species showing that the stress of handling and injecting captive shearwaters caused a simultaneous strong increase in heart rate and drop in PNS-mediated heart rate variability (Carravieri et al., 2016). A principle role for the PNS in the autonomic stress response can also be inferred from the change in 'SDNN' during stress (a heart rate variability index that reflects combined SNS+PNS activity). If 'SDNN' reflected purely PNS activity, it would have decreased to the same extent as the 'rMSSD' did during stress; if it reflected more SNS activity than PNS activity, it would be expected to increase. The decrease in 'SDNN' (indicated by a negative effect size for 'SDNN' in Fig. 4), indicates a larger decrease in PNS activity than increase in SNS activity during stress.

4.2 Changes in SNS activity during the autonomic stress response

We also found evidence for a weaker and shorter-lived increase in 'fight-or-flight' SNS activity during stress. We observed this SNS response in the 'SDNN' values (SNS+PNS activity) immediately post-handling: 'SDNN' was higher in stressed birds restrained in bags than in birds recovering inside burrows (Fig. 3B). Because the 'SDNN' reflects combined SNS and PNS activity, acute stress both augments the 'SDNN' via increases in SNS drive and reduces the 'SDNN' via decreases in PNS drive. Thus, 'SDNN' values can only be higher during stress (relative to non-stressful situations) due to higher SNS activity. Moreover, in this case, PNS activity was the same for birds restrained in bags as it was for birds recovering inside burrows ('rMSSD', Fig. 3A) so any differences in 'SDNN' between groups should be due to differences in SNS activity. Indeed, stressed, bag-restrained birds, show a net higher rather than lower 'SDNN' (and also 'SDNN:rMSSD' ratio) than birds recovering inside the burrow, which indicates that the 'SDNN' immediately post-handling in bag-restrained birds reflects an increase in SNS activity.

This elevation in SNS activity appears to have disappeared after 90 minutes post-handling, because by this time birds inside burrows had significantly higher PNS activity than did bag-restrained birds ('rMSSD, Fig. 3A), yet 'SDNN' did not differ between groups. Therefore, the increase in PNS in the 'SDNN' at 90

minutes was likely obscured by a similar-sized decrease in SNS in the 'SDNN' (Fig. 3B).

Additional evidence for an increase in SNS activity just after handling comes from the fact that the decrease in the SNS+PNS index 'SDNN' during stress is smaller compared to the decrease in the PNS index 'rMSSD' (Fig. 4). This indicates that the large decrease in PNS contribution to the 'SDNN' during stress is partially offset by an increase in SNS activity, resulting in a smaller net decrease in 'SDNN'.

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4.3 Duration of the autonomic stress response

We found that 'rest-and-digest' PNS activity ('rMSSD'), which was initially very low just after handling, recovered (by increasing and stabilising at baseline values) after two hours in the nest burrow (Figs. 2A, 3A, A.2A). Both indexes reflecting SNS:PNS balance, however, recovered (by decreasing and stabilising at baseline values) more rapidly post-handling: the 'SDNN:rMSSD' ratio recovered after only one hour and heart rate recovered after 90 minutes (Figs. 2D, 2C, 3D, 3C, A.2D, A.2C). The fact that these SNS:PNS indexes recovered faster than the PNS indexes did, suggests concomitant decreases in 'fight-or-flight' SNS activity with the increase in PNS activity.

The autonomic stress response begins with a rapid decrease in PNS activity that occurs within one or two heartbeats (von Borell et al., 2007), followed by the slower SNS response that is initiated within up to 5 s and gradually increases to reach a maximum response after about 20-30 s (Hainsworth, 1995; Malliani, 1995; von Borell et al., 2007). Even the slower-acting sympatho-adrenal-medullary SNS response mediated by circulating catecholamine concentrations can be rapidly modulated within minutes (e.g. half-life of norepinephirine in rats is 1.5 min, Benedict et al., 1978; in humans ca. 2 min, Eliasson, 1984; and epinephrine in humans ca. 3 min Dimsdale and Moss, 1980). Thus, heart rate and heart rate variability can in principle be adjusted much more rapidly than the relatively long recovery period we observed in the birds inside their burrows.

This raises the question, why does the large, and fast-acting PNS response require so much time to fully recover? One possibility is that animals continue to experience psychogenic stress long after they have 'escaped' handling, which may maintain adaptive vigilance and immobility. However, this freezing-type defensive behavior actually tends to occur by increased, rather than decreased, PNS activity (via the more primitive dorsal vagus nerve rather than the ventral vagus nerve, Jänig, 2006; Porges, 2009; Porges et al., 2007). In fact very little is known about the mechanisms or function of the PNS component of the autonomic stress response. Until now, most attention in stress research, including in humans and laboratory animals, has focused on glucocorticoid responses and the SNS branch of the autonomic system. Although the PNS has been acknowledged to be important, as PNS activity is high in most vertebrates (reviewed in Taylor et al., 2014; Carravieri et al., 2016) and it is becoming increasingly evident that it plays an important role in mediating the autonomic stress response not only in humans (Porges et al., 1992) but also in other vertebrates (Carravieri et al., 2016), very little attention has been given to the role of the PNS in stress research.

4.4 The autonomic stress response exhibited during a capture-and-restraint stress protocol

The capture-and-restraint protocol is a widely-used standard method for comparing hormonal stress reactivity within and among individuals (Wingfield, 1982; Romero and Wingfield, 2016), and is especially well-suited for field research on free-living vertebrates as it requires only a single capture, a rapid blood sample (within three minutes) for measuring baseline glucocorticoids, and then confining the animal (usually in a bag) for easily obtained additional samples (e.g. at 20, 60 and 90 minutes post-capture) that reveal stress-induced glucocorticoid elevation and reflect the responsiveness of the animal's HPA-axis to what it most likely perceives as a predator attack. We performed this test on birds and recorded ECGs (rather than blood sampling), to understand the effect of bag confinement on the autonomic stress response and to disentangle the effect of bag confinement from that of handling.

Our study showed that birds confined in a cloth bag post-handling perceived bag confinement as stressful because 'rest-and-digest' PNS activity was lower (Fig. 3A) and SNS:PNS balance was higher than in birds recovering in their own burrows (Fig. 3C, 3D). But the birds appeared to perceive bag confinement as less stressful than handling, because PNS parameters increased post-handling over the course of 90 minutes in the bag (Fig. 3A), and SNS:PNS indexes also decreased during this time (Fig. 3C, 3D).

Our heart rate and heart rate variability analyses therefore revealed that confined birds already begin recovering from acute stress during a time when glucocorticoids would still be rapidly increasing (or at least be present at very high concentrations in the blood, e.g. Wingfield et al., 1982; Cockrem, 2007; Romero and Wingfield, 2016). An advantage of studying stress responses using heart rate and heart rate variability is that it provides a continuous record of the dynamics of the stress response that can reflect changes in the animal's perception of stress within seconds (see also Cyr, et al. 2009; Dickens and Romero, 2009). Another advantage of using ECG loggers is that it avoids the need for prolonged confinement or repeated handling for blood samples (though it requires recapturing the animal to retrieve the logger), which may make the response to the simulated stressor more ecologically realistic.

4.5 Comparison of heart rate in free-living birds with those in captivity

Resting heart rate of Streaked Shearwaters, measured after 3 hours of recovery in the burrow following ECG logger attachment, was 162 bpm (N=12, 95% CIs of 145 and 178 bpm), which is similar to the baseline value of 167 bpm (N=8, 95% CIs of 120 and 216) measured from birds that had been incubating eggs inside their burrows for 18 hours, and higher than the resting heart rate of 134 bpm (N=5, upper and lower 95% CIs of 131 and 138, respectively) measured in captive Streaked Shearwaters reported in Carravieri et al. (2016). This is surprising because Carravieri et al. (2016) measured heart rate from ECGs recorded only 30 minutes after handling, a time at which the animals in our study would have had a heart rate between 248 and 196 bpm (heart rate averages for 20 min and 60 min post-handling, respectively, Fig. A.2C). Though the general findings in Carravieri et al. (2016) were broadly congruent to those reported in our study (in that PNS drive dominated heart rate and heart rate variability during rest and during acute stress), some notable differences were evident, such as the substantially lower heart rate and the lack of SNS activity in the

SNS+PNS index 'SDNN' in the captive birds in the study of Carravieri et al. (2016). These differences suggest that sympathetic fatigue occurs during prolonged capture, which underscores the importance of performing more studies of autonomic stress responses on wild animals in their natural environment. Furthermore, the higher baseline heart rate in our study compared to that of the captive shearwaters in Carravieri et al. (2016) suggests the presence of a considerable degree of SNS activity in the free-living birds.

4.6 Conclusion

We have shown for the first time in a free-living wild animal that a short period of handling is enough to induce a strong autonomic response that is (1) driven mainly by a strong decrease in 'rest-and-digest' PNS activity that requires up to 2 hours to recover to that of a resting state, and (2) also a smaller, more short-lived increase in 'fight-or-flight' SNS activity. We also found that confinement in a cloth bag, a standard protocol in stress research, is less stressful than handling but more stressful than when birds are returned to their nest burrows. Though the PNS clearly dominates autonomic activity in shearwaters, the higher baseline heart rate measured in free-living birds in this study, compared to that of chronically stressed newly captured birds (Carravieri et al., 2016) points to the presence of substantial SNS activity in shearwaters during resting conditions.

Our study demonstrates that quantifying autonomic activity from heart rate and heart rate variability is an effective non-invasive approach for studies of stress physiology in wild animals, applicable to behavioural and physiological research and for studies of animal welfare and conservation. Comparing glucocorticoid-mediated stress responses within and between individuals, populations and species has become a very productive field of inquiry in behavioural ecology. We encourage future studies to consider also autonomic stress response, in particular investigating the role of the important and thus far little-studied parasympathetic nervous system.

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Figure legends

Figure 1. Heart rate variability at different levels of analysis. A. ECG wave of an adult Streaked Shearwater. The timing of each R peak (in the PQRS complex of a single heart beat) used for calculating heart rate and heart rate variability indexes. B. Oscillations in heart rate in beats per minute (bpm) over time (tachogram). C. Color-coded power (i.e. amplitude squared) of oscillations in heart rate over frequencies and time (spectrogram), with lighter tones indicating larger amplitude (i.e. stronger oscillations). The two light-coloured bands indicate the presence of a distinct higher and lower frequency oscillation driven by PNS or combined SNS+PNS activity (frequencies indicated on x-axis in both 'Hz' and in 'seconds between oscillations').

Figure 2. Baseline heart rate and heart rate variability indexes reflecting PNS and SNS activity over the course of 24 hours of birds incubating eggs in their nest burrows. Means +/-95% CIs of heart rate and log-transformed heart rate variability indexes immediately after handling (0 hrs) and after 1, 2, 3, 6, 12, 18 and 24 hours inside burrow (*N*=8). Red point at 18 hours indicates baseline reference value used for comparing with stress responses in Fig. 3. Black points indicate values compared with red point at 18 hrs in Table 2. A. log 'rMSSD' values reflect parasympathetic (PNS) activity. B. log 'SDNN' values reflect combined sympathetic (SNS) and PNS activity. C. Heart rate reflects the balance between SNS and PNS activity. D. log 'SDNN:rMSSD' also reflects the balance between SNS and PNS activity.

Figure 3. Comparison of PNS and SNS activity between birds returned to nest burrows after handling and birds confined in cloth bags. Means +/-95% CIs of raw (uncorrected) heart rate and log-transformed heart rate variability indexes calculated from ECGs recorded from birds placed into an opaque cloth back for 90 minutes after handling and birds placed into their burrows. Bag: *N*=60 at 0 min, 59 at 20 min, 36 at 90 min. Burrow: *N*=213 at 0 min, 214 at 20 min, 194 at 90 min. * indicates significant differences between bag and burrow treatment from models including random effect of individual ID. Red lines indicate baseline values (from birds incubating inside nest burrow for 18 hours, Fig. 2). A. log 'rMSSD' values reflect parasympathetic (PNS) activity. B. log 'SDNN' values reflect combined sympathetic (SNS) and PNS activity. C. Heart rate reflects the balance between SNS and PNS activity. D. log 'SDNN:rMSSD' also reflects the balance between SNS and PNS activity.

Figure 4. Comparisons of effect sizes reflecting change in various heart rate and heart rate variability indexes during stress. Effect sizes are slopes (*b*) +/-95% CIs from mixed models of log-transformed standardised data, containing individual ID as a random factor. For easier interpretation, this figure shows the

degree of change in heart rate and heart rate variability indexes with the onset of stress (between unstressed state quasi-baseline values at 90 min post-handling compared to stressed state at 0 min post-handling). It shows that during stress, heart rate ('HR') is higher and the PNS index 'rMSSD' and the SNS+PNS index 'SDNN' are lower than when birds are in a resting state. Arrows indicate contributions of the PNS and SNS (and their directions of change during stress) to various indexes. For example, the decrease in 'rMSSD' during stress is entirely due to a decrease in PNS, indicated by the dark grey arrow. The decrease in 'SDNN' is due to the same size decrease in PNS but mitigated by a increase in SNS. The difference in decrease in 'rMMSD' and 'SDNN' reveals the proportional contribution of an SNS increase to the stress response (as visually approximated by the light grey arrow). We then also show these (estimated) proportional PNS and SNS contributions next to the heart rate increase, showing that the HR increase is due to both strongly decreasing PNS activity and more mildly increasing SNS activity. *N*=188 from 93 different birds for all tests. Non-overlapping CIs indicate significant differences between effect sizes, CIs not overlapping with 0 indicate effect sizes differ from 0. Units for heart rate are bpm.

Table 1. Differences in baseline heart rate and heart rate variability indexes of incubating adults (*N*=8 birds) after 18 hours of resting inside nest burrow compared to those calculated at (A) 0 hrs post-handling, (B) 1 hr post-handling and (C) 24 hrs post-handling. See Fig. 2.

ANS branch(es)	Parameter	*b*	*s. e.*	*T*	*P*
A. 0 hrs					
PNS	'rMSSD'	1.673	0.284	5.900	<0.001
SNS+PNS	'SDNN'	0.816	0.175	4.667	0.00230
SNS:PNS balance	'SDNN:rMSSD'	-0.857	0.265	-3.237	0.00597
	Heart rate	-148.367	25.694	-5.774	<0.001
B. 1 hr					
PNS	'rMSSD'	0.140	0.229	0.613	0.559
SNS+PNS	'SDNN'	0.118	0.193	0.613	0.559
SNS:PNS balance	'SDNN:rMSSD'	-0.0220	0.238	-0.092	0.929
	Heart rate	-1.421	16.455	-0.086	0.934
C. 24 hrs					
PNS	'rMSSD'	-0.0752	0.207	-0.364	0.727
SNS+PNS	'SDNN'	-0.197	0.194	-1.011	0.345
SNS:PNS balance	'SDNN:rMSSD'	-0.121	0.163	-0.746	0.468
	Heart rate	-18.154	13.780	-1.317	0.229

Table 2. Changes in heart rate and heart rate variability indexes at different time intervals within a 3-hour period of recovery inside the nest burrow after the acute stress of handling (A – C). *N*=24 for 0, 20, 60, 90 min, 23 for 120 min, 12 for 180 min. Comparison of stabilised index values with baseline index values (D, after 18 hrs in burrow, *N*=8). See Fig. A.2.

ANS branch(es)	Parameter	*b*	*s. e.*	*T*	*P*
A. Changes over entire 180 min in burrow post-handling					
PNS	'rMSSD'	0.0065	7.43E-04	8.75	<0.001
SNS+PNS	'SDNN'	0.0041	6.11E-05	6.71	< 0.001
SNS:PNS balance	'SDNN:rMSSD'	-0.00241	5.57E-04	-4.338	< 0.001
	Heart rate	-0.759	0.0478	-15.87	< 0.001
B. Changes between 120 and 180 min in burrow post-handling					
PNS	'rMSSD'	-1.57E-04	0.00118	-0.133	0.897
SNS+PNS	'SDNN'	0.00109	0.00108	1.005	0.335
SNS:PNS balance	'SDNN:rMSSD'	6.15E-04	0.00123	0.502	0.622
	Heart rate	-0.134	0.0707	-1.89	0.0831
C. Changes between 90 min and 120 min in burrow post-handling					
PNS	'rMSSD'	0.00616	0.00170	3.62	< 0.001
SNS+PNS	'SDNN'	0.00701	0.00174	4.03	< 0.001
SNS:PNS balance	'SDNN:rMSSD'	3.02E-04	2.19E-03	0.138	0.892
	Heart rate	-0.200	0.111	-1.80	0.085
D. Stabilised HR and HRV indexes compared with baseline (18 hrs in burrow)					
120 min	'rMSSD'	0.236	0.237	0.998	0.325
120 min	'SDNN'	0.187	0.170	1.097	0.280
60 min	'SDNN:rMSSD'	-0.026	0.163	-0.160	0.874
90 min	Heart rate	17.010	18.450	0.922	0.364

Table 3. Differences in autonomic activity between birds placed inside their nest burrow after handling vs. those placed inside a cloth bag for 90 min. Positive *b* indicates higher values for burrow than bag treatment. Bag: *N*=60 at 0 min, 59 at 20 min, 36 at 90 min. Burrow: *N*=213 at 0 min, 214 at 20 min, 194 at 90 min. See Fig. 3.

Time	*b*	*s. e.*	*T*	*P*
A. PNS – 'rMSSD'				
0 min	-0.00492	0.0915	-0.0540	0.957
20 min	0.173	0.0914	1.90	0.0593
90 min	0.353	0.108	3.28	0.00128
B. SNS + PNS – 'SDNN'				
0 min	-0.161	0.0664	-2.42	0.0163
20 min	-0.140	0.0749	-1.87	0.0630
90 min	0.0540	0.0926	0.583	0.561
C. SNS:PNS balance - 'SDNN:rMSSD'				
0 min	-0.161	0.0792	-2.04	0.0427
20 min	-0.320	0.0714	-4.48	< 0.001
90 min	-0.313	0.0673	-4.64	< 0.001
D. SNS:PNS balance – Heart rate				
0 min	9.40	7.0020	1.34	0.181
20 min	-27.9	7.49	-3.73	< 0.001
90 min	-32.5	6.64	-4.89	< 0.001

Appendices

Fig. A.1. Neurologger 2A for ECG (0.625 ms sampling interval, voltage range +/- 3 mV, 20g weight, 1 GB memory and Li-Po battery of 3.7 V and 240 mAh, Evolocus LLC) and 100 JPY coin (diameter of 22.6mm). Three wires extending from the ECG logger soldered to small safety pins that are attached subcutaneously to the skin under the feathers, and function as electrodes. Wires are wrapped around one side of the bird and the attached logger was firmly taped to the dorsal feathers.

Fig. A.2. Mean (+/- 95% CIs) of HRV indexes and HR in adult Streaked Shearwaters over the course of 2-3 hours as they recover in their nest burrow after handling. A. 'rMSSD' reflects PNS activity. B. 'SDNN' reflects combined SNS and PNS activity. C. 'SDNN:rMSSD' reflects the balance between SNS and PNS activity. D. HR reflects the balance between the reciprocal actions of the SNS and PNS. N = 24 for 0, 20, 60, 90 min, 23 for 120 min, 12 for 180 min.

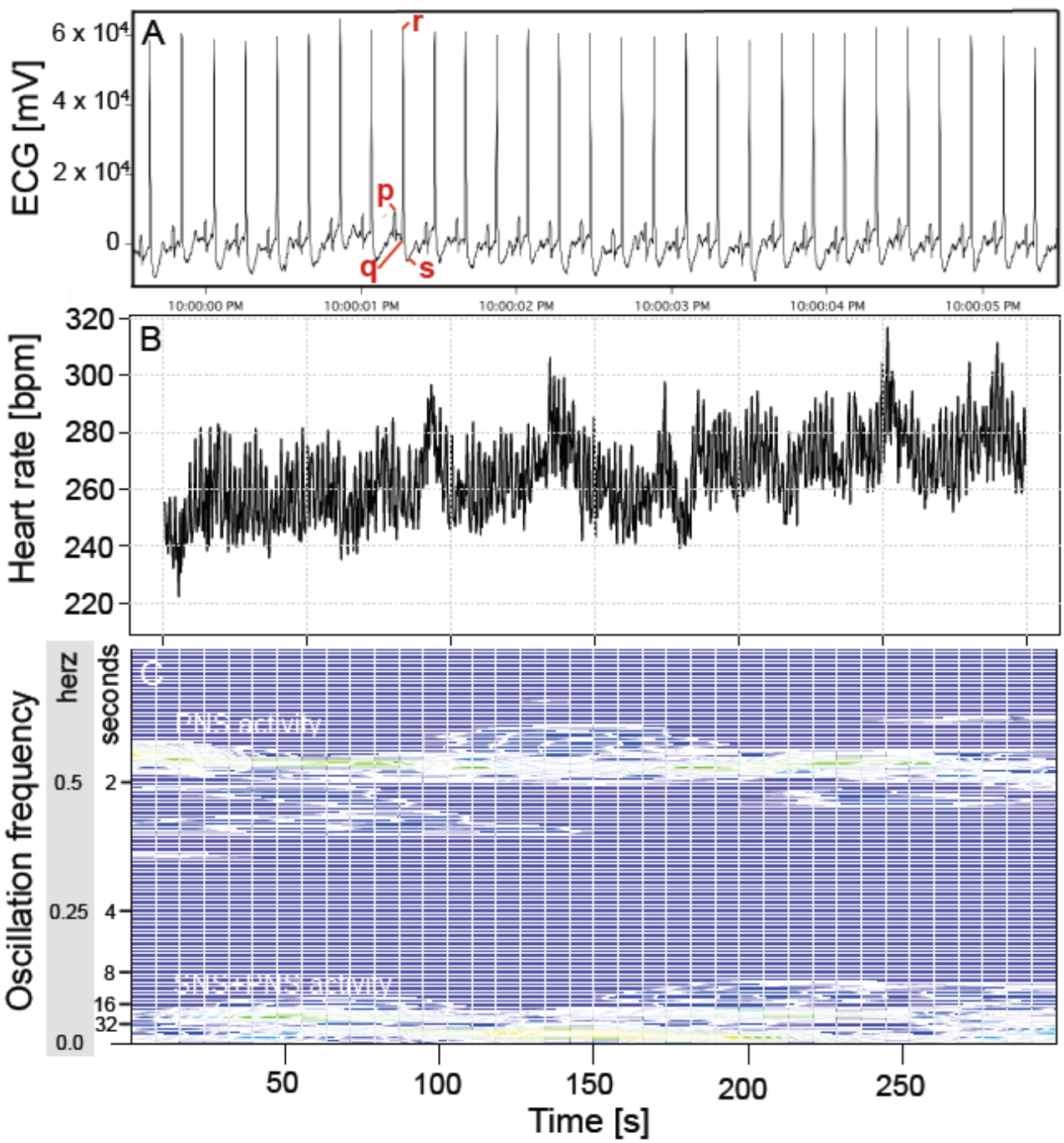
A
ECG [mV]
6 x 10⁴
4 x 10⁴
2 x 10⁴
0
r
p
q
s
10:00:00 PM
10:00:01 PM
10:00:02 PM
10:00:03 PM
10:00:04 PM
10:00:05 PM
B
Heart rate [bpm]
320
300
280
260
240
220
C
Oscillation frequency
herz
seconds
0.5
2
0.25
4
8
16
32
0.0
PNS activity
SNS+PNS activity
50
100
150
200
250
Time [s]

Figure 1

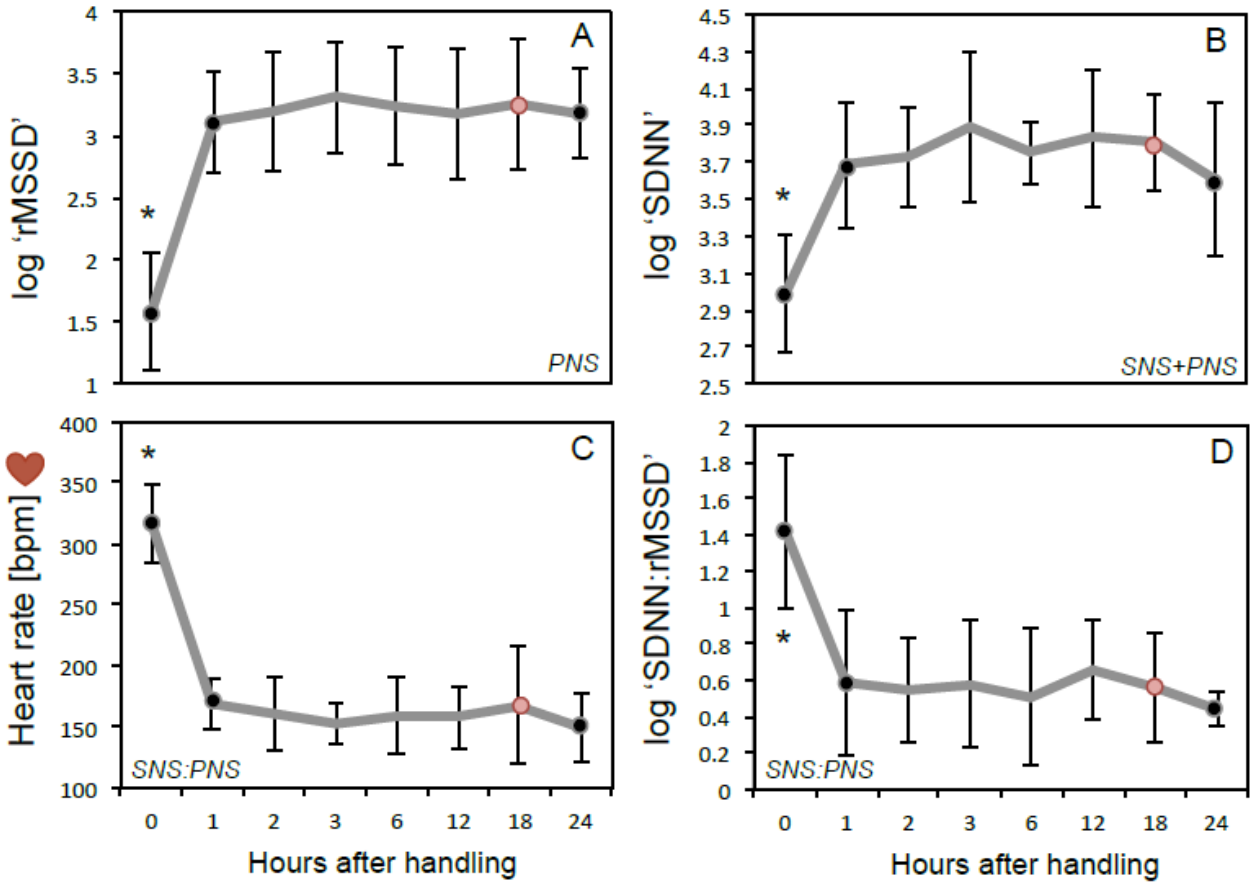

Figure 2

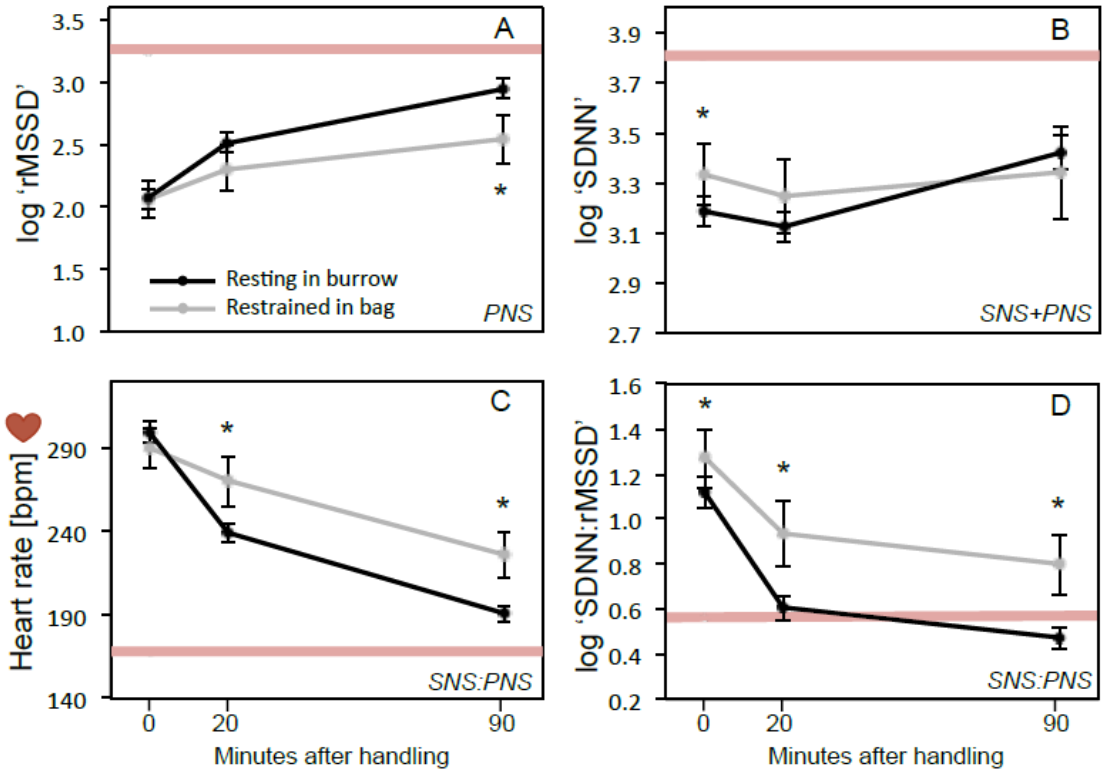

Figure 3

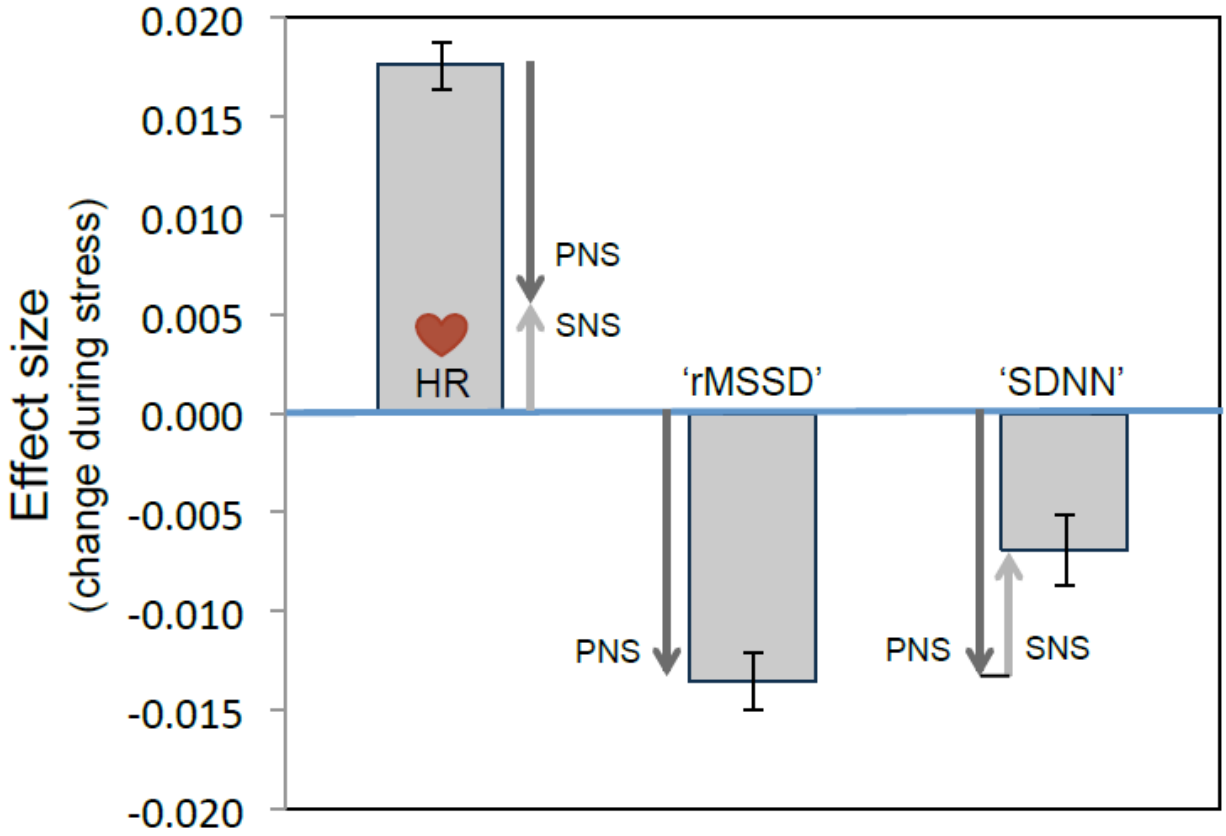

Figure 4

Figure A.1

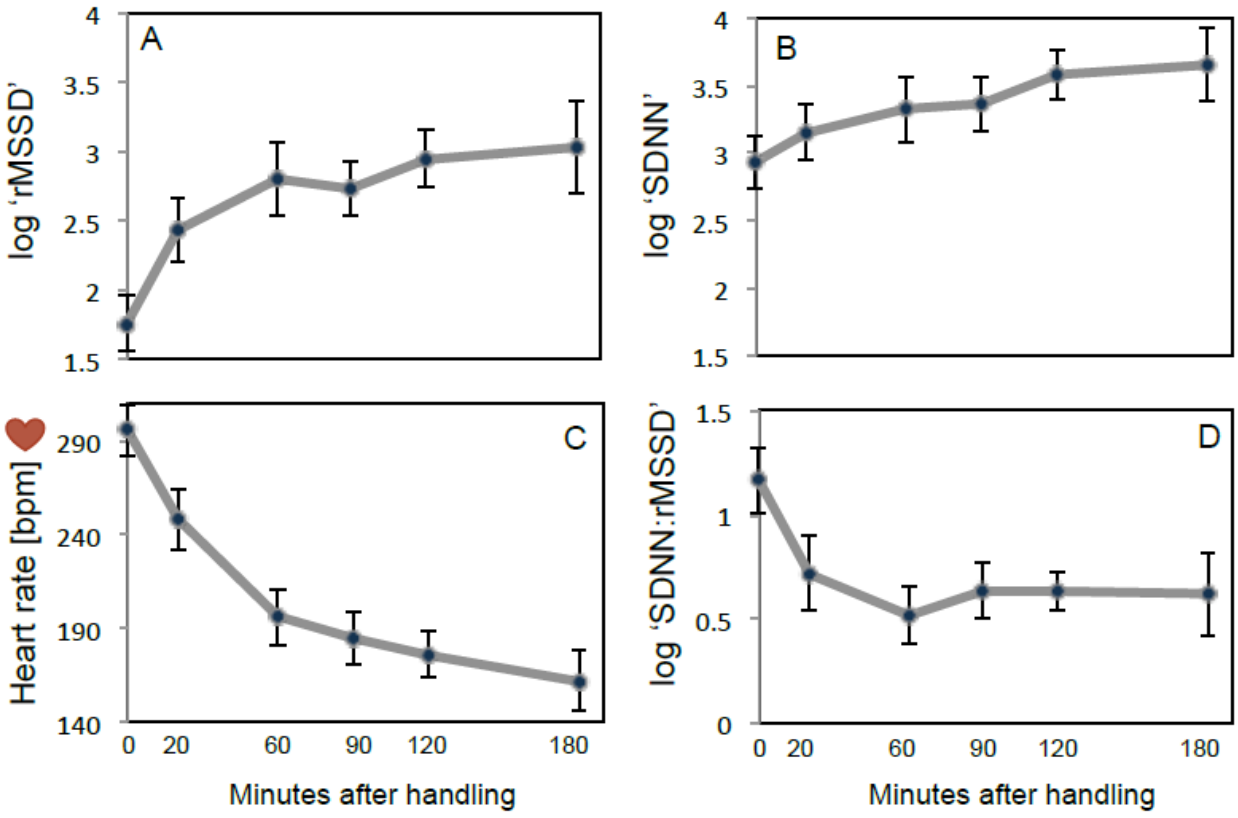

Figure A.2