Supplementary Material

Frequency domain analyses

Beat positions were used to produce a tachogram which was then interpolated to create an identical wave made of equally-spaced data points which was analysed by fast Fourier transform (over 1024 consecutive data points, i.e. 102.4 sec, with a step of 5.12 sec) that produced a power spectrum separating oscillations in the tachogram wave occurring at different frequencies. From the power spectrum, we quantified the power (or amplitude squared) of the different oscillations in heart rate occurring within the 5-minute interval of heartbeats being analysed. High-frequency (HF) oscillations (between 0.3 and 2 Hz in this species, or every 0.5 - 3.3 s) reflect variability in HR that is modulated by the PNS. The power of HF oscillations in HR is therefore an index of PNS activity. Low-frequency (LF) oscillations (0.04 - 0.3 Hz in this species, or every 3.3 - 25 s) are modulated by both the SNS and PNS, so LF power can be used as an SNS+PNS index (von Borell et al., 2007; Malik et al., 1996), and the ratio between LF:HF power therefore an index of SNS:PNS ratio (Pagani, 1984, 1986; von Borell et al., 2007).

Results

Baseline PNS and SNS activity in incubating birds inside their burrows

All indexes (except for LF:HF ratio, which never changed) calculated just after handling differed from those calculated at 18 hours. 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 S1, Fig. S1).

PNS and SNS activity after handling stress and recovery in burrow

When birds were returned to their burrows after handling, the PNS index HF power increased significantly over the course of 90 min (b=0.0120, *s. e.*=0.000779, T=15.4, P<0.001; Fig. S2A). The SNS+PNS index LF power also increased significantly, (b=0.0126, *s. e.*=0.000801, T=15.7, P<0.001; Fig. S2B). The SNS:PNS index LF:HF did not change (b=-0.00107, *s. e.*=0.000900, T=-1.189, P=0.235, Fig. S2C).

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 HF power increased over the course of 0, 20 and 90 minutes post-handling (b=0.00916, *s. e.*=0.0269, T=3.40, P<0.001, Fig. S2A). The SNS+PNS index LF power increased over time (b=0.0112, *s. e.*=0.00246, T=4.57, P<0.001, Fig. S2B). The SNS:PNS index LF:HF did not change over time (b=0.00123, *s. e.*=0.00202, T=0.611, P=0.543, Fig. S2C).

Comparing PNS and SNS activity between bag vs. burrow

PNS activity was higher in birds returned to their burrows than in birds confined in cloth bags, but only after 90 min had passed post-handling (Table S2A, Fig. S2A). The SNS+PNS index LF power did not differ between groups, either immediately or 90 min post-handling (Table S2B, Fig. S2B). The SNS:PNS index LF:HF ratio was higher in birds inside a bag for 90 minutes than in those placed inside their burrows, (Table S2C, Fig. S2C).

Discussion

LF power appeared to reflect purely PNS activity in most cases, which we inferred from the following patterns. First, the decrease in HF power (a PNS index) during handling stress (Fig. S3) is very similar to the decrease in LF power (an SNS+PNS index) reflected in a similar effect size, or degree of change for both 'HF' and 'LF' in Fig. S3. As HF power reflects purely PNS drive, the similar-sized decrease in LF power must entirely be due to PNS drive, with little contribution from the SNS (which would increase during stress, not decrease). This deduction is also supported by the fact that during stress, the LF:HF ratio does not significantly change (the change in LF:HF ratio due to stress, as indicated by the value of the effect size, does not differ from 0, Fig. S3), which indicates that LF and HF power decrease at the same rate via a strong, dominating decrease in PNS drive. The only context in which we found meaningful patterns in LF:HF ratio was in birds placed inside a bag for 90 minutes after handling, in which LF:HF ratio was elevated at 20 min and 90 min post-handling compared to birds recovering in their burrows, suggesting higher SNS activity confined in a bag (Table S2C, Fig. S2C).

Why did we find a signal from the SNS in the SDNN but not in LF power, if both are supposed to reflect SNS+PNS activity? The degree to which LF power reflects SNS activity, and therefore its utility as an SNS+PNS index, has generated some controversy in the medical literature with several studies suggesting that in humans at rest, the LF band primarily reflects PNS-mediated transmissions between the heart and the brain to regulate blood pressure and so should actually not be used as an index of SNS activity (discussed in Shaffer et al., 2014). Indeed, in line with our findings, a recent autonomic blocker experiment on the same shearwater species found little contribution of the SNS to LF power, with a small SNS contribution detectable only in the LF:HF ratio (Carravieri et al., 2016). Experimental evidence suggests that in resting humans, the SNS actually modulates the amplitude of oscillations at a lower frequency occurring between ca. 0.0033 - 0.04 Hz, or every 25 to 300 s (compared to the 0.04 - 0.15 Hz LF band, or every 6.6 to 25 s in humans, Shaffer et al., 2014). These "very low frequency" or VLF oscillations would require longer ECG recordings of a resting animal than the five-minute intervals used in our analyses, to accurately quantify their spectral power. Shaffer et al. (2014) point out that the frequency of these SNS-modulated VLF oscillations can vary according to several factors including physical activity (Bernardi et al., 1996) or stress, and can actually cross over into the lower region of the LF band during locomotion or during substantial stress, so identifying the correct frequency range may require visually inspecting power spectral density plots to find the location of the peak for each individual. Although VLF power might be a good indicator of SNS activity in resting animals, it would require longer ECG recordings, and therefore may not be practical for tests of free-living animals. The SDNN index, however, correlates well with VLF power over a 24-h period in humans (Shaffer et al., 2014), and considering that we found evidence that the SDNN does indeed reflect SNS activity, perhaps time domain indexes are more suitable than frequency domain indexes for quantifying SNS activity in free-living animals.

References

Bernardi, L., Valle, F., Coco, M., Calciati, A., Sleight, P., 1996. Physical activity influences heart rate variability and very-low-frequency components in Holter electrocardiagrams. Cardiovasc. Res. 32, 234-237.

Carravieri, A., Müller, M.S., Yoda, K., Hayama, S., Yamamoto, M., 2016. Dominant parasympathetic modulation of heart rate and heart rate variability in a wild-caught seabird. Physiol. Biochem. Zool. 89, 263-276.

Malik, M., Bigger, J.T., Camm, A.J., Kleiger, R.E., Malliani, A., Moss, A.J., Schwartz, P.J., 1996. Heart rate variability: standards of measurement, physiological interpretation, and clinical use. Eur. Heart J. 17, 354–381.

Pagani M., Lombardi, F., Guzzetti, S., Rimoldi, O., Furlan, R., Pizzinelli, P., Sandrone, G., et al., 1986. Power spectral analysis of heart rate and arterial pressure variabilities as a marker of sympatho-vagal interaction in man and conscious dog. Circ. Res. 59,178–193.

Pagani, M., Lombardi, F., Guzzetti, S., Sandrone, G., Rimoldi, O., Malfatto, G., Cerutti, S., Malliani, A., 1984. Power spectral density of heart rate variability as an index of sympatho-vagal interaction in normal and hypertensive subjects. J. Hypertens. 2, 383–385.

Shaffer, F., McCraty, R., Zerr, C.L., 2014. A healthy heart is not a metronome: an integrative review of the heart's anatomy and heart rate variability. Front. Psychol. 5, 1–19.

von Borell, E., Langbein, J., Depres, G., Hansen, S., Leterrier, C., Marchant-Forde, J., Marchant-Forde, R., Minero, M., Mohr, E., Prunier, A., Valance, D., Veissier, I., 2007. Heart rate variability as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals – a review. Physiol. Behav. 92, 293-316.

Table S1. Differences in baseline heart rate variability indexes from power spectrum analysis 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. S2. HF power refers to the power (i.e. amplitude) of high frequency oscillations in heart rate driven by parasympathetic (PNS) activity. LF power refers to power of low frequency oscillations driven by combined activity of PNS and sympathetic (SNS) branches. LF:HF refers to the ratio between LF and HF power and reflects the balance between SNS and PNS activity.

ANS branch(es)	Parameter	*b*	*s. e.*	*T*	*P*
A. 0 hrs					
PNS	HF power	2.544	0.571	4.458	**0.00294**
SNS+PNS	LF power	2.595	0.401	6.475	**0.0000146**
SNS:PNS balance	LF:HF	0.0505	0.525	0.0960	0.926
B. 1 hr					
PNS	HF power	0.0251	0.458	0.0550	0.958
SNS+PNS	LF power	0.316	0.293	1.079	0.316
SNS:PNS balance	LF:HF	0.291	0.429	0.678	0.509
C. 24 hr					
PNS	HF power	-0.125	0.323	-0.388	0.710
SNS+PNS	LF power	-0.340	0.353	-0.963	0.368
SNS:PNS balance	LF:HF	-0.215	0.256	-0.839	0.429

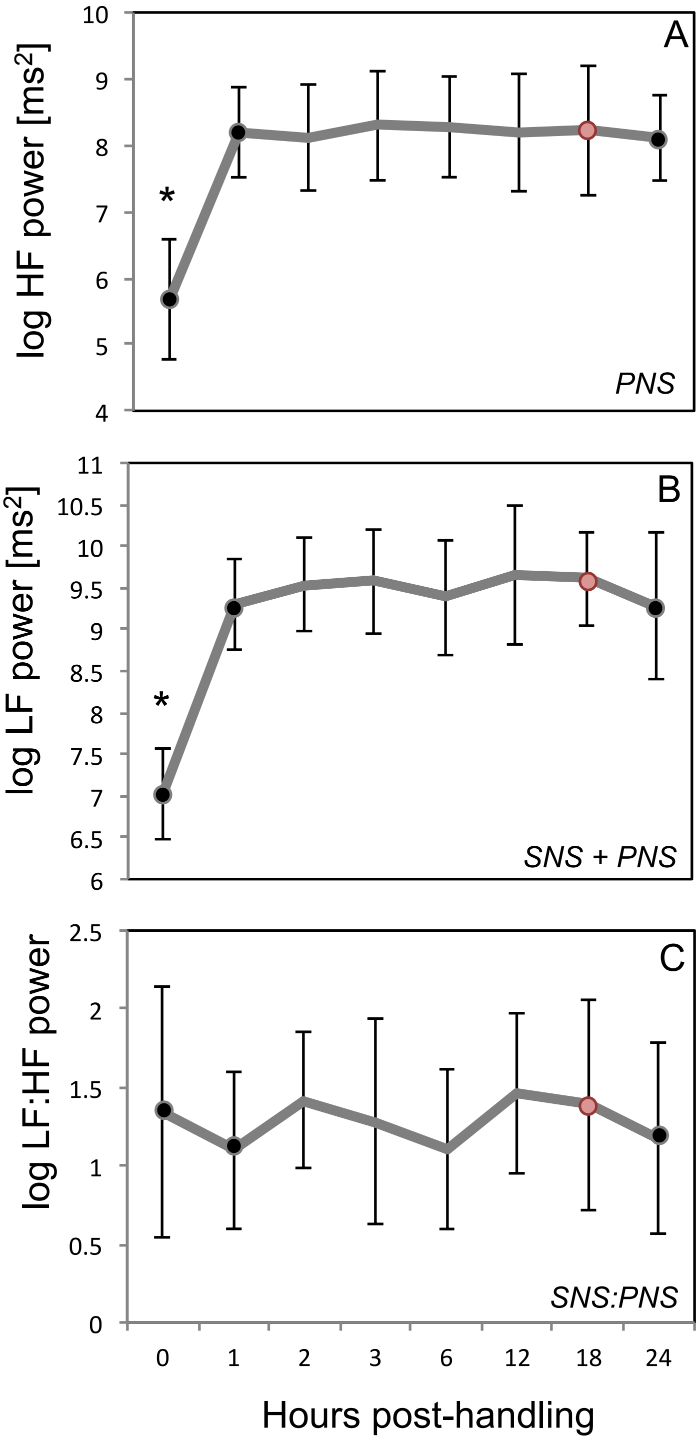
Table S2. Differences in autonomic activity between birds placed inside their nest burrow after handling vs. those placed inside a cloth bag for 90 min. Positive *b* indicates higher values for burrow than bag treatment. Bag: *N*=60 at 0 min, 59 at 20 min, 36 at 90 min. Burrow: *N*=213 at 0 min, 214 at 20 min, 194 at 90 min. See Fig. S2. HF power refers to the power (i.e. amplitude) of high frequency oscillations in heart rate driven by parasympathetic (PNS) activity. LF power refers to power of low frequency oscillations driven by combined activity of PNS and sympathetic (SNS) branches. LF:HF refers to the ratio between LF and HF power and reflects the balance between SNS and PNS activity.

Time	b	$s. e.$	T	P
A. PNS (HF power)				
0 min	0.0443	0.194	0.229	0.819
20 min	0.274	0.172	1.60	0.111
90 min	0.533	0.191	2.79	0.00589
B. SNS + PNS (LF power)				
0 min	0.00110	0.164	0.0070	0.995
20 min	-0.0720	0.144	-0.500	0.618
90 min	0.192	0.180	1.07	0.287
C. SNS:PNS balance (LF:HF ratio)				
0 min	-0.0482	0.136	-0.353	0.724
20 min	-0.357	0.127	-2.81	0.00548
90 min	-0.370	0.149	-2.48	0.0143

Figure S1. Baseline heart rate variability indexes from power spectrum analysis 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. S2. Black points indicate values compared with red point at 18 hrs in Table S2. A. log HF power values reflect parasympathetic (PNS) activity. B. log LF power values reflect combined sympathetic (SNS) and PNS activity. C. log LF:HF power also reflects the balance between SNS and PNS activity.

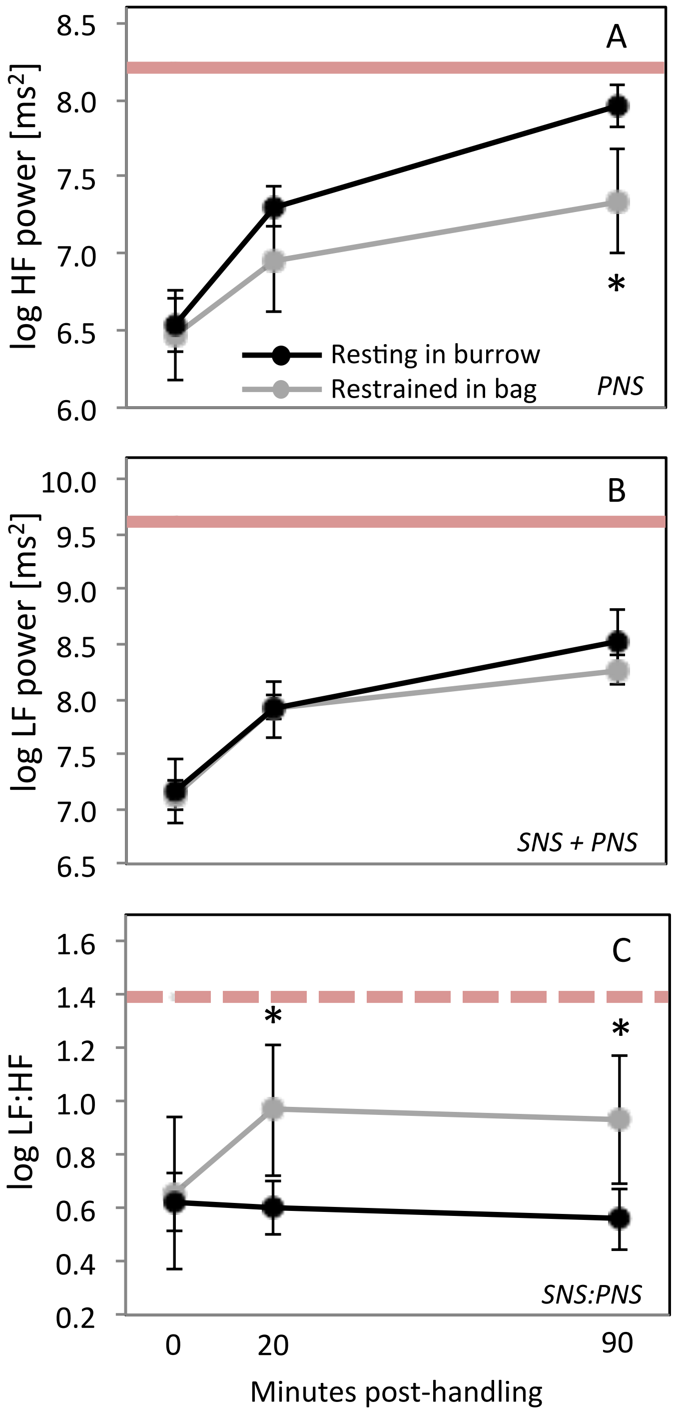
Figure S2. Comparison of PNS and SNS activity between birds returned to nest burrows after handling and birds confined in cloth bags. Means +/-95% CIs of raw (uncorrected) log-transformed heart rate variability (power spectrum analysis) indexes calculated from ECGs recorded from birds placed into an 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. S1). A. log HF power values reflect parasympathetic (PNS) activity. B. log LF power values reflect combined sympathetic (SNS) and PNS activity. C. log LF:HF also reflects the balance between SNS and PNS activity.

Figure S3. 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. Open symbols indicate indexes reflecting the balance between sympathetic (SNS) and parasympathetic (PNS) activity. Black symbols indicate indexes reflecting PNS activity. Grey symbols indicate indexes reflecting combined SNS+PNS 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. Units for HF and LF power are ms^2.



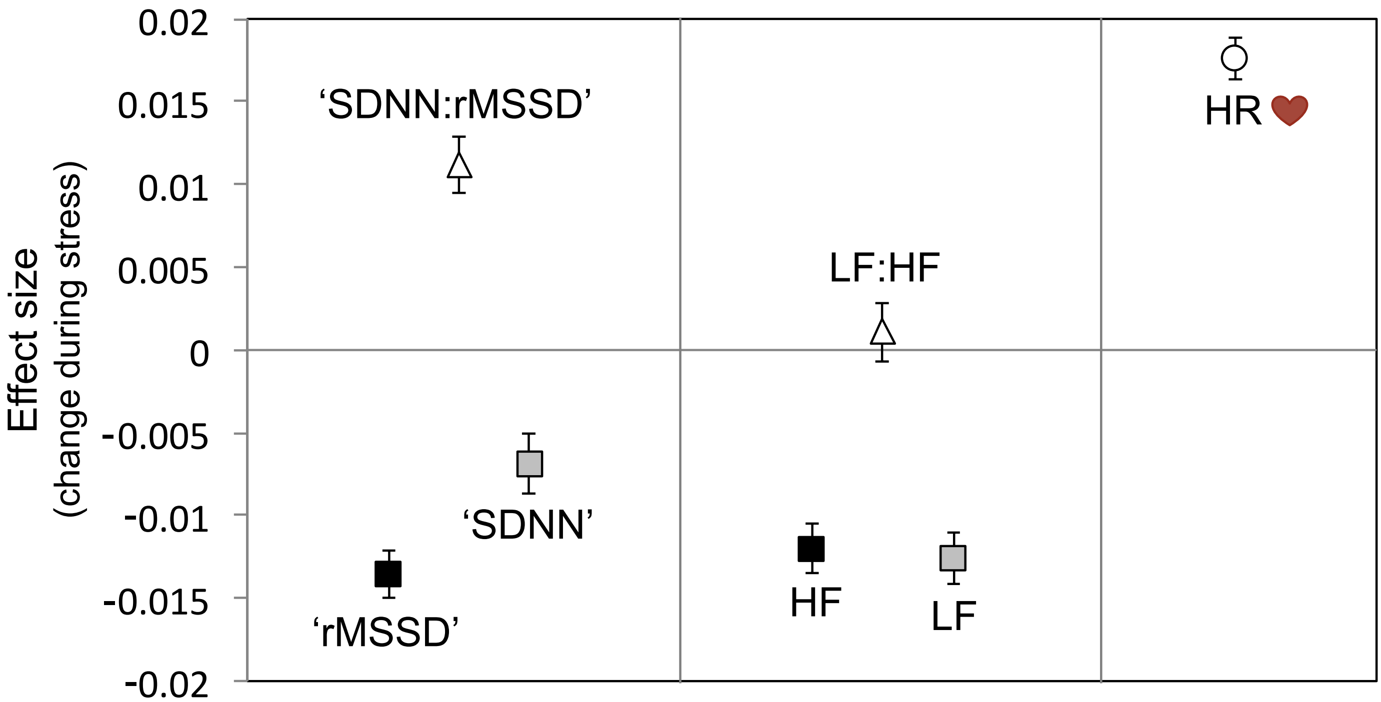
A
PNS
log HF power [ms2]
10
9
8
7
6
5
4
*
B
SNS + PNS
log LF power [ms2]
11
10.5
10
9.5
9
8.5
8
7.5
7
6.5
6
*
C
SNS:PNS
log LF:HF power
2.5
2
1.5
1
0.5
0
0
1
2
3
6
12
18
24
Hours post-handling

Fig. S1



A
B
C
log HF power [ms²]
log LF power [ms²]
log LF:HF
8.5
8.0
7.5
7.0
6.5
6.0
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
*
Resting in burrow
Restrained in bag
PNS
SNS + PNS
SNS:PNS
0
20
90
Minutes post-handling

Fig. S2



Effect size
(change during stress)
0.02
0.015
0.01
0.005
0
-0.005
-0.01
-0.015
-0.02
‘SDNN:rMSSD’
‘SDNN’
‘rMSSD’
LF:HF
HF
LF
HR

Fig. S3