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# Individual differences in heart rate reveal a broad range of autonomic phenotypes in a free-living seabird population

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## ABSTRACT

Animals in the same population consistently differ in their physiology and behaviour, but the underlying mechanisms remain poorly understood. As the autonomic nervous system regulates wide-ranging physiological functions, many of these phenotypic differences may be generated by autonomic activity. We investigated for the first time in a free-living animal population (the long-lived seabird Streaked Shearwater, *Calonectris leucomelas*), whether individuals consistently differ in autonomic activity, over time and across contexts. We repeatedly recorded electrocardiograms from individual shearwaters, and from heart rate and heart rate variability quantified sympathetic activity, which drives the 'fight-or-flight' response, and parasympathetic activity, which promotes 'rest-and-digest' processes. We found a broad range of autonomic phenotypes that persisted even across years: heart rate consistently differed among individuals during stress and non-stress and these differences were driven by parasympathetic activity, thus identifying the parasympathetic 'rest-and-digest' system as a central mechanism that can drive broad phenotypic variation in natural animal populations.

**KEY WORDS: stress, heart rate, autonomic nervous system, individual differences, seabird, fight-or-flight**

## INTRODUCTION

Animals in the same population consistently differ from each other in their physiological and behavioural responses to the environment (Carere and Maestripieri, 2013), but the proximate causes of these differences, particularly the underlying neural mechanisms, remain poorly understood (Snell-Rood 2013; Duckworth, 2015). The autonomic nervous system regulates wide-ranging physiological functions such as heartbeat, blood pressure, gastrointestinal activity, immunity, metabolism, and reproductive functions to support the physical demands of behaviour (e.g. locomotion, eating, sex) or other internal changes (e.g. hemorrhage, infection), while maintaining homeostasis (Jänig, 2006; Kuenzel, 2015). Much of the phenotypic variation observed in wild populations may therefore be generated by individual differences in autonomic activity, but this has not yet been studied. Moreover, autonomic activity, due to its direct synaptic connections to the central nervous system, provides a window into an animal's brain while it activates behavioural responses (Thayer et al., 2012; Beissner et al., 2013) and can give new insight into the stability vs. plasticity of the neural processes underlying physiology and behaviour.

The autonomic nervous system is comprised of two independently regulated neural branches extending from the brain to the body where they have largely opposing effects. The sympathetic branch drives the 'fight-or-flight' response (including an increase in heart rate), which helps prepare an animal for danger, and the parasympathetic branch promotes 'rest-and-digest' processes and self-maintenance (including a decrease in heart rate, Jänig, 2006; Kuenzel, 2015). Heart rate therefore reflects the balance between sympathetic and parasympathetic activity. In addition, the sympathetic and parasympathetic branches generate oscillations in heart rate at different frequencies, so heart rate variability can be analysed to separately measure the activity of each of the two autonomic branches (Carravieri et al., 2015; Müller et al., 2017).

We investigated for the first time, in a free-living animal population (the long-lived pelagic seabird Streaked Shearwater, *Calonectris leucomelas*), whether individuals consistently differ in autonomic activity, over time and across contexts. We repeatedly recorded electrocardiograms from individual shearwaters, and quantified individual repeatability of heart rate and heart rate variability indexes that reflect parasympathetic and sympathetic activity, within and across years, and across the different contexts of stress, recovery from stress, and a non-stress baseline, to assess the stability vs. plasticity of individual autonomic responses. We found a wide range of autonomic phenotypes in the shearwater population that persisted across contexts and remained stable even across years.

## MATERIALS AND METHODS

### Study system

We performed fieldwork on breeding adult streaked shearwaters in a large colony (84,000 breeding pairs, M. Yamamoto, unpublished data) on Awashima Island (38° 18′N, 139° 13′E) in the Sea of Japan during the chick-rearing season (Aug–Oct) in 2014 and 2015. Shearwaters build nests inside narrow burrows excavated in the soil on a steep coastal slope facing the sea. Burrows are typically 10–20 cm wide and ca. 0.5–1 m deep. Streaked shearwaters lay only one egg per season and both parents contribute equally to parental care (Ogawa et al., 2015). During the chick-rearing period, adults spend the entire day at sea foraging for fish. They return to the colony after sunset to feed their chicks (spending most of this time inside their nest burrows), and depart for the sea again just before sunrise.

### Data collection

Fieldwork was performed at night, between 8pm and 4am, when adults were present in the colony. Adults were captured from their nest burrows, identified by their permanent metal rings (unringed birds were given a ring), equipped with externally attached miniaturized electrocardiogram (ECG) data loggers and then returned to their nest burrows, so we could measure heart rate, parasympathetic (‘rest-and-digest’) and sympathetic (‘fight-or-flight’) indexes of heart rate variability during handling stress (just after return to the burrow, 0 min post-handling), during recovery from stress (after 20 and 90 minutes of resting in the burrow post-handling) and at baseline (120 min post-handling). We performed 55 tests in 2014 (49 individuals) and 140 tests in 2015 (69 individuals). Twenty-three birds were captured at least once in both years.

We used Little Leonardo ECG loggers (model W400-ECG, 21 × 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, 20 g, 1 GB memory, Evolocus LLC; for more details see Müller et al., 2017). Though the two logger types differ in sampling interval, they both produce an ECG trace that shows a clear signal for each heartbeat; measurements of heart rate and heart rate variability did not differ between records from the two types of loggers. Three lead wires extend from the ECG logger, with small safety pins (electrodes) soldered to the ends, that we subcutaneously attached to the skin of the birds on the breast (Yamamoto et al., 2009). We wrapped the wires around one side of the bird and secured the logger to the dorsal feathers with Tesa tape (Yamamoto et al., 2009). Compared to gluing, using subcutaneous pins has several advantages: it requires no feather removal, results in quicker logger attachment (and therefore reduced handling time), and it causes no lasting damage (birds that are repeatedly tested and recaptured within a few days of a previous test, exhibit no skin wounds or irritation from previous ECG logger attachments, Müller et al., 2017). It has therefore become the standard method for seabirds (e.g. Ropert-Coudert et al., 2006; Carravieri et al., 2016; Müller et al., 2017). We cleaned pins and skin with alcohol wipes before attaching loggers and replaced the pins several times during each season. After logger attachment (after a total handling

time of 7–12 min), birds were placed back into their burrows for 2 h. At the end of each test, we retrieved the bird from the burrow, removed the logger and measured bill length with calipers, and with a Pesola spring scale (5 g accuracy). All fieldwork was authorized by the Ministry of the Environment. All procedures were approved by the Animal Experimental Committee of Nagoya University.

**ECG data processing**

We analysed ECG data using Igor Pro version 6.37 (Wavemetrics, USA) in five-minute intervals, based on (von Borell et al., 2007). In the PQRS complex, which is the cluster of graphical deflections that comprise a single heartbeat in an ECG wave, the R peak (occurring with the depolarization of the right and left ventricles of the heart), in particular is very prominent in this species (Müller et al., 2017). We identified R peaks in ECG recordings primarily using the software Ethographer (Sakamoto et al., 2009), which permits smoothing of the wave and enhances the length of R peaks to facilitate peak detection. We manually identified R peaks when necessary. We created a data frame of the timing of each heartbeat (in milliseconds).

Using the RHRV package (Mendez et al., 2014) in R software (version 3.2.1), we filtered the beat positions to eliminate spurious beats from other prominent (non-R) peaks in the wave caused by muscle noise. The filtered dataset was then used to calculate inter-beat intervals (IBIs). Heartbeat positions plotted over time reveal oscillations in heart rate caused by the autonomic nervous system, which generates most of the heart rate variability (Müller et al., 2017). Oscillations occurring at a high frequency (between 0.3 and 2 Hz in this species, or every 0.5–3.3 s, Müller et al., 2017) reflect variability in heart rate generated by the parasympathetic nervous system and correspond to respiration - during inhalation heart rate accelerates, during exhalation heart rate slows, making oxygen delivery more efficient (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 parasympathetic activity. Oscillations occurring at low frequency (0.04–0.3 Hz in this species, or every 3.3–25 s, Müller et al., 2017) are generated by both the sympathetic and parasympathetic nervous system, and the amplitude is an index of combined sympathetic and parasympathetic activity (Malik et al., 1996; von Borell et al., 2007; Yamamoto et al. 2009; Carravieri et al., 2016).

We calculated these indexes from IBI data, using RHRV. We calculated the standard deviation of the differences between successive IBIs (‘rMSSD’), which reflects the amplitude of high frequency oscillations and therefore parasympathetic activity, the standard deviation of all IBIs (‘SDNN’), which reflects the amplitude of low frequency oscillations and therefore the combined sympathetic and parasympathetic activity (hereafter sympathetic + parasympathetic index), and the ratio between the ‘SDNN’ and ‘rMSSD’ (‘SDNN:rMSSD’) which therefore is approximately a sympathetic:parasympathetic ratio (Malik et al., 1996; von Borell et al., 2007; Kjaer and Jørgensen, 2011; Shaffer et al., 2014; Carravieri et al., 2016, see Müller et al., 2017 for more details about heart rate variability analysis in this species). We also used RHRV to compute average heart rate (which reflects the balance between sympathetic and parasympathetic activity, increasing or decreasing, respectively, when their level of activity increases) over the course of each five-minute interval.

**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. To test how autonomic activity changed between acute stress just after handling (0 min post-handling), during recovery in the nest burrow (20 and 90 min post-handling), and at baseline (120 min post-handling), we performed mixed models on heart rate and heart rate variability indexes using the *lmer* function from the *lme4* package (Bates et al., 2015) and *lmerTest* (Kuznetzova et al., 2015) to determine statistical significance. Time interval (0, 20, 90 and 120 min post-handling) was included in the model as a continuous predictor. Fifty-five birds were tested more than once, so we included individual ID as a random factor in all models ($n$=780 observations from 197 tests from 97 different individuals).

Repeatability is a standardized index that reflects the proportion of the variation in a phenotypic trait that comes from between-individual variation (Lessells and Boag, 1987). Thus high repeatability values (closer to 1) indicate large and consistent differences between individuals in a trait, due to large between-individual differences relative to within-individual variability whereas values closer to zero indicate that differences between individuals are small and intra-individual variability is high.

We calculated repeatability for each autonomic parameter (Heart rate, 'rMSSD', 'SDNN', 'SDNN:rMSSD'), for each time interval (0, 20, 90, 120 min), within and across years. Repeatability was calculated as the between-individual variance component divided by the sum of the within-individual and between-individual variance components, which were derived from linear mixed models (LMMs, with restricted maximum likelihood, Nakagawa and Schielzeth, 2010). We also included additional potentially confounding variables (timing in season, year) in the LMMs that could incorrectly inflate within- or between-variance estimates and could therefore bias our repeatability values, therefore, we calculated 'adjusted repeatability' *sensu* Nakagawa and Schielzeth (2010). The LMMs were constructed in the following way: they contained an autonomic index (e.g. heart rate) as a dependent variable, and the random factor 'Individual ID', the fixed covariate 'Calendar date' (timing in the season) and the random factor 'Year'. The variance of the random factor 'Individual ID' represents the between-individual variance component, and the 'residual variance' component represents the within-individual variance. Calendar date corrected for changes in autonomic activity in all birds across the season that could bias results, either by artificially increasing or reducing consistency estimates for individuals if they were repeatedly tested at a similar time or at very different times, respectively, or artificially increasing between-individual differences if all tests for one bird was performed at a very different time in the season than all tests for another bird were performed. Year was included in case autonomic activity differed between years, as some birds were tested only in 2014 and others tested only in 2015 (effects of Calendar date and Year on autonomic activity reported elsewhere). The repeatability estimate was further corrected based on the recommendations of Nakagawa and Schielzeth (2010). As we used mean values of heart rate or heart rate variability over a continuous 5-minute interval and heartbeats from a five-minute interval are not independent, it was more appropriate to use a repeatability

estimate of measurement means. Whether a trait was significantly repeatable or not, did not differ depending on whether we used uncorrected repeatability or corrected repeatability for measurement means and therefore the type of repeatability estimate we use does not qualitatively change results or interpretation.

We performed two sets of analyses: within-year repeatability and between-year repeatability. In our models producing variance components for within-year repeatability ($n$=45 individuals, 120–123 observations, see sample size details below), for birds that were tested in both years, we included only data from the year with the most tests for that individual. We also included only data from individuals that were tested two or more times within a year. The average time between consecutive tests from the same individual within a year was 6.72 days (s.d.=6.13, range of 1–30 days). The average time span between first and last test from the same individual within the same year was 11.67 days (s.d.=8.59, range of 2–41 days).

For our analyses of between-year repeatability ($n$=23 individuals, 46 observations) if birds were tested more than once in one or more of the years, we selected data points from each year that were collected on the most similar calendar date. The difference between calendar dates of tests from the two different years, were 9.21 days (s.d.=8.68, range of 1–37 days between dates of tests).

Repeatability estimates were adjusted using the equation for unequal sample sizes from different individuals (Lessells and Boag, 1987). We estimated 95% confidence intervals directly from a simulated distribution of repeatabilities generated by parametric bootstrapping (1000 iterations, described in detail by Faraway, 2006; as recommended by Nakagawa and Schielzeth, 2010). We performed likelihood ratio tests to test for statistical significance of variance of the random effect of individual ID (Bolker et al., 2009).

To visually compare the size of within- vs. between-individual variance among autonomic indexes, we performed the same linear mixed models on the data after it had been standardized (x-mean)/s.d. and extracted variance components (Figs 1B, 2B, and 2D). Data were standardized after removing outliers. Outliers were individuals that showed heart rates higher than 335 bpm during the recovery (20 or 90 minutes post-handling) or non-stress (120 minutes post-handling) phase, as such high heart rates indicate a stress response. Only 1 outlier was removed from recovery at 20 minutes, and 3 outliers were removed from non-stress at 120 minutes. 24-hour ECG recordings of birds at rest (incubating inside their nests) revealed no detectable circadian rhythm in heart rate or heart rate variability (Müller et al., 2017) so we did not correct for time of night in our analyses.

## RESULTS AND DISCUSSION

Shearwaters are very flexible in their autonomic responses, evident in large changes in sympathetic (‘fight-or-flight’) and parasympathetic (‘rest-and-digest’) activity across the contexts of stress, recovery from stress and non-stress: heart rate (which reflects the balance between sympathetic and parasympathetic activity) decreased from circa 300 beats per minute (bpm) during stress to circa 180 bpm at baseline (b=-0.8466, s.e.=0.0267, $P$<0.001, Fig. 1A). Heart rate variability indexes also revealed large changes in the activity of individual autonomic branches: parasympathetic activity increased sharply between the contexts of stress and

baseline ('rMSSD', the standard deviation of the differences between successive inter-beat intervals, Fig. 2A, b=0.0073, s.e.=0.0003, $P<0.001$), sympathetic+parasympathetic activity ('SDNN', the standard deviation of all inter-beat intervals, Fig. 2C, b=0.0038, s.e.=0.0003, $P<0.001$) also increased to baseline, and sympathetic:parasympathetic balance ('SDNN:rMSSD', b=-0.0035, s.e.=0.0003, $P<0.001$) decreased toward baseline, in line with the expectation that 'rest-and-digest' activity increases and 'fight-or-flight' activity decreases as an animal goes from a state of stress to a baseline resting state.

Despite this flexibility in autonomic activity, individual autonomic responses in a given context consistently differed from each other, revealing a wide range of autonomic phenotypes in this free-living population. Repeated recordings within the same year from 45 individuals showed that their heart rates consistently differed from each other and these differences persisted across contexts (Fig. 1B, Table 1A): during stress (0 min post-handling, Fig. 1C), during recovery from stress (after 20 and 90 min in the nest post-handling), and at baseline (after 120 min in the nest, Fig. 1D). Heart rate variability analysis showed that the wide range of differing autonomic phenotypes in this population measured from heart rate, were driven by individual differences in parasympathetic 'rest-and-digest' activity ('rMSSD'), which was also highly repeatable across the different contexts (Table 1B, Fig. 2B), and showed repeatability even across years (Table 1B). Sympathetic activity, on the other hand, is elevated only during stress in this species (Müller et al., 2017) and did not show consistent individual differences mainly due to high within-individual flexibility—the 'SDNN' index, which reflects combined sympathetic+parasympathetic activity, produced significant repeatability only after birds had fully recovered from stress at a time when sympathetic activity is negligible (120 min, Table 1C, Fig. 2D).

The sympathetic and parasympathetic branches are regulated—through direct synaptic connections—by two, mostly separate, and inversely activated, brain networks (Thayer et al., 2012; Beissner et al. 2013) that are well studied in humans (Fox et al., 2005; Buckner et al., 2008), present in other mammals (Rilling et al., 2007; Vincent et al., 2007; Lu et al., 2012), and have functionally and anatomically homologous structures also in birds (Shanahan et al., 2013). These brain networks regulate not only physiology (via the autonomic nervous system) but also behaviour (via the somatic nervous system). The activity of the two autonomic branches, measured from heart rate and heart rate variability, therefore provides a real time window into the activation of these brain networks and the mental state of the animal while it interacts with its environment (Jänig et al., 2006; Beissner et al., 2013). Examining consistency vs. plasticity of individual autonomic responses over time and across contexts, thus provides insight into the stability vs. flexibility of the neural circuitry that regulates physiology and behaviour. Though we found the birds to be very flexible in their autonomic responses between contexts (evident in large changes in sympathetic and parasympathetic activity between stress and non-stress, Figs 1A, 2A, and 2C), individual birds consistently differed from each other in their responses in the same context (Figs 1B and 2B), demonstrating significant stability in the activation of one or more brain networks in a given situation. This stability was not evident for the network regulating the sympathetic branch, which is activated during focused attention on specific tasks/events including threats (Thayer et al., 2012; Beissner et al., 2013): sympathetic activity exhibited high within-individual variability in

sympathetic-active contexts (stress and recovery) even within years (Fig. 2D, Table 1C). On the other hand, activation of the 'default-mode' brain network, which regulates the parasympathetic branch and a mental state of broadly-tuned outward watchfulness and monitoring of the external environment (Thayer et al., 2012; Beissner et al., 2013), appeared to be very stable within individuals even across years (Fig. 2B, Table 1B).

Our demonstration of consistent individual autonomic phenotypes in a wild free-living animal points to autonomic activity as a key neural mechanism driving broad phenotypic variation in natural populations, which has important ecological and evolutionary implications. Many eco-physiological and life history traits and trade-offs hinge on the allocation of limited resources. Because the autonomic nervous system regulates metabolism (reflected in heart rate, Romero and Wingfield, 2016), distinct autonomic phenotypes can mediate different solutions to resource allocation trade-offs. Divergent phenotypes in the same population are often favored in competitive environments and can make way for adaptive individual niche specialization (Bergmüller and Taborsky, 2010; Dall et al., 2012). Continued success of such phenotypic variants is a step toward evolutionary change (Fusco and Minelli, 2010). Autonomic phenotypes in humans are 50% heritable (Neijts et al., 2015) and show a partial genetic basis in laboratory animals as well (Koolhaas et al., 1999; Korte et al., 1999; Kjaer and Jorgensen, 2011). Our study identifies the autonomic nervous system as a potentially important mediator of life history evolution.

**Competing interests**

We have no competing interests.

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**Tables**

**Table 1. Consistent individual differences, or repeatability of autonomic activity in streaked shearwaters in different contexts**: acute stress (0 min post-handling), recovery (20, 90 min post-handling while recovering in nest burrow) and non-stress (120 min post-handling), within/between years. Within-year: *n*=120–123 observations (45 individuals); between-year: *n*=46 observations (23 individuals).

| | Within-year | | Between-year | |
|---|---|---|---|---|
| | Repeatability | *P* | Repeatability | *P* |
| A. Sympathetic:Parasympathetic balance (Heart rate) | | | | |
| 0 | 0.463 | 0.005** | 0.301 | 0.439 |
| 20 | 0.440 | 0.018* | 0.286 | 0.520 |
| 90 | 0.523 | 0.007** | 0.642 | 0.016* |
| 120 | 0.541 | 0.001** | 0.195 | 0.762 |
| B. Parasympathetic activity (log 'rMSSD') | | | | |
| 0 | 0.673 | <0.001*** | 0.149 | 1.000 |
| 20 | 0.618 | <0.001*** | 0.412 | 0.187 |
| 90 | 0.611 | <0.001*** | 0.764 | 0.001** |
| 120 | 0.686 | 0.001** | 0.149 | 1.000 |
| C. Sympathetic+Parasympathetic activity (log 'SDNN') | | | | |
| 0 | 0.289 | 0.120 | 0.134 | 1.000 |
| 20 | 0.354 | 0.051 | 0.268 | 0.513 |
| 90 | 0.365 | 0.058 | 0.642 | 0.011* |
| 120 | 0.559 | <0.001*** | 0.269 | 0.544 |
| D. Sympathetic:Parasympathetic balance (log 'SDNN:rMSSD') | | | | |
| 0 | 0.506 | 0.001** | 0.151 | 1.000 |
| 20 | 0.676 | <0.001*** | 0.372 | 0.286 |
| 90 | 0.690 | <0.001*** | 0.192 | 0.796 |
| 120 | 0.710 | <0.001*** | 0.150 | 1.000 |

**Figures**

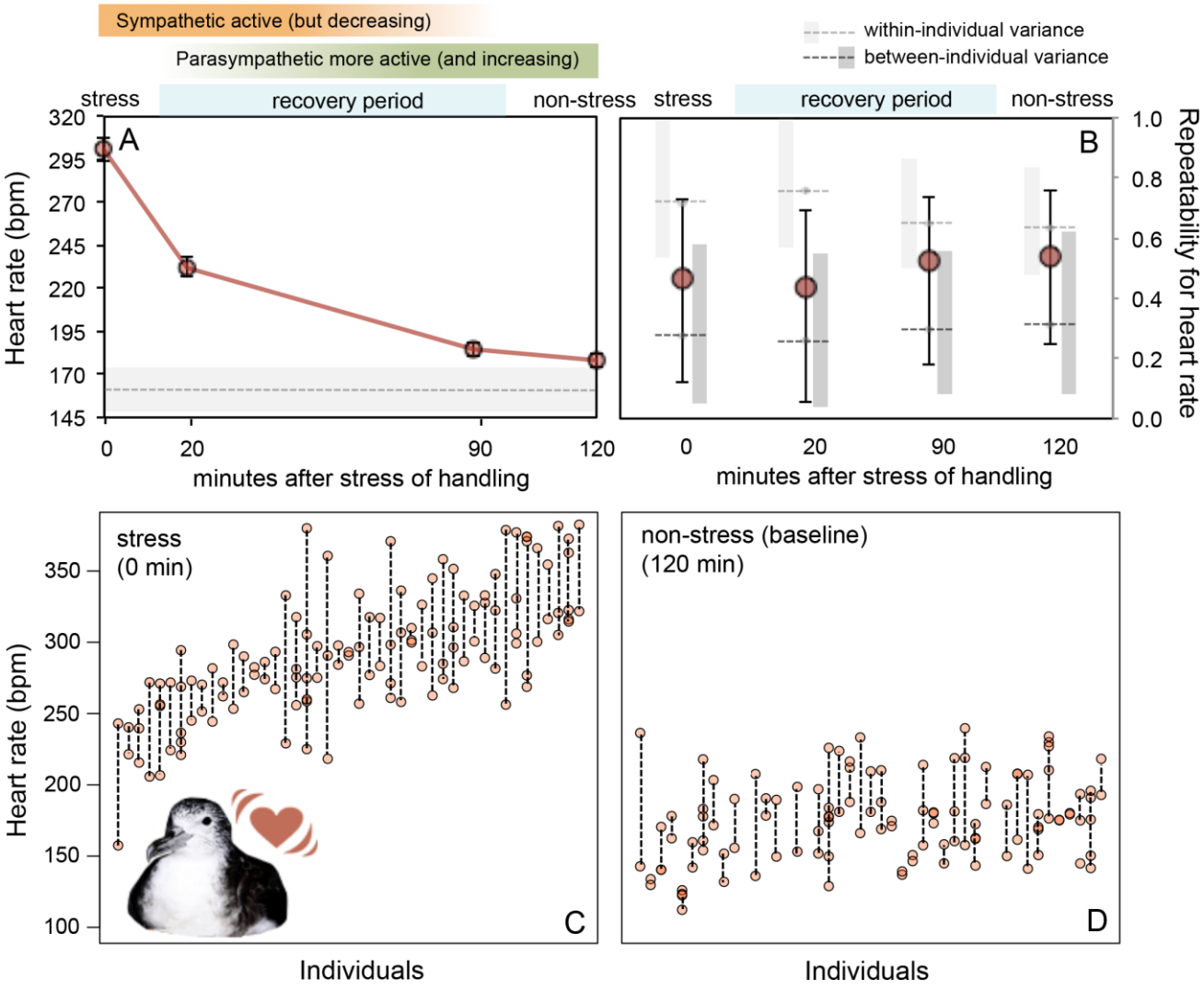


**Figure 1. Changes in heart rate between stress and non-stress in streaked shearwaters and consistent individual differences. (A)** Means ±95% CIs of heart rate (beats per minute, bpm) post-handling, during two-hour recovery period in nests (*n*=195). Grey dotted line reflects baseline mean ±95% CIs from a previous study where loggers were kept on incubating birds for 24 hours (Muller et al. 2017). **(B)** Repeatability (R) is a standardized index ranging between 0 and 1, that reflects consistent individual differences, and is calculated as between-individual variation divided by the sum of between-individual and within-individual variation, in a phenotypic trait. Individual within-year repeatability (circles) ±95% CIs and within-/between-individual standardized variance components ±95% CIs for heart rate post-handling (*n*=123, 122, 123, 120 for 0 min, 20 min, 90 min and 120 min, respectively). **(C-D)** Individual differences in heart rate. Vertically connected points are heart rate values from repeated tests of 45 individuals (same year), 0 min (**C**) and 120 min (**D**) post-handling.

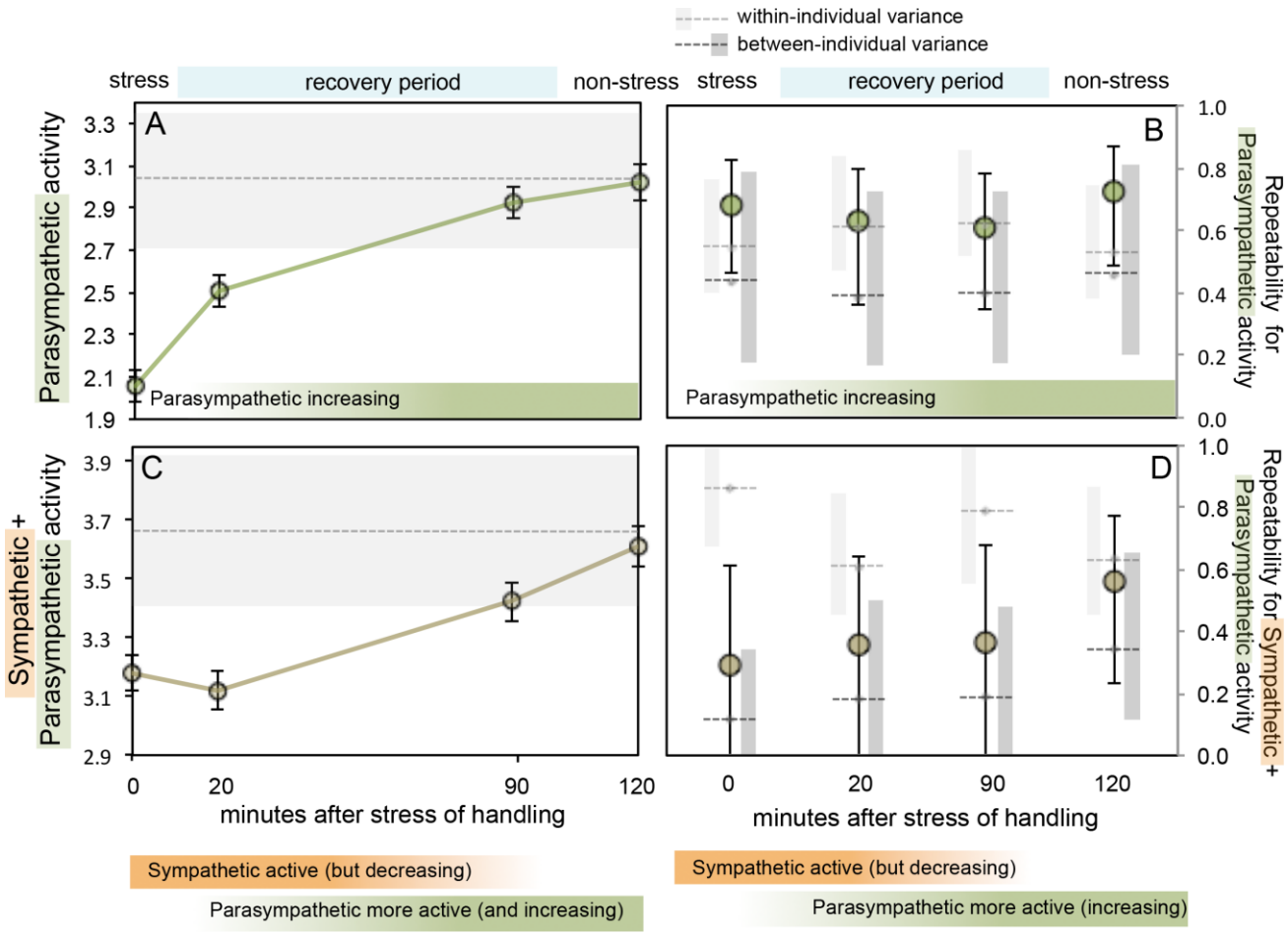


**Figure 2. Changes in autonomic indexes (from heart rate variability) between stress and non-stress in streaked shearwaters and consistent individual differences. (A)** Means ±95% CIs of parasympathetic 'rest-and-digest' activity post-handling (log 'rMSSD' index of heart rate variability on y-axis), during two-hour recovery period in nests (*n*=195). Grey dotted line reflects baseline mean ±95% CIs. **(B)** Individual within-year repeatability (circles) ±95% CIs and within-/between-individual standardized variance components ±95% CIs for parasympathetic activity (log 'rMSSD') post-handling (*n*=120–123). **(C)** Means ±95% CIs of combined sympathetic 'fight-or-flight' + parasympathetic 'rest-and-digest' activity (represented by log 'SDNN' index of heart rate variability) post-handling, during two- hour recovery period in nests (*n*=195). Grey dotted line reflects baseline mean ±95% CIs. **(D)** Individual within-year repeatability (circles) ±95% CIs and within-/between-individual standardized variance components ±95% CIs for combined sympathetic 'fight-or-flight' + parasympathetic 'rest-and-digest' activity (log 'SDNN') post-handling (*n*=123, 122, 123, 120 for 0 min, 20 min, 90 min and 120 min, respectively).