

Wake respirometry may quantify stress and energetics of free-living animals

Kayleigh Rose^{1*}, Rory P Wilson¹, Claudia Ramenda², Hermina Robotka,² Martin Wikelski^{3,4}, Emily L C Shepard^{1,3*}

Addresses

¹ Biosciences, Swansea University, Singleton Park, Swansea, SA2 8PP, UK

² Max Planck Institute for Biological Intelligence, Seewiesen, Germany

³ Max Planck Institute for Animal Behavior, Radolfzell, Germany

⁴ Centre for the Advanced Study of Collective Behavior, University of Konstanz, Konstanz, Germany.

*Co-corresponding authors: k.a.r.rose@swansea.ac.uk, e.l.c.shepard@swansea.ac.uk

SUMMARY

Quantifying activity-specific energy expenditure in free-living animals is a major challenge as current methods require calibration in the lab and animal capture. We propose “wake respirometry”, a new method of quantifying fine-scale changes in CO₂ production in unrestrained animals, using a non-dispersive infrared CO₂ sensor positioned downwind of the animal i.e. in its wake. We parameterise the dispersion of CO₂ in a wake using known CO₂ concentrations, flow rates and wind speeds. Tests with three bird species in a wind tunnel demonstrated that the system can resolve breath-by-breath changes in CO₂ concentration, with clear exhalation signatures increasing in period and integral with body size. Changes in physiological state were detectable following handling, flight and exposure to a perceived threat. We discuss the potential of wake respirometry to quantify stress and respiratory patterns in wild animals and estimate activity-specific metabolic rates through the full integration of CO₂ production across the wake.

Keywords: Metabolic rate, respirometry, stress response, flight costs

INTRODUCTION

Determination of the energy expenditure of free-living animals is pivotal for understanding the costs and rewards of behaviours and elucidating strategies that enhance lifetime reproductive success (Lemon 1991; Shaffer, Costa & Weimerskirch 2003). However, quantification of activity-specific metabolic rate in freely moving individuals is a major challenge (Wilson & Culik 1993; Butler *et al.* 2004). Methods that have provided key insight in this regard include ‘heart rate’ (Nolet *et al.* 1992; Bevan *et al.* 1994; Bevan *et al.* 1995; Ward *et al.* 2002; Green 2011) and ‘dynamic body acceleration’ as proxies for energy expenditure (Wilson *et al.* 2006; Halsey *et al.* 2009; Halsey, Shepard & Wilson 2011; Wilson *et al.* 2020). Heart rate loggers can inform us of relative costs in animals both at rest and during activity, while dynamic body acceleration allows the interrogation of costs associated with movement. However, each of these methods has to be calibrated by indirect calorimetry (Halsey *et al.* 2009; Gleiss, Wilson & Shepard 2011; Halsey, Shepard & Wilson 2011; Halsey & Bryce 2021) or doubly labelled water (Nolet *et al.* 1992; Bevan, Speakman & Butler 1995; Elliott *et al.* 2013). Both indirect calorimetry and doubly labelled water work by assessing the rate of CO₂ production (and, in indirect calorimetry, sometimes O₂ consumption (Lighton 2019)). A limitation of indirect calorimetry is that it necessitates animals to be confined to boxes (e.g. (Hawkins, Butler & Speakman 2000)) or equipped with masks (Ward *et al.* 2001; Morris, Nelson & Askew 2010; Langman *et al.* 2012), which prohibits or constrains expression of many behaviours and can induce stress in the study animal (Tucker 1972). In contrast, doubly labelled water allows animals to move freely within their natural environment, but does not allow easy assessment of activity-specific metabolic rate (Nagy, Siegfried & Wilson 1984; Wilson & Culik 1993) although judicious use of sophisticated animal-attached tags is providing a way forward (Sutton *et al.* 2021)).

During the years that these processes have been developed and refined, our ability to determine CO₂ concentration with high accuracy, even at low concentrations, has advanced dramatically. In particular, non-dispersive infrared spectroscopy (NoDIS) has been shown to resolve CO₂ concentrations as low as 0.01 ppm (https://www.licor.com/env/products/gas_analysis/LI-7000/specifications.html). Exhaling (air-breathing) animals have CO₂ concentrations around 4% when their respiratory gases leave their body, with this concentration being diluted with distance from the source due to diffusion and wind. Nonetheless, the accuracy of NoDIS means that the CO₂ signal should be detectable at some

distance from the CO₂ source using a system that does not interact with the study animal in any physical way. We propose that it might be possible to determine metabolic rate by positioning these NoDIS sensors close to unrestrained, and possibly even free-living, animals and examining CO₂ concentrations over time, providing there is directional flow of air. This would necessitate mapping out a full 2D cross-section of the CO₂ wake. We term this approach ‘wake respirometry’ because the CO₂ signal from an animal is drifted downwind and over the sensor. The concentration of CO₂ at any point around an animal, and therefore the ability of a NoDIS to quantify it, will depend on the concentration of CO₂ being emitted in the exhaled air, the position of the sensor relative to the source, and the speed and direction of the wind passing over the animal (Figure 1), as well as the expiratory flow rate, and the rate of diffusion and dilution of CO₂ in air and water vapour.

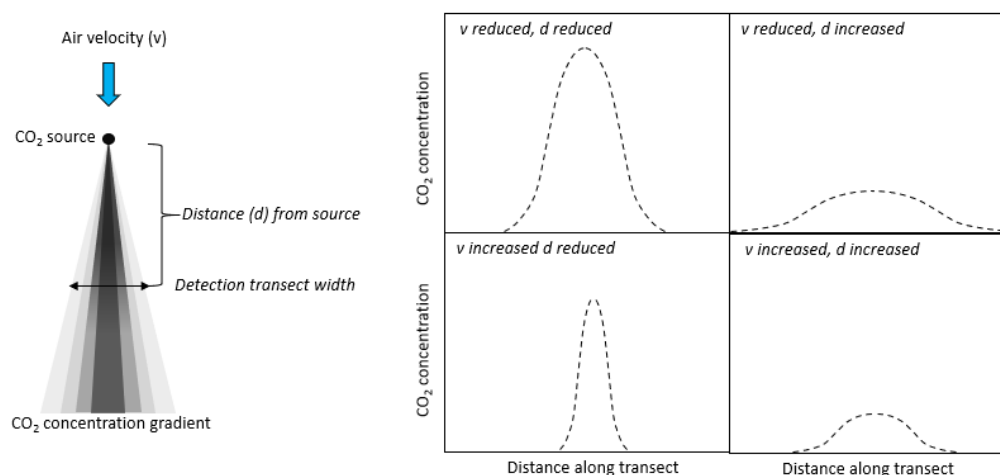


Figure 1. Expected changes in CO₂ concentration in relation to sensor distance from the source and wind speed. The distribution of CO₂ emitted at a constant rate from a point source in space is roughly expected to follow an increasing radius of the iso-concentrations as the gas diffuses out, modulated by air flow, which will tend to distribute the CO₂ downwind of the source, with distance-dependent iso-concentration radii decreasing with increasing air speed.

In this work, we take a first step towards this goal by describing the use of the NoDIS method in wind tunnels behind perched captive, but unrestrained, pigeons *Columba livia domestica*, a starling *Sturnus vulgaris*, and a zebra finch *Taeniopygia guttata*. Our aims are to; (i) demonstrate detection of a CO₂ signal downwind of unrestrained animals, and (ii) examine how

this signal is affected by rate of CO₂ emission, source-sensor distance, lateral position across the wake and wind speed. We also (iii) examine what the approach can tell us about animal state and respiratory physiology in perched pigeons, post-flight, post-handling and in response to a perceived threat. Finally, (iv) we map out future directions for the method to integrate the full CO₂ shadow downwind of a resting animal and even behind a bird flying in a wind tunnel to derive figures for metabolic rates from animals undertaking activities that are currently assessed using conventional means, with associated limitations.

RESULTS

Definition of the CO₂ wake downwind of a source

We used a defined gas mix of 4% CO₂ /air (BOC) to carry out our calibrations in an open jet style wind tunnel custom designed for bird flight (test section width 1.8 m, length 2.2 m, height 1.5 m) in Swansea University, UK. Across a range of emission rates (0.5 L min⁻¹ and 1 L min⁻¹), source-sensor distances (10, 30 and 50 cm) and windspeeds (1, 5 and 10 m s⁻¹), the width of the CO₂ wake was measured using a NoDIS, LI-7500A Open Path CO₂/H₂O Analyzer (Lincoln, Nebraska, USA), and ranged from 5-14 cm (Fig 2). CO₂ concentrations increased from the periphery of the wake towards a maximum in the centre and decreased with increasing distance from the source and increasing windspeed (Fig. 2). Transects across the downwind wake of a constant CO₂ source showed that transect width increased with increasing distance from the source, apart from at the greatest windspeed of 10 m s⁻¹ where the transect width remained narrow (Fig 2).

Using limits of detectable CO₂ concentrations (here, 2 ppm) to define potential operational areas indicated that at an emission rate of 1 L min⁻¹, the NoDIS sensor detected a clear CO₂ signal up to 50 and 10 cm from the source at wind speeds of 1 and 10 m s⁻¹, respectively (Fig. 2A-C). The same was found when the emission rate was halved (0.5 L min⁻¹) (Fig. 2D-F).

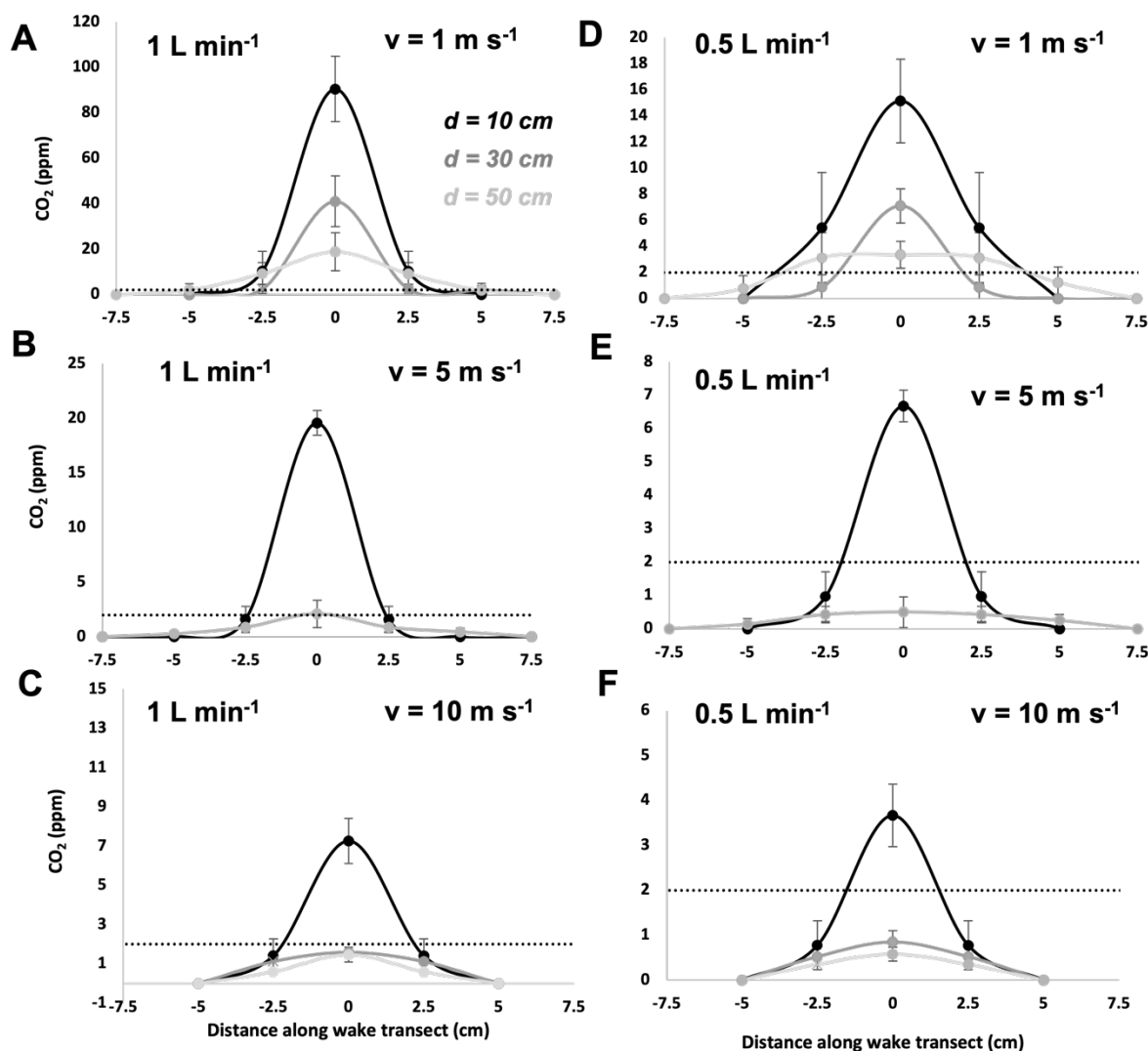


Figure 2. CO₂ concentrations (mean \pm S.D.) measured across the wake at different distances (d) from the source and windspeeds (v). (A-C) with a 4% CO₂/air mix emission rate of 1 L min⁻¹. (D-F) with an emission rate of 0.5 L min⁻¹. The dotted line shows the position of the 2 ppm CO₂ concentration. N.B data were collected at -7.5, -5, 0, 2.5, 5 and 7.5 cm from the midline. At -2.5 cm means \pm S.D. mirror those on the opposite side of the wake.

Bird CO₂ wake exhalation signatures

In a closed system wind tunnel at the Max Planck Institute for Biological Intelligence, Germany, we positioned the sensor 46 cm behind a zebra finch, starling and pigeon with the windspeed of set to 2 m s⁻¹. Clear and regular peaks in CO₂ were detectable for all three (Fig. 3). These signals allow calculation of breathing frequency and the integral under the signal. However, as indicated by the calibration work, the quality of the signal depends on the rate of CO₂ emission, and smaller species had less consistency in their peaks (Fig. 3). The signal was clearest when the infrared path was aligned with the tail and body (as opposed to the head) indicating that the exhaled CO₂ attached to the body. Some variation in the signal amplitude is expected due to movement of the head, which would influence integral calculations, but birds moved their heads less with the tunnel air turned on. Measures of breathing frequency should not be affected by head movement unless an exhalation is directed away from the sensor path.

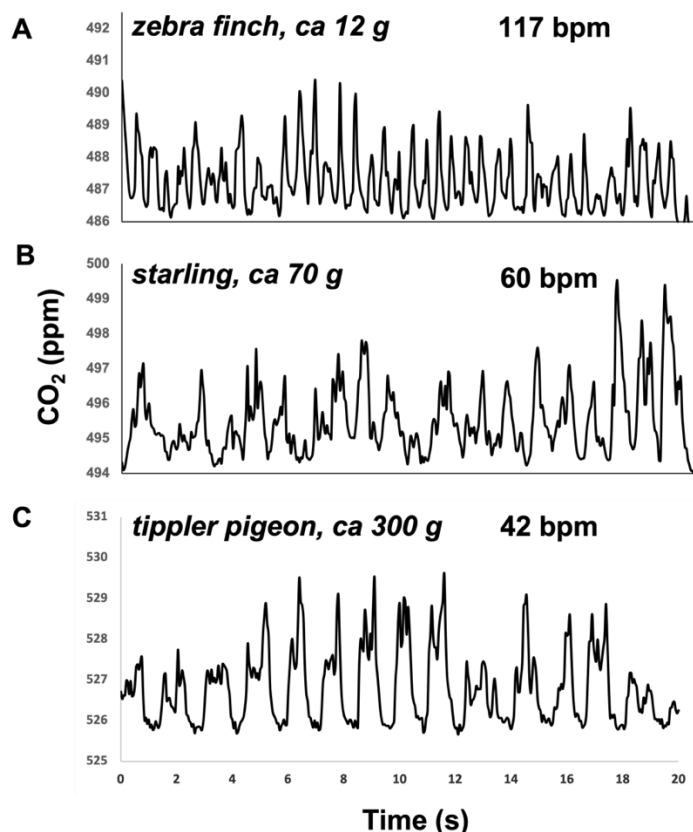


Figure 3. Raw CO₂ exhalation signatures of three bird species of different body mass.

In rested pigeons, CO₂ signatures were typically M-shaped (Fig 3C and Fig 4), with the second of the two peaks often being the greatest. Here, the rate of change in CO₂ was typically greater leading up to the second CO₂ peak, compared to the first, and lowest during the decline following the second peak. In rested pigeons, there were short plateaus in CO₂ concentration between exhalations that were equal to baseline measurements. In contrast, immediately post handling, and post exercise, or in smaller birds, the waveform had only a single peak and lacked plateaus between exhalations and concentration minima exceeded background CO₂ concentrations.

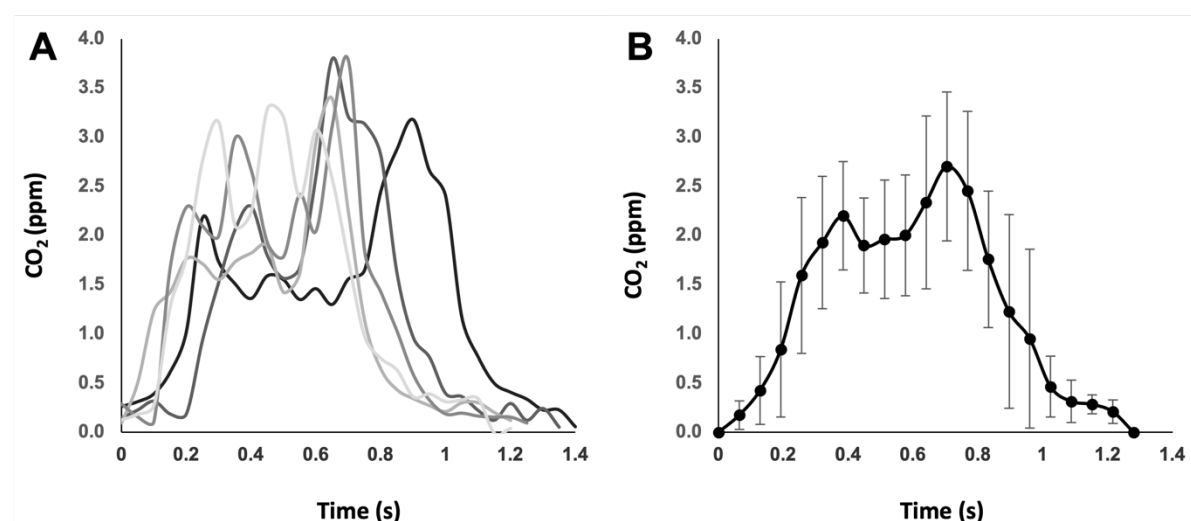
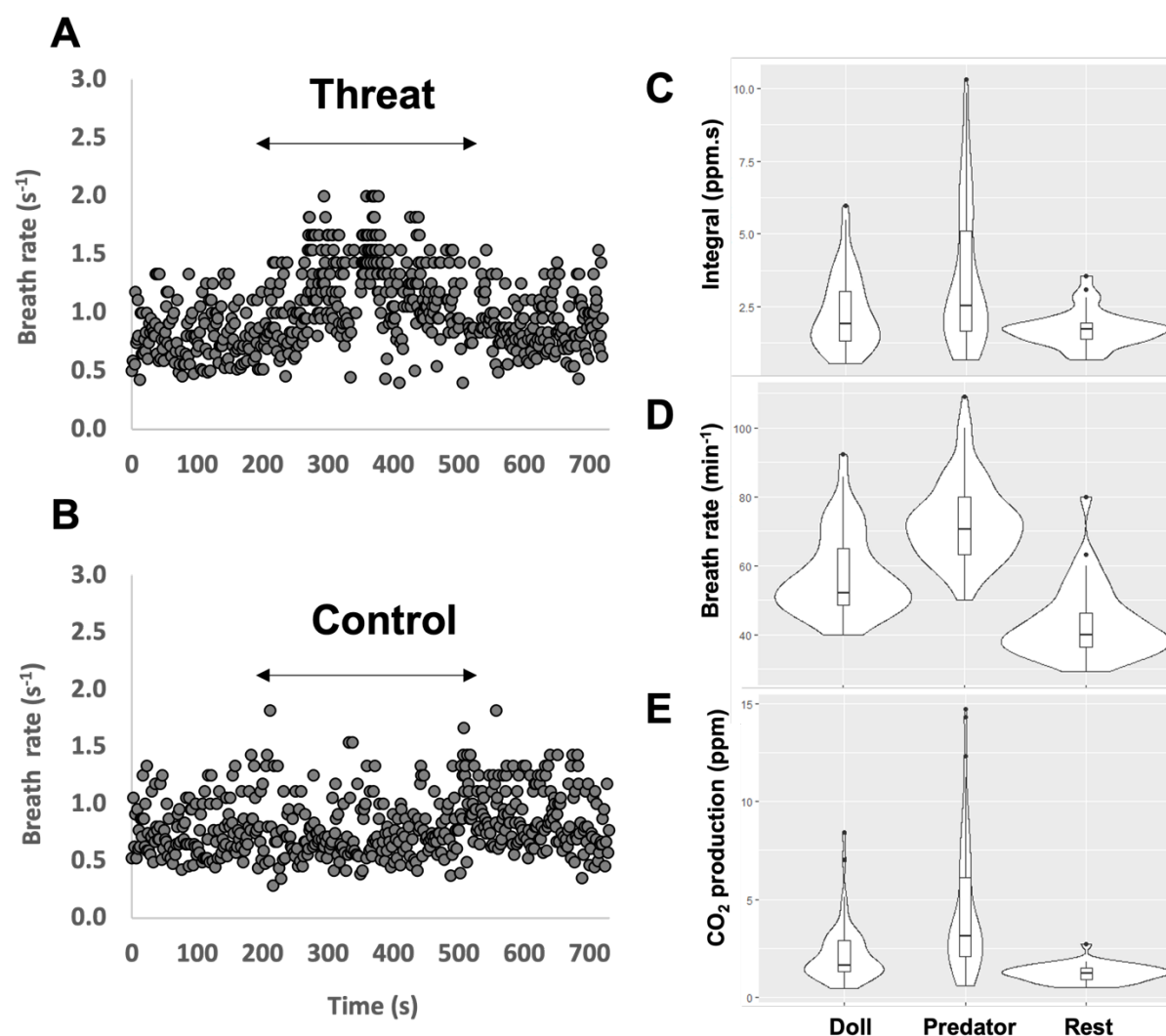


Figure 4. CO₂ concentration over time for 5 consecutive breaths in a tippler pigeon stationary on a perch in a wind tunnel with an air speed of 7 m s⁻¹. A) raw data sampled at 20 Hz. B) Means \pm S.D every 20th of a breath.

Animal state and respiratory physiology

In tippler and homing pigeons, we observed within-individual responses to different treatments. Respiration rates and, in most instances, CO₂ production, increased during the period of exposure to a stuffed buzzard relative to a rested state, while either no response, or a smaller response, was observed when presented with a control novel object, a doll (e.g., Fig. 5, see tables S1 and S2 for statistical results for homing and tippler pigeons, respectively).

179



180

181 **Figure 5. A homing pigeon's breathing parameters in response to a perceived**
 182 **threat, control novel object and at rest** (A) changes in respiration rate of a rested
 183 individual exposed to a stuffed buzzard and (B) a control novel object (doll), where data
 184 points represent single breaths and arrows indicate the duration of exposure to the
 185 stimulus. Associated changes in the (C) integral of each breath, D) breath rate and E)
 186 CO₂ production (the product of the breath rate and integral) for 1 min of data per condition
 187 (see tables S1 and S2 for statistical results).
 188

189 Immediately after handling, maximum breathing rates in tippler pigeons ranged from 1.5 - 3.3
 190 breaths s⁻¹. This decreased to minimum breathing rate ranging 0.28 - 1 breaths s⁻¹ within 1 minute,
 191 with most of the decline occurring within the first 10 s. Figures 6A-D show example post handling

data from a homing pigeon. The integral under the exhalation peaks increased over time, although this was highly variable (Fig. 6B), and CO₂ production decreased (Fig 6C). A negative curvilinear relationship was observed between the total CO₂ concentration measured per breath and breath rate (Fig 6D). Figure 6E shows inter-individual variation in breath rate over time post-handling.

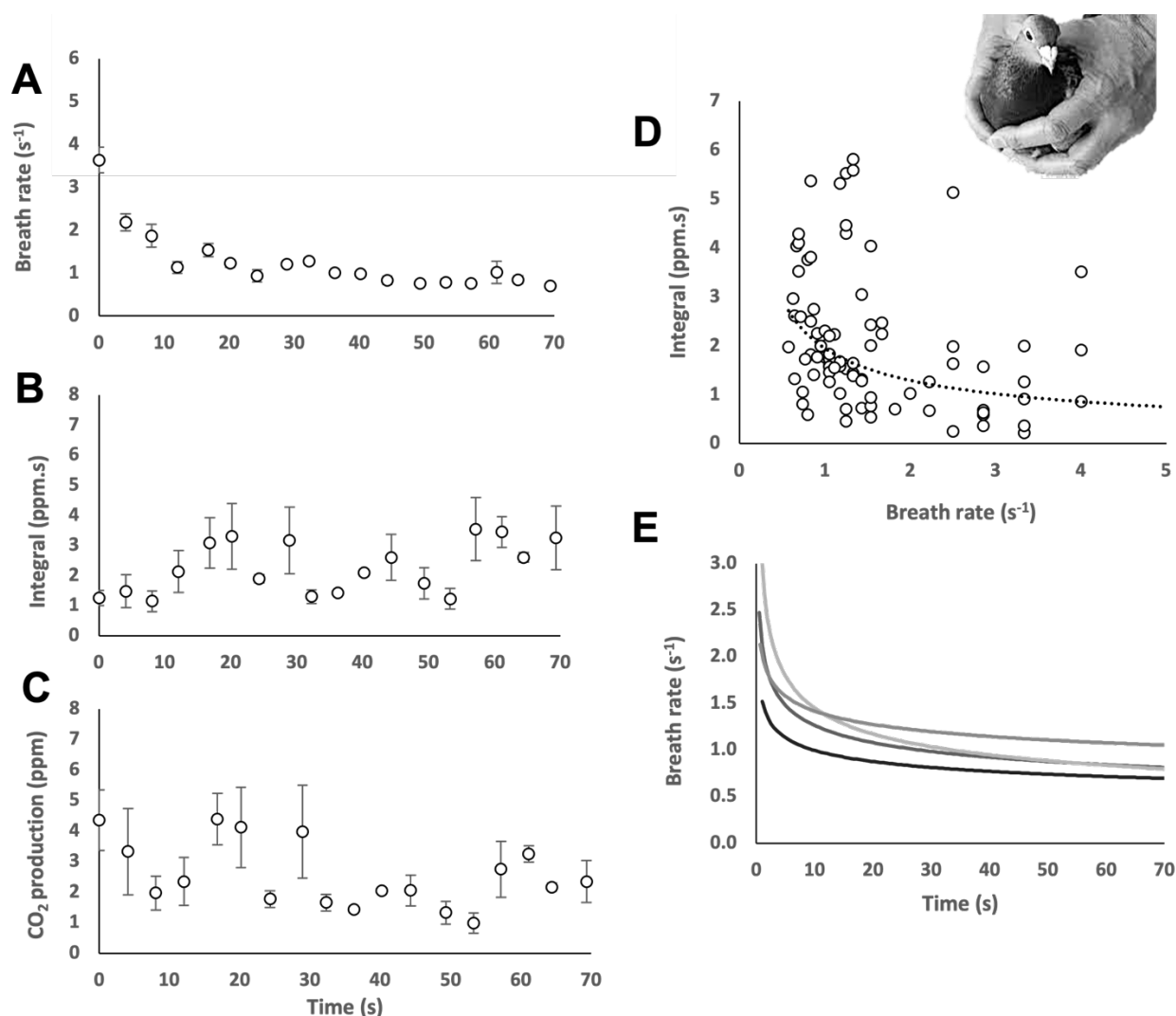


Figure 6. Recovery post handling and introduction to the tunnel. **A)** Respiration rate decreased to resting values within one minute, **B)** The integral increased over that minute, **C)** CO₂ production decreased over a minute. Data points for A-C are means \pm S.E over 4 seconds for a single tippler pigeon. **D)** Total CO₂ concentration measured per breath increased with decreasing breath rate. **E)** Individual variation in breath rate over time. Each line is the best fit for an individual tippler pigeon.

In an example of post-flight recovery in a homing pigeon, breath rate declined from a maximum of 6.7 breaths s^{-1} to a minimum of 0.6 breaths s^{-1} within 1 minute (Fig. 7A). The integral of the exhalation peaks increased gradually with recovery time (Fig. 7B), whereas CO_2 production decreased rapidly (within 10 s) (Fig. 7C). In all examples, there was a negative correlation between the integral and breath rate post flight (Fig. 6D).

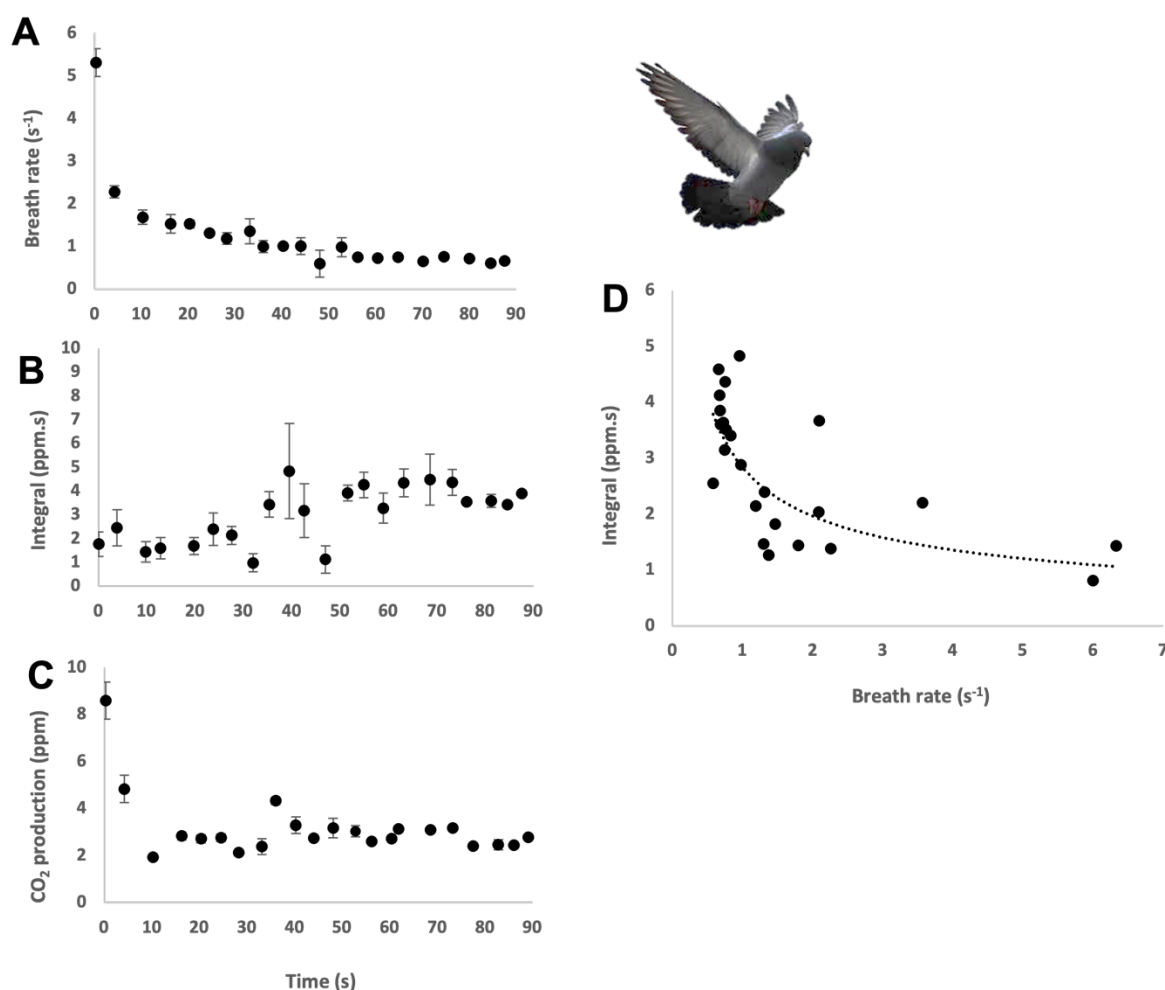


Figure 7. Recovery of a homing pigeon after 10 minutes of flight at 10 m s^{-1} . (A) breath rate (s^{-1}), (B) integral under the exhalation peak (ppm.s), (C) CO_2 production (ppm), (D) integral versus breath rate. Datapoints represent a mean over 4 seconds and error bars in A-C are S.E..

DISCUSSION

There is abundant literature on how amniote breathing frequency, together with tidal volume, modulates metabolic rate (Hallam & Dawson 1993; Kohin, Williams & Ortiz 1999); on its uses in the measurement of stress responses (Greenacre & Lusby 2004; Fucikova *et al.* 2009; Torné-Noguera, Pagani-Núñez & Senar 2013; Doss & Mans 2016; Doss & Mans 2017; Liang *et al.* 2018); its involvement in temperature regulation ((El Hadi & Sykes 1982; Brent *et al.* 1984; Bucher & Bartholomew 1986); entering, and arousing from, torpor (Withers 1977); and how it scales with body mass (Frappell & Baudinette 1995; Frappell, Hinds & Boggs 2001; Mortola & Seguin 2009). Here, we demonstrate that a NoDIS system can be used to quantify real-time changes in respiration rate with breath-by-breath resolution of CO₂ concentration when the sensor is positioned in the wake of an animal, rather than integrated into a mask or alternative system that requires restraint or tethering (Butler, West & Jones 1977; Franz & Goller 2003; Wilson *et al.* 2019). In fact the system is so sensitive that, for birds as large as pigeons, two sub-peaks in CO₂ were evident within each exhalation signature, suggesting the anterior and posterior air sacs of the respiratory system empty slightly out of phase with one another (Bretz & Schmidt-Nielsen 1971; Maina 2005; Perry, Lambertz & Shmitz 2019). Furthermore, by multiplying the frequency by the integral under the CO₂ exhalation signature to provide a proxy for CO₂ production, we were able to document responses to, and recovery from, stressors or exercise over fine-scales (cf (Franz & Goller 2003)).

Limitations in detection of CO₂ downwind of a constant CO₂ source

The performance of the system depends on there being discernible pulses in CO₂, and here we describe the operational limits for this. The NoDIS system that we used is reported to have an RMS noise of 0.16 ppm, which we could confirm with our baseline measurements. The variability that we obtained in our CO₂ signals in our calibration trials in the wind tunnel (Fig. 2) was due to inconsistencies in both the rate of expulsion of the CO₂ and in the air flow (although the wind tunnel had a high degree of laminar flow) and increased with the magnitude of the signal.

As predicted, varying wind speed affected the measured CO₂ concentrations according to the position of the NoDIS sensor relative to the gas source and the rate of gas emission. The

detection limits of the sensor (Fig. 2) indicate that, for CO₂ emission rates of 1 L min⁻¹, measurements can be made up to 50 cm immediately downwind of the source if the wind speed is 1 m s⁻¹ but this reduces to 10 cm at wind speeds 10 m s⁻¹. The detectable CO₂ wake transect widths in these instances are 10 and 6 cm, respectively, indicating angles of 5.71 and 3.43° either side of a perpendicular line from the source to the sensor. With half the emission rate (0.5 L min⁻¹), transect widths become 5 and 4 cm and angles become 14.03 and 11.31°.

These calibration figures provide broad working limits for researchers wishing to work with animals the size of the birds used in our study and go some way to helping define when the system might operate for different study animals and conditions. However, beyond these calibrations, further variability in the CO₂ signal will occur due to; (i) movement of the animal (effectively displacing the CO₂ source relative to the NoDIS sensor), (ii) the pulsed nature of CO₂ expiration during breathing, and (iii) as a result of the air flow variability itself (direction and speed) if the system is used outside.

Limitations in detection of CO₂ downwind of an animal-based CO₂ source

Animal movement is a major factor in modulating the CO₂ signal and we suggest that this can be broadly broken down into three categories; whole body movement (where the animal moves from one site to another), body rotation (where the body turns about its vertical axis only) and head movement only. Ideally, a means to capture a full transect of the wake is required to minimise these sources of variation, whether that be achieved by moving the sensor closer to the study animal or working with a greater NODIS emitter distance or more sensors. In all cases, we recommend that researchers using NoDIS systems film their study animal if possible so that the role of movement can be ascertained. Clearly, for an animal that moves laterally, such as a bird on a perch, thereby displacing the CO₂ source, this can result in the sensor operating outside the detection plume. In fact, such animal movement would preclude our approach for many animals for much of their time.

Complementary to the methodology demonstrated here, however, there are situations where animals may remain immobile, or at least stay in one spot for extended periods. For example from incubating (Gabrielsen *et al.* 1991), sleeping (Tillmann 2009) or torpid (Nowack, Stawski & Geiser 2017) animals, to basking reptiles (Mukherjee *et al.* 2017), to birds such as flycatchers

(Muscicapidae) and raptors which may regularly use particular look-out posts as vantage points (Fitzpatrick 1980), and territorial birds singing (Odom *et al.* 2014). Body rotation is expected to change the CO₂ signal in a predictable manner because it effectively results in either a lateral displacement of the CO₂ source (for example if an incubating bird facing upwind rotates 90°) and/or changes the distance between source and sensor (if the bird rotates 180°), both of which have nominally predictable effects on the concentration of the CO₂ reaching the sensor if properly calibrated. Head movement, especially in long-necked study animals such as swans, can obviously lead to appreciable CO₂ source displacement and the extent to which this changes the signal will depend greatly on the situation. A resting swan, for example, is predicted to have an expiration emission rate that is some 7.5 times that of a pigeon (Frappell & Baudinette 1995) which may make an immediately downwind, but distanced, NoDIS sensor, less sensitive per cm degree of lateral movement (see the extent of the flat top to the distribution in Fig. 2) than it would be in a pigeon although, of course, the pigeon would move its head absolutely less.

The carefully controlled wind conditions of the wind tunnel standardize an important element of the protocol and the value of the CO₂ concentration data over time in the wild will be critically dependent on the variability in wind speed and the turbulence. Variation in wind speed (gustiness) increases with overall mean wind speed so the CO₂ pulses detected by the NoDIS system will vary accordingly, specifically having the period of the exhalent pulse contracted or expanded, with accompanying changes in pulse height. Overall, this should not change respiration rate values measured over a number of cycles, but it will alter interpretation of patterns of air exhalation (see below) and may alter the values of the integrals of the CO₂ concentration under the expiration pulse (see below), although expanded exhalation periods should be accompanied by decreased CO₂ production. These issues may be largely mitigated by having high resolution measurement of wind speed (and vector) at the site so that, if necessary, corrections could be applied or at least data filtered to exclude aberrant gusts or periods of calm.

Another option, for specific cases where there is no wind, is to blow air at an appropriate rate past the study animal. This is easiest for animals in prescribed hollows, such as bats in their roosting boxes but may also work for animals outside. The noise, general disturbance and potential for wind effects on metabolic rate may severely limit this option though.

Wake respirometry: Future developments

Given the accuracy of the NoDIS system in measuring CO₂ concentrations in precise locations downwind of the study animal, it is tantalising to speculate whether this approach might enable researchers to determine metabolic rate in free-living animals. Effectively, the integral of every CO₂ pulse should be proportional to the sum of the CO₂ expired in that exhalation, and should indicate relative changes in metabolic rate. It could therefore be used to examine recovery in animals that have engaged in movement outside the sample area before returning, in a manner similar to our pigeons (Fig. 7). Birds flying back to their nest would be an obvious example.

Derivation of absolute metabolic rate is more challenging. However, data from multiple NoDIS sensors within the CO₂ footprint could be integrated and summed to provide the complete CO₂ footprint per exhalation and over longer time periods. Alternatively, a series of inhalant tubes could sample the wake and mix the air for analysis by a single NoDIS sensor to attempt derivation of total CO₂ emission. Either way, some sort of calibration would be desirable but simulation of CO₂ emissions from the study animal on site *post hoc* by bleeding gas from models may help.

Overall, this work has demonstrated that the new, portable generation of CO₂ sensors can provide insight into stress and respiratory patterns in unrestrained animals, and as a result, that they could be used to document stress responses in wild animals. With suitable consideration of the limitations imposed by factors such as animal movement and wind variability, wake respirometry should have a future that will help with a diverse suite of issues, such as determination of the extent to which urbanisation (Charmantier *et al.* 2017), tourism (Mullner, Linsenmair & Wikelski 2004), or natural disasters (Nowack, Stawski & Geiser 2017) might affect target animals, and indeed, comparison of the effects of different stressors (Clinchy, Sheriff & Zanette 2013). Beyond that, it also opens the way for potential measurements of the metabolic rate of unrestrained birds, both resting, and in the case of wind tunnels, in flight.

STAR METHODS

Definition of the CO₂ shadow downwind of a source

Apparatus

Non-dispersive infrared spectroscopy uses infrared radiation that is emitted across an (open) path of defined length, across which the CO₂ is to be measured, with the radiation being detected at the distal end of the path by a lead selenide sensor. Both water vapour and CO₂ absorb the radiation so gas densities can be determined by considering the absorption with respect to a reference. We used the LI-7500A Open Path CO₂/H₂O Analyzer (Lincoln, Nebraska, USA), which has an emitter-sensor distance of 125 mm, resolution 0.01 ppm, and error within 1% of reading. During our use of this system, we deployed it in a vertical orientation and set it to sample at 20 Hz (RMS noise 0.16 ppm at 370 ppm CO₂).

Calibrations

We assessed the viability of our approach by conducting trials under varying conditions. We used a defined gas mix of 4% CO₂ /air (BOC) to carry out our calibrations in an open jet style wind tunnel custom designed for bird flight (test section width 1.8 m, length 2.2 m, height 1.5 m) in Swansea University, UK. Rubber tubing (10 mm outer diameter) and a variable flow metre were used to release the gas mix at two fixed flow rates (0.5 and 1 L min⁻¹), via a metal tube inserted through the ceiling of the tunnel, which extended to a central position 60 cm inside the test section. The NoDIS sensor was positioned on a stand at the same height as the source to record CO₂ (ppm) at three source-sensor distances (10, 30, 50 cm) and three wind speeds (1, 5 and 10 m s⁻¹). At each emission rate, windspeed, and distance (d) from the source, ten seconds of CO₂ (ppm) data was logged (20 Hz) every 5 or 2.5 cm along the wake transect until the signal was no longer detectable.

CO₂ signals from captive birds

Captive birds used in wind tunnel trials

Data were recorded from adult captive tippler pigeons (n=4, *ca.* 300 g), homing pigeons (n=5, *ca.* 400 g), a starling (*ca.* 70 g) and a zebra finch (*ca.* 10 g) in a closed system wind tunnel at the Max Planck Institute for Biological Intelligence, Germany. Birds were kept in aviaries beside a closed system wind tunnel according to §11 Permission (§11 TierSchG) and were accustomed to being inside the tunnel for flight training. Animal experiments performed in Seewiesen, Germany, were conducted according to the regulations of the government of Upper Bavaria (Germany protocol numbers: AZ 55.2-1-54-2532-86-2015; 311.5-5682.1/1-2014-021). At Swansea University, data

were recorded from a hand-reared homing pigeon (female, *ca.* 350 g) accustomed to flight training in the open jet wind tunnel. Here, homing pigeons were housed in an outdoor loft with aviary under an establishment licence. Experiments were carried out under the project licence (X5770C662) and ethical permission for this work was given by Swansea University AWERB (200418/65).

Measurements from birds in different physiological states in wind tunnels

We investigated whether our set-up could detect responses of the tippler and homing pigeons to the following treatments; i) release after being handled, which was assessed by keeping birds in a darkened box before they were held by an experimenter for 2 minutes and then introduced to the tunnel perch, and proximity to ii) a potential threat (stuffed buzzard *Buteo buteo*) and iii) a similar-sized control novel object (rag doll). Within-individual responses to the buzzard and doll were assessed when the birds were in a rested state on the perch.

The perch (55 cm tall, 8 cm wide) was positioned in the centre of the test section while the NoDIS was fixed to a stand positioned 46 cm downstream. The perch width was relatively small to prevent birds moving to the left or right relative to the sensor. Birds always faced into the wind when the tunnel was on. Fixed windspeeds (7 and 10 m s⁻¹ for tippler and homing pigeons, respectively) were chosen to ensure a clear signal with minimal variation in CO₂ concentration between exhalations.

The wind tunnel room was 17-19°C. Lights in the study room were dimmed to a low level and noise additional to that of the wind tunnel was kept to a minimum. Two experimenters were in the room during all trials, which were conducted during normal active hours. A typical trial consisted of a bird being placed inside a darkened carrier box (dimensions 50 x 35 x 30 cm) for 10 minutes in the wind tunnel room. The bird was then removed from the box and held for 2 minutes with fingers around both sides of the body. The same experimenter restrained all birds (Rabdeau *et al.* 2019). At the same time, a baseline CO₂ trace was recorded in the absence of the bird with the tunnel on. The bird was then placed on the perch upstream of the sensor and left to sit quietly. After 20 min, either the taxidermy buzzard or rag doll was presented outside the test section and upstream of the study bird and held there for 2 minutes. Another 20 minutes of quiet time followed before the second stimulus was presented. The order in which the stimuli were introduced within trials was randomised and birds were presented with each stimulus only once to avoid habituation.

For a comparison of the CO₂ exhalation signal between birds of different body size at a single wind speed and bird-sensor distance (46 cm and 2 m s⁻¹), resting data were collected from a perched tipler pigeon, starling, and zebra finch in the Max Planck wind tunnel. Twelve starlings were flown together at 10 m s⁻¹ for 10 minutes as part of their usual training regime, after which, one individual was kept within the tunnel to recover on a perch. Similarly, ten zebra finches were flown together at 8 m s⁻¹ for 10 minutes, and one individual was kept within the tunnel for breath recordings.

Data were also collected behind a homing pigeon in the test section of the tunnel at Swansea University after 11 minutes of flight training at 10 m s⁻¹ for post-flight recovery data. The bird was perched 20 cm upstream of the NoDIS (perch height 60 cm) and the wind speed remained at 10 m s⁻¹ during data collection after the flight training. Lights were dimmed at the end of a training session for respirometry measurement and experimental temperatures ranged from 18.8-19.2°C.

Data processing and extraction

CO₂ data (ppm) were corrected for baseline drift in OriginLab 2021 using linear interpolation between baseline data collected in the absence of a bird at the beginning and end of trials or at regular intervals throughout calibration experiments in the absence of CO₂ emission. Calibration measurements, exhalation and breath cycle parameters were examined using in house software (DDMT, Wildbyte Technologies, <http://wildbytetechnologies.com>). Changes in CO₂ over time were isolated for each expiration from drift-corrected and baseline-corrected data. Breathing frequency (breaths per unit time) was calculated as the reciprocal of the breath cycle period. A proxy for CO₂ production was calculated by multiplying breathing frequency by the integral of the CO₂ signal.

Statistical analyses

Statistical analyses were conducted in R Studio using R version 4.0.3 (R Development Core Team 2019). Non-parametric Kruskal-Wallis and Dunn (holm adjusted) post-hoc tests were conducted to investigate within-individual differences in pigeon breath rate, integral of the CO₂ signature and CO₂ production (breath rate x integral) under three different conditions: in a rested and undisturbed state; when exposed to a control novel object (rag doll); and when exposed to a potential threat (stuffed buzzard). In all cases, assumptions for parametric one-way ANOVAs were not met and

this was confirmed by examining qq plots and histograms of model standardised residuals as well as Shapiro Wilk tests to confirm a distribution significantly different from normal. For each pigeon, one minute of breath-by-breath data was used per condition. Data were investigated separately for each pigeon because of the variability of signal strength that is expected due to each bird's varying body form and head movement, our small sample size, and additional variation in measurements expected due to individual differences in body mass.

463
464
465

Table S1 Results of Kruskal-Wallis tests and Dunn (holm adjusted) post hoc tests to investigate differences in the respiratory parameters of individual homing pigeons exposed to different stimuli

ID	Parameter	Median	ϵ^2	X^2 (df)	P	n	Comparison	z	P.unaj	P.adj
HP1	Integral (ppm.s)	Rest: 1.72 Doll: 1.92 Predator: 2.52	0.089	16.71 (2)	<u>0.0002</u>	169	doll - predator doll - rest predator - rest	-1.47 0.70 2.43	0.142 0.486 0.015	0.283 0.486 <u>0.046</u>
	Breath rate (/min)	Rest: 40 Doll: 52.17 Predator: 70.59	0.555	94.19 (2)	<u><0.001</u>	169	doll - predator doll - rest predator - rest	-5.62 4.09 9.44	<0.001 <0.001 <0.001	<u><0.001</u> <u><0.001</u> <u><0.001</u>
	CO ₂ production (ppm)	Rest: 1.24 Doll: 1.65 Predator: 3.14	0.310	53.49 (2)	<u><0.001</u>	169	doll - predator doll - rest predator - rest	-4.58 1.73 7.10	<0.001 0.083 <0.001	<u><0.001</u> 0.083 <u><0.001</u>
HP2	Integral (ppm.s)	Rest: 1.71 Doll: 1.47 Predator: 1.94	n/a	3.26 (2)	0.196	219	n/a	n/a	n/a	n/a
	Breath rate (/min)	Rest: 40 Doll: 50 Predator: 63	0.183	41.52 (2)	<u><0.001</u>	219	doll - predator doll - rest predator - rest	-6.44 -3.84 3.42	<0.001 <0.001 <0.001	<u><0.001</u> <u><0.001</u> <u><0.001</u>
	CO ₂ production (ppm)	Rest: 1.76 Doll: 1.23 Predator: 2.00	0.037	10.06 (2)	<u>0.007</u>	219	doll - predator doll - rest predator - rest	-2.74 -2.88 0.18	0.006 0.004 0.854	<u>0.012</u> <u>0.011</u> 0.854
HP3	Integral (ppm.s)	Rest: 2.18 Doll: 2.80 Predator: 2.39	0.035	8.38 (2)	<u>0.016</u>	184	doll - predator doll - rest predator - rest	2.57 2.66 -0.10	0.010 0.007 0.918	<u>0.020</u> <u>0.023</u> 0.918
	Breath rate (/min)	Rest: 41.38 Doll: 40 Predator: 57.14	0.440	81.61 (2)	<u><0.001</u>	184	doll - predator doll - rest predator - rest	-7.84 -1.53 7.74	<0.001 0.126 <0.001	<u><0.001</u> 0.126 <u><0.001</u>
	CO ₂ production (ppm)	Rest: 1.53 Doll: 1.88 Predator: 1.95	0.045	10.22 (2)	<u>0.006</u>	184	doll - predator doll - rest predator - rest	-0.78 1.89 3.08	0.435 0.058 0.002	0.435 0.117 <u>0.006</u>
HP4	Integral (ppm.s)	Rest: 2.67 Doll: 3.46 Predator: 3.98	0.054	11.27 (2)	<u>0.003</u>	174	doll - predator doll - rest predator - rest	-1.43 1.72 3.32	0.154 0.087 <0.001	0.154 0.173 <u>0.003</u>
	Breath rate (/min)	Rest: 41.28 Doll: 46.15 Predator: 46.15	0.195	35.30 (2)	<u><0.001</u>	174	doll - predator doll - rest predator - rest	0.98 5.40 4.30	0.033 <0.001 <0.001	0.033 <u><0.001</u> <u><0.001</u>
	CO ₂ production (ppm)	Rest: 1.75 Doll: 2.60 Predator: 2.76	0.140	25.86 (2)	<u><0.001</u>	174	doll - predator doll - rest predator - rest	-0.78 3.72 4.60	0.435 <0.001 <0.001	0.435 <u><0.001</u> <u><0.001</u>

466

467
468

Table S2 Results of Kruskal-Wallis tests and Dunn (holm adjusted) post hoc tests to investigate differences in the respiratory parameters of individual tippler pigeons exposed to different stimuli

ID	Parameter	Median	σ^2	X ² (df)	P	n	Comparison	z	P.unaj	P.adj
Emil	Integral	Rest: 2.68 Predator: 2.08	0.065	6.90 (1)	<u>0.009</u>	93	Rest-predator only	n/a	n/a	n/a
	Breath rate	Rest: 38.11 Predator: 54.55	P	51.91 (1)	<u><0.001</u>	93	Rest-predator only	n/a	n/a	n/a
	CO ₂ production	Rest: 1.58 Predator: 1.86	0.034	4.13 (1)	<u>0.042</u>	93	Rest-predator only	n/a	n/a	n/a
Emils_son_2	Integral	Rest: 3.52 Doll: 2.91 Predator: 1.77	0.199	30.22 (2)	<u><0.001</u>	145	doll - predator doll - rest predator - rest	3.35 -2.13 -5.44	<0.001 0.033 <0.001	<u>0.002</u> <u>0.033</u> <u><0.001</u>
	Breath rate	Rest: 44.44 Doll: 52.17 Predator: 47.08	n/a	1.91 (2)	0.385	145	n/a	n/a	n/a	n/a
	CO ₂ production	Rest: 2.73 Doll: 2.25 Predator: 1.35	0.018	26.95 (2)	<u><0.001</u>	145	doll - predator doll - rest predator - rest	3.35 -1.79 -5.09	<0.001 0.073 <0.001	<u>0.002</u> 0.071 <u><0.001</u>
TF1	Integral	Rest: 5.15 Doll: 3.66 Predator: 4.60	0.159	19.29 (2)	<u><0.001</u>	112	doll - predator doll - rest predator - rest	-2.47 -4.37 -2.08	0.013 <0.001 0.038	<u>0.027</u> <u><0.001</u> <u>0.038</u>
	Breath rate	Rest: 31.18 Doll: 36.36 Predator: 41.38	0.304	35.13 (2)	<u><0.001</u>	112	doll - predator doll - rest predator - rest	-2.47 -4.37 -2.08	0.014 <0.001 0.038	<u>0.027</u> <u><0.001</u> <u>0.038</u>
	CO ₂ production	Rest: 2.74 Doll: 2.22 Predator: 3.03	0.063	8.91 (2)	<u>0.012</u>	112	doll - predator doll - rest predator - rest	-2.98 -1.61 1.20	0.003 0.108 0.230	<u>0.008</u> 0.216 0.230
TF2	Integral	Rest: 1.72 Doll: 1.34 Predator: 2.18	0.070	13.95 (2)	<u><0.001</u>	174	doll - predator doll - rest predator - rest	-3.73 -1.94 1.59	<0.001 0.053 0.112	<u><0.001</u> 0.106 0.112
	Breath rate	Rest: 43.48 Doll: 50 Predator: 50	0.126	23.55 (2)	<u><0.001</u>	174	doll - predator doll - rest predator - rest	0.30 4.42 4.11	0.767 <0.001 <0.001	0.767 <u><0.001</u> <u><0.001</u>
	CO ₂ production	Rest: 1.35 Doll: 1.12 Predator: 1.73	0.077	15.18 (2)	<u><0.001</u>	174	doll - predator doll - rest predator - rest	-3.73 -0.76 2.76	<0.001 0.447 0.006	<u><0.001</u> 0.447 <u>0.011</u>
TF3	Integral	Rest: 2.95 Doll: 3.21 Predator: 3.64	n/a	1.33 (2)	0.515	142	n/a	n/a	n/a	n/a
	Breath rate	Rest: 45.15 Doll: 41.28 Predator: 50	0.210	31.13 (2)	<u><0.001</u>	142	doll - predator doll - rest predator - rest	-5.60 -2.79 2.83	<0.001 0.005 0.005	<u><0.001</u> <u>0.005</u> <u>0.009</u>
	CO ₂ production	Rest: 2.19 Doll: 2.13 Predator: 3.04	n/a	5.76	0.056	142	doll - predator doll - rest predator - rest	n/a	n/a	n/a

469

ACKNOWLEDGEMENTS: This work was supported by the European Research Council under the European Union's Horizon 2020 research and innovation program (starting grant 715874 to ELCS) and a Max Planck Sabbatical Fellowship (to ELCS).

AUTHOR CONTRIBUTIONS

Conceptualization, ELCS, RPW, KARR; Methodology, ELCS, RPW, KARR; Investigation, KARR, HR, CR; Resources, MW, Formal Analysis, KARR; Writing – Original Draft, ELCS, RPW, KARR; Writing – Review & Editing, ELCS, RPW, KARR, MW, HR, CR; Project Administration, ELCS, KARR; Funding Acquisition, ELCS.;

REFERENCES

- Bevan, R.M., Speakman, J.R. & Butler, P.J. (1995) Daily Energy-Expenditure of Tufted Ducks - a Comparison between Indirect Calorimetry, Doubly Labeled Water and Heart-Rate. *Functional Ecology*, **9**, 40-47.
- Bevan, R.M., Woakes, A.J., Butler, P.J. & Boyd, I.L. (1994) The Use of Heart-Rate to Estimate Oxygen-Consumption of Free-Ranging Black-Browed Albatrosses *Diomedea Melanophrys*. *Journal of Experimental Biology*, **193**, 119-137.
- Bevan, R.M., Woakes, A.J., Butler, P.J. & Croxall, J.P. (1995) Heart-Rate and Oxygen-Consumption of Exercising Gentoo Penguins. *Physiological Zoology*, **68**, 855-877.
- Brent, R., Pedersen, P.F., Bech, C. & Johansen, K. (1984) Lung Ventilation and Temperature Regulation in the European Coot *Fulica atra*. *Physiological Zoology*, **57**, 19-25.
- Bretz, W.L. & Schmidt-Nielsen, K. (1971) Bird respiration: flow patterns in the duck lung. *Journal of Experimental Biology*, **54**, 103-118.
- Bucher, T.L. & Bartholomew, G.A. (1986) The early ontogeny of ventilation and homeothermy in an altricial bird, *Agapornis roseicollis* (Psittaciformes). *Respir Physiol*, **65**, 197-212.
- Butler, P.J., Green, J.A., Boyd, I.L. & Speakman, J.R. (2004) Measuring metabolic rate in the field: the pros and cons of the doubly labelled water and heart rate methods. *Functional Ecology*, **18**, 168-183.
- Butler, P.J., West, N.H. & Jones, D.R. (1977) Respiratory and cardiovascular responses of the pigeon to sustained level flight in a wind-tunnel *Journal of Experimental Biology*, **71**, 7-26.
- Charmantier, A., Demeyrier, V., Lambrechts, M., Perret, S. & Grégoire, A. (2017) Urbanization Is Associated with Divergence in Pace-of-Life in Great Tits. *Frontiers in Ecology and Evolution*, **5**.
- Clinchy, M., Sheriff, M.J. & Zanette, L.Y. (2013) Predator-induced stress and the ecology of fear *Functional Ecology*, **27**, 56-65.
- Doss, G.A. & Mans, C. (2016) Changes in Physiologic Parameters and Effects of Hooding in Red-tailed Hawks (*Buteo jamaicensis*) During Manual Restraint. *J Avian Med Surg*, **30**, 127-132.
- Doss, G.A. & Mans, C. (2017) The Effect of Manual Restraint on Physiological Parameters in Barred Owls (*Strix varia*). *J Avian Med Surg*, **31**, 1-5.
- El Hadi, H. & Sykes, A.H. (1982) Thermal panting and respiratory alkalosis in the laying hen. *Br Poult Sci*, **23**, 49-57.

- Elliott, K.H., Le Vaillant, M., Kato, A., Speakman, J.R. & Ropert-Coudert, Y. (2013) Accelerometry predicts daily energy expenditure in a bird with high activity levels. *Biology Letters*, **9**.
- Fitzpatrick, J.W. (1980) Foraging behaviour of neotropical tyrant flycatchers. *The Cooper Ornithological Society* **82**, 43-57.
- Franz, M. & Goller, F. (2003) Respiratory patterns and oxygen consumption in singing zebra finches. *Journal of Experimental Biology*, **206**, 967-978.
- Frappell, P.B. & Baudinette, R.V. (1995) Scaling of respiratory variables and the breathing pattern in adult marsupials. *Respir Physiol*, **100**, 83-90.
- Frappell, P.B., Hinds, D.S. & Boggs, D.F. (2001) Scaling of respiratory variables and the breathing pattern in birds: an allometric and phylogenetic approach. *Physiol Biochem Zool*, **74**, 75-89.
- Fucikova, E., Drent, P.J., Smits, N. & van Oers, K. (2009) Handling Stress as a Measurement of Personality in Great Tit Nestlings (*Parus major*). *Ethology*, **115**, 366-374.
- Gabrielsen, G.W., Mehlum, F., Karlsen, H., Andersen, O. & Parker, H. (1991) Energy cost during incubation and thermoregulation in the female common eider *Somateria mollissima* *Norsk Polarinstitutt Skrifter*, **195**, 51-62.
- Gleiss, A.C., Wilson, R.P. & Shepard, E.L. (2011) Making overall dynamic body acceleration work: on the theory of acceleration as a proxy for energy expenditure. *Methods in Ecology and Evolution*, **2**, 23-33.
- Green, J.A. (2011) The heart rate method for estimating metabolic rate: review and recommendations. *Comp Biochem Physiol A Mol Integr Physiol*, **158**, 287-304.
- Greenacre, C.B. & Lusby, A.L. (2004) Physiologic responses of Amazon parrots (*Amazona* species) to manual restraint. *Journal of Avian Medicine and Surgery*, **18**, 19-22.
- Hallam, J.F. & Dawson, T.J. (1993) The pattern of respiration with increasing metabolism in a small dasyurid marsupial. *Respir Physiol*, **93**, 305-314.
- Halsey, L.G. & Bryce, C.M. (2021) Proxy problems: Why a calibration is essential for interpreting quantified changes in energy expenditure from biologging data. *Functional Ecology*, **35**, 627-634.
- Halsey, L.G., Shepard, E.L., Quintana, F., Gomez Laich, A., Green, J.A. & Wilson, R.P. (2009) The relationship between oxygen consumption and body acceleration in a range of species. *Comp Biochem Physiol A Mol Integr Physiol*, **152**, 197-202.
- Halsey, L.G., Shepard, E.L.C. & Wilson, R.P. (2011) Assessing the development and application of the accelerometry technique for estimating energy expenditure. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, **158**, 305-314.
- Hawkins, P.A.J., Butler, P.J. & Speakman, J.R. (2000) Estimation of the rate of oxygen consumption of the common eider duck (*Somateria mollissima*), with some measurements of heart rate during voluntary dives. *The Journal of Experimental Biology*, **203**, 2819-2832.
- Kohin, S., Williams, T.M. & Ortiz, C.L. (1999) Effects of hypoxia and hypercapnia on aerobic metabolic processes in northern elephant seals. *Respir Physiol*, **117**, 59-72.
- Langman, V.A., Rowe, M.F., Roberts, T.J., Langman, N.V. & Taylor, C.R. (2012) Minimum cost of transport in Asian elephants: do we really need a bigger elephant? *Journal of Experimental Biology*, **215**, 1509-1514.
- Lemon, W.C. (1991) Fitness consequences of foraging behaviour in the zebra finch. *Nature*, **352**, 153-155.
- Liang, D., He, C., Luo, X., Liu, Y., Goodale, E. & Pagani-Nunez, E. (2018) Breath rate of passerines across an urbanization gradient supports the pace-of-life hypothesis and suggests diet-mediated responses to handling stress. *Ecol Evol*, **8**, 9526-9535.
- Lighton, J.R.B. (2019) *Measuring metabolic rates : a manual for scientists*, Second edition. edn. Oxford University Press, Oxford ; New York, NY.
- Maina, J.N. (2005) *The lung-air sacs system of birds*. Springer, Berlin.

- Morris, C.R., Nelson, F.E. & Askew, G.N. (2010) The metabolic power requirements of flight and estimations of flight muscle efficiency in the cockatiel (*Nymphicus hollandicus*). *Journal of Experimental Biology*, **213**, 2788-2796.
- Mortola, J.P. & Seguin, J. (2009) Resting breathing frequency in aquatic birds: a comparative analysis with terrestrial species. *Journal of Zoology*, **279**, 210-218.
- Mullner, A., Linsenmair, K.E. & Wikelski, M. (2004) Exposure to ecotourism reduces survival and affects stress response in hoatzin chicks (*Opisthocomus hoazin*). *Biological Conservation*, **118**, 549-558.
- Nagy, K.A., Siegfried, W.R. & Wilson, R.P. (1984) Energy-Utilization by Free-Ranging Jackass Penguins, *Spheniscus-Demersus*. *Ecology*, **65**, 1648-1655.
- Nolet, B.A., Butler, P.J., Masman, D. & Woakes, A.J. (1992) Estimation of Daily Energy-Expenditure from Heart-Rate and Doubly Labeled Water in Exercising Geese. *Physiological Zoology*, **65**, 1188-1216.
- Nowack, J., Stawski, C. & Geiser, F. (2017) More functions of torpor and their roles in a changing world. *J Comp Physiol B*, **187**, 889-897.
- Odom, K.J., Hall, M.L., Riebel, K., Omland, K.E. & Langmore, N.E. (2014) Female song is widespread and ancestral in songbirds. *Nature Communications*, **5**.
- Perry, S.F., Lambert, M. & Shmitz, A. (2019) *Respiratory biology of animals: evolutionary and functional morphology*. Oxford University Press.
- Rabadeau, J., Badenhausser, I., Moreau, J., Bretagnolle, V. & Monceau, K. (2019) To change or not to change experimenters: caveats for repeated behavioural and physiological measures in Montagu's harrier. *Journal of Avian Biology*, **50**.
- Shaffer, S.A., Costa, D.P. & Weimerskirch, H. (2003) Foraging effort in relation to the constraints of reproduction in free-ranging albatrosses. *Functional Ecology*, **17**, 66-74.
- Sutton, G.J., Botha, J.A., Speakman, J.R. & Arnould, J.P.Y. (2021) Validating accelerometry-derived proxies of energy expenditure using the doubly labelled water method in the smallest penguin species. *Biology Open*, **10**.
- Tillmann, J. (2009) Fear of the dark: night-time roosting and anti-predation behaviour in the grey partridge (*Perdix perdix* L.). *Behaviour*, **146**, 999-1023.
- Torné-Noguera, A., Pagani-Núñez, E. & Senar, J.C. (2013) Great Tit (*Parus major*) breath rate in response to handling stress: urban and forest birds differ. *Journal of Ornithology*, **155**, 315-318.
- Tucker, V.A. (1972) Metabolism during flight in the laughing gull, *Larus atricilla*. *American Journal of Physiology* **222**.
- Ward, S., Bishop, C.M., Woakes, A.J. & Butler, P.J. (2002) Heart rate and the rate of oxygen consumption of flying and walking barnacle geese (*Branta leucopsis*) and bar-headed geese (*Anser indicus*). *Journal of Experimental Biology*, **205**, 3347-3356.
- Ward, S., Moller, U., Rayner, J.M.V., Jackson, D.M., Bilo, D., Nachtigall, W. & Speakman, J.R. (2001) Metabolic power, mechanical power and efficiency during wind tunnel flight by the European starling *Sturnus vulgaris*. *Journal of Experimental Biology*, **204**, 3311-3322.
- Wilson, R.P., Borger, L., Holton, M.D., Scantlebury, D.M., Gomez-Laich, A., Quintana, F., Rosell, F., Graf, P.M., Williams, H., Gunner, R., Hopkins, L., Marks, N., Gerdali, N.R., Duarte, C.M., Scott, R., Strano, M.S., Robotka, H., Eizaguirre, C., Fahlman, A. & Shepard, E.L.C. (2020) Estimates for energy expenditure in free-living animals using acceleration proxies: A reappraisal. *Journal of Animal Ecology*, **89**, 161-172.
- Wilson, R.P. & Culik, B.M. (1993) Activity-specific metabolic rates from doubly labelled water studies: are activity costs underestimated? *Ecology*, **74**, 1285-1287.
- Wilson, R.P., Holton, M., Wilson, V.L., Gunner, R., Tysse, B., Wilson, G.I., Quintana, F., Duarte, C. & Scantlebury, D.M. (2019) Towards informed metrics for examining the role of human-induced animal responses in tag studies on wild animals. *Integrative Zoology*, **14**, 17-29.

- Wilson, R.P., White, C.R., Quintana, F., Halsey, L.G., Liebsch, N., Martin, G.R. & Butler, P.J. (2006) Moving towards acceleration for estimates of activity-specific metabolic rate in free-living animals: the case of the cormorant. *Journal of Animal Ecology*, **75**, 1081-1090.
- Withers, P.C. (1977) Respiration, metabolism, and heat exchange of euthermic and torpid poorwills and hummingbirds. *Physiological Zoology*, **50**, 43-52.