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RESEARCH ARTICLE

Experimental reduction in blood oxygen-carrying capacity alters foraging behaviour in a colonial waterbird

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ABSTRACT

Oxidative metabolism is a key component of organismal physiology and it is primarily determined by aerobic capacity, which depends on the capacity of blood to carry oxygen. However, experimental manipulations of blood oxygen-carrying capacity are rarely implemented to test ecophysiological hypotheses in vertebrate populations. Here, we combined an experimental manipulation of blood oxygen-carrying capacity with GPS tracking to test whether suboptimal (reduced) haematological performance affects foraging behaviour in a colonial waterbird, the black-headed gull, *Chroicocephalus ridibundus*. First, a validation of phenylhydrazine (PHZ) treatment in gulls revealed a 9–18% reduction in haematocrit and blood haemoglobin concentration (via oxidative denaturation and haemolysis of erythrocytes). Then, GPS tracking of experimental (PHZ-treated) and control (saline-treated) gulls during the incubation period provided no support for reduced or suspended engagement in energetically costly activities (long-distance foraging trips) by experimental birds. Instead, we found evidence for fine-scale alterations in foraging behaviour of PHZ-treated individuals, which resulted in fewer foraging trips per unit time, but trips that were longer in duration and distance compared with those of control birds. This suggests reduced foraging performance of experimental birds (e.g. lower capacity to find and collect food during trips) or evasion of social competition, although no differences in the total investment in foraging may also suggest compensatory physiological responses to haemolytic anaemia. Our study contributes to a better understanding of the physio-ecological nexus in non-diving colonial avian species. Whether behavioural effects of reduced aerobic capacity have any implications for gull condition and reproductive performance should be the subject of further investigation.

KEY WORDS: Birds, Oxidative metabolism, Foraging flights, GPS tracking, Haematocrit, Haemoglobin concentration

INTRODUCTION

Blood oxygen-carrying capacity is a key component of oxidative metabolism and oxygen consumption, as, in conjunction with cardiac output (the product of heart rate and stroke volume) it determines the amount of oxygen delivered to tissues per unit time (Campbell, 1995). Traditional proxies of blood oxygen-carrying capacity include haematocrit (i.e. the relative volume of red blood cells in blood; Hct) and total blood haemoglobin (Hb) concentration, both forming an important part of the oxygen transport cascade. Although other physiological parameters (e.g. cardiac output or Hb oxygen affinity) contribute to the aerobic performance and oxygen consumption rate of an organism (according to the Fick equation; Milsom et al., 2021), low Hct and Hb are primarily indicative for relative anaemia and their elevated levels can enhance oxygen transport by increasing the total erythrocyte number (per plasma volume) and mean erythrocyte Hb content. Although any alterations in Hct and Hb can be compensated for by other components of the oxygen cascade (Scott, 2011; Laguë, 2017), these quick and simple measurements can be easily applied both in the laboratory and directly in the field, and are thus commonly used to quantify blood oxygen-carrying capacity in wild animals, including birds (reviewed in Fair et al., 2007; Minias, 2015). While Hct and Hb are based on different biological principles (blood cytology and blood biochemistry, respectively) and may show fine-scale differences in sensitivity to ecological or environmental factors (Bańbura et al., 2007), they often correlate well at the intra- and inter-specific levels (Velguth et al., 2010; Yap et al., 2019; Minias, 2020), and may provide reliable complementary information on the potential of an organism to satisfy its oxygen demand.

Hct and Hb levels have been shown to correspond with a broad spectrum of condition-related and reproductive traits in natural bird populations (reviewed in Fair et al., 2007; Minias, 2015). However, most of this research on avian Hct and Hb is purely correlative. The primary focus of experimental research has been on testing how ecological manipulations affect haematological parameters, rather than vice versa. For example, experimental manipulations of diet quality have shown a negative impact of nutritional stress (low quality diet) on Hct and Hb in two estrildid finches (Pryke et al., 2012; Pryke and Rollins, 2012). An experimental infestation of captive birds with either blood-sucking ectoparasites or haematozoans revealed reduced Hct levels in infested compared with control birds (Heylen and Matthysen, 2008; Cornet et al., 2014) and, similarly, experimental parasite reductions in natural conditions (e.g. via nest sterilization or medication treatment) enhanced haematological performance of experimental birds (Słomczyński et al., 2006; Schoenle et al., 2017). Finally, experimental manipulation of resource allocation in reproduction (e.g. by experimentally increased egg production) resulted in decreased haematological parameters (Kalmbach et al., 2004), whereas an increase in reproductive effort (e.g. by experimental brood enlargement or mate removal) and flight costs (e.g. by feather or foraging behaviour manipulations) often led to upregulation of Hct and Hb, reflecting responses to elevated energy requirements and oxygen demands (Saino et al., 1997; Hõrak et al., 1998;

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Kilpimaa et al., 2004; Yap et al., 2021; but see Navarro and González-Solís, 2007; Fowler and Williams, 2017).

While we seem to have a thorough knowledge on the sources of variation in avian haematology, experimental studies investigating how haematology affects bird performance and behaviour are generally lacking. This kind of experimental framework requires reversible manipulation of blood oxygen-carrying capacity, e.g. by the application of treatments that cause denaturation or haemolysis of red blood cells. Although the protocols for the application of these experimental procedures in wild birds have been established and validated (Williams et al., 2012), their implementation in avian research is far from ubiquitous. In fact, we are aware of experimental manipulations of blood oxygen-carrying capacity in only a couple of wild passerine birds, showing effects of reduced Hct on nesting behaviour and parental reproductive performance (Fronstin et al., 2016; Griebel and Dawson, 2020). Also, an assessment of flight performance in a hypobaric wind tunnel showed that yellow-rumped warblers, *Setophaga coronate*, with reduced Hct had lower flight capacity at low altitudes (Yap et al., 2018). Notably, these results strongly suggest that birds performing long flights to search for food may alter their foraging behaviour dependent on their haematological status.

Here, we combined an experimental manipulation of blood oxygen-carrying capacity with GPS tracking to test whether suboptimal (reduced) haematological performance affects foraging behaviour of a wild colonial waterbird, the black-headed gull, *Chroicocephalus ridibundus* (Linnaeus 1766). This species nests in large colonies (as many as several thousand birds), usually on inland water bodies and wetlands, and it performs long foraging flights during the breeding season (up to ∼60 km of foraging trip distance; Jakubas et al., 2020). Following Williams et al. (2012), we used phenylhydrazine hydrochloride (PHZ) to induce a reversible reduction of Hct and Hb, primarily caused by oxidative denaturation and haemolysis of red blood cells, with no reported acute side-effects in vertebrates (Fronstin et al., 2016). We expected that PHZ-treated birds should reduce the total investment in foraging (by reducing the frequency, duration or distance of their foraging trips) to keep energy expenditure at low levels under a handicapped aerobic performance and flight capacity (activity reduction hypothesis). Alternatively, we expected that an application of PHZ treatment may increase the duration and distance of individual foraging trips, compensating for poorer flight/foraging performance of experimental birds and reflecting greater time investments required to satisfy energetic demands (compensation hypothesis).

MATERIALS AND METHODS

Study area

We conducted all experimental procedures in 2021–2022 in the black-headed gull colony near Przykona village in central Poland (52°00′20.6″N; 18°39′28.2″E). The colony was located on a small (3.5 ha) islet on an artificial lake (165 ha) established in 2004 by flooding a former open-pit lignite mine. Colony size estimates showed strong fluctuations between years, ranging from 1500 to 3500 breeding pairs of black-headed gulls, mixed with a small number of Caspian gulls, *Larus cachinnans* (ca. 10 pairs). The landscape around the colony was primarily composed of agricultural areas, barren vegetation and pine forests (Fig. 1) and our previous research using GPS tracking indicated that black-headed gulls from the study colony show little selectivity in habitat use during foraging flights, except for the selective avoidance of artificial areas (forests were excluded from the analyses as they are generally unfavourable for gull foraging; Jakubas et al., 2020).

Validation of experimental procedures

We experimentally reduced blood oxygen-carrying capacity in the black-headed gull using PHZ, following methodology developed by Williams et al. (2012). Among wild and captive birds, experimental use of PHZ has only been validated for a couple of passerines, including common starling, *Sturnus vulgaris* (Fronstin et al., 2016), tree swallow, *Tachycineta bicolor* (Griebel and Dawson, 2020), and zebra finch, *Taeniopygia guttata* (Williams et al., 2012), and thus we first aimed to validate experimental procedures in our study species. For this purpose, during the early breeding season (May) in 2021, we captured 20 black-headed gulls nesting in our study colony (henceforth referred to as the validation group). We captured all birds during the incubation period using nest traps (nylon loops). At capture, we punctured the ulnar vein of each bird with a disposable needle to collect blood samples for the measurement of Hct and Hb, which we used as proxies for blood oxygen-carrying capacity. To measure Hct, we collected 40 µl of blood into heparinized capillary tubes. We kept all tubes cooled and centrifuged them at 10,000 rpm for 5 min within 8 h of blood collection. We determined Hct using a microhaematocrit reader. To measure Hb, we collected 5 µl of blood into a disposable HemoCue microcuvette and used a portable photometer HemoCue Hb 201+ (HemoCue, Ängeholm, Sweden) for an immediate Hb measurement. The HemoCue photometer uses the azide-methaemoglobin method for spectrometric Hb measurements and has been widely used in a broad spectrum of wild and captive avian species (e.g. Velguth et al., 2010; Amos et al., 2013; Ishtiaq and Barve, 2018). After blood sampling, we measured body mass with an electronic balance (to the nearest 1 g) and subjected each bird to PHZ treatment by intramuscular (pectoral muscle tissue) injection of 1.25 µl of saline solution with 12.5 µg PHZ (Sigma-Aldrich, St Louis, MO, USA) per 1 g body mass. We marked all PHZ-treated birds from the validation group with metal and plastic rings with alpha-numeric codes (to facilitate targeted recapture) and released them with no GPS loggers within 20 min of capture. We carried out all the procedures in accordance with guidelines for the use of animals in research (Buchanan et al., 2012) and report results following the recommendations of the ARRIVE guidelines (Kilkenny et al., 2010). We conducted all experiments by permission of the Local Bioethical Commission for Experiments on Animals in Łódź and the Regional Environmental Protection Directorate in Poznań, Poland. All values are reported as means±s.e.m.

Previous validation of PHZ treatment in zebra finches and European starlings showed that Hct and Hb returned to pre-injection levels within 5–10 days of treatment (Williams et al., 2012), while in a laboratory study of Japanese quails, *Coturnix japonica*, PHZ-induced haemolytic anaemia was still apparent after 72 h (3 days) (Clark et al., 1988). Thus, to assess PHZ effects in gulls, we aimed to recapture PHZ-treated gulls at 2–7 days post-treatment. We successfully recaptured 18 individuals from the validation group and repeated Hct and Hb measurements. We found a significant reduction in both Hct and Hb (*t*-test for dependent samples: t=6.31, d.f.=17, P<0.001 for Hct; t=14.03, d.f.=17, P<0.001 for Hb) and post-treatment values were on average 9.3±1.4% (Hct) and 16.4±1.1% (Hb) lower compared with pre-treatment levels (Fig. 2). Individuals from the final experimental group (i.e. PHZ treated and GPS logger equipped, see details below) showed a similar significant reduction in Hct (9.4±2.7%; t=3.44, d.f.=10, P=0.006) and Hb (18.3±1.3%; t=15.13, d.f.=14, P<0.001) within 2–3 days post-treatment (Fig. 2), when most data on foraging trips

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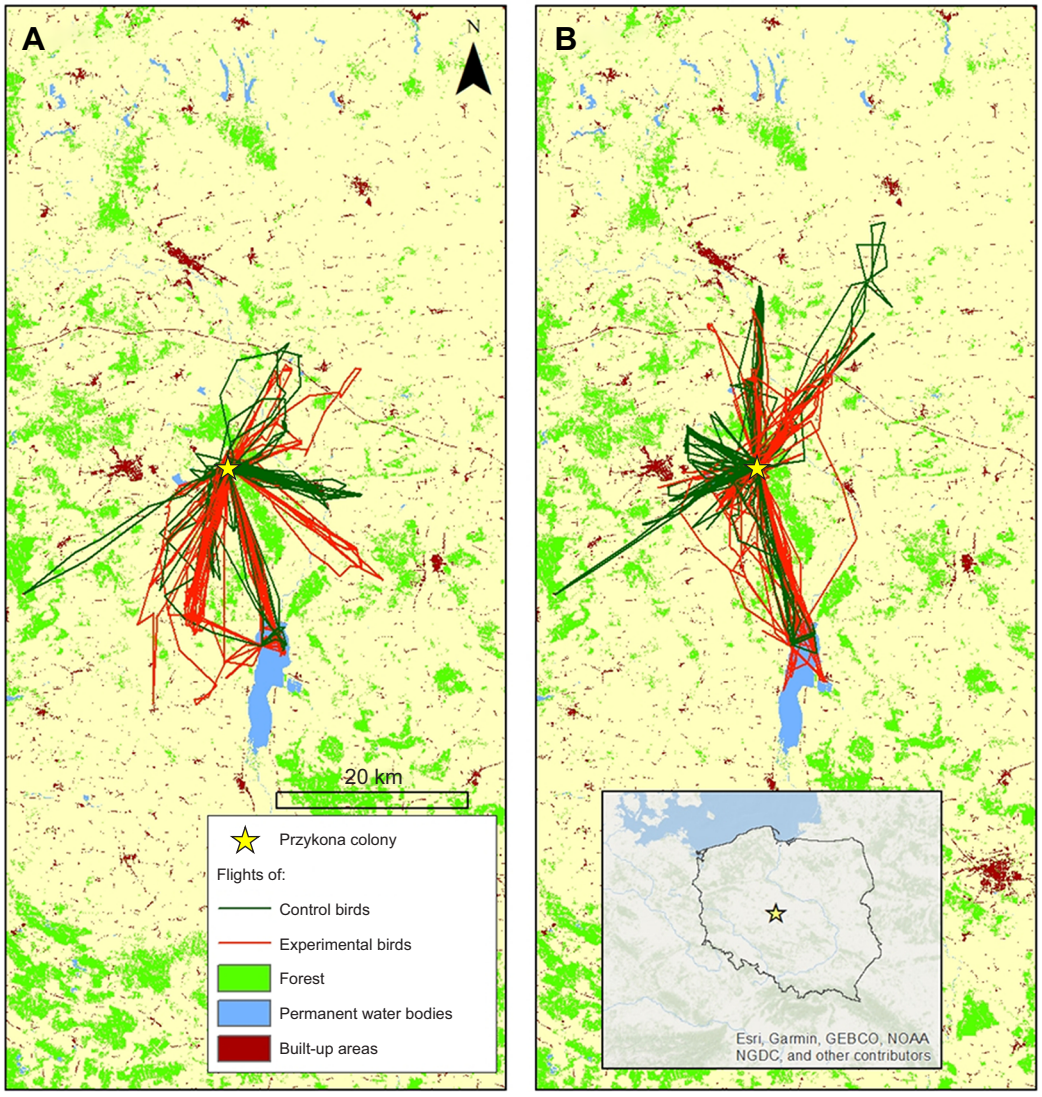

Fig. 1. A map of the foraging trips of GPS-tracked black-headed gulls. (A) 2021; (B) 2022. Trips of experimental [phenylhydrazine (PHZ)-treated] and control (saline-treated) birds are marked with red and green lines, respectively. Maps were created based on data from Pan-European High Resolution Layer: Imperviousness Degree (IMD) 2012, Forest Type (FTY) 2012, and Permanent Water Bodies (PWB) 2012 [European Environment Agency (EEA); https://land.copernicus.eu/pan-european/high-resolution-layers]. The inset in B shows the location of the study black-headed gull colony (Przykona) in central Poland (52°00′20.6″N; 18°39′28.2″E).

were collected (see details below). In contrast, control individuals (i.e. saline treated and GPS logger equipped, see details below) showed no significant reduction in Hct within 2–3 days post-treatment (1.9±1.2%; t=1.66, d.f.=20, P=0.11) (Fig. 2). Although control birds showed a significant reduction in Hb within 2–3 days post-treatment (5.9±1.3%; t=4.60, d.f.=17, P<0.001), possibly facilitated by blood sampling, the magnitude of this reduction was significantly lower compared with that in experimental birds (t-test for independent samples: t=6.70, d.f.=31, P<0.001) (Fig. 2). Post-treatment Hct and Hb levels stayed within the physiological range established for the black-headed gull (Mostaghni et al., 2005), but average Hb did not exceed the lower quartile values reported for this species during the breeding period in central Europe (Minias et al., 2019). In conclusion, all analyses of pre- and post-treatment Hct and Hb measurements provided clear support for an effective short-term reduction in blood oxygen-carrying capacity as a result of our experimental procedures (PHZ treatment). Despite a wide application of PHZ treatment to induce anaemia in fish and mammals (reviewed in Berger, 2007), no side-effects (unrelated to red blood cells) of this manipulation are known, except for chronic mutagenic and genotoxic effects (e.g. Zeljezic et al., 2016), which are thought to be unlikely to affect individual performance over short time scales. While we cannot rule out an occurrence of acute side-effects of PHZ treatment in black-headed gulls, previous research in wild avian species deemed this possibility unlikely (Fronstin et al., 2016) and, thus, we primarily attributed all the differences in foraging behaviour between our experimental and control groups to experimentally reduced blood oxygen-carrying capacity.

Experimental manipulations

After successful validation of the PHZ treatment, we selected 50 adult individuals for our final experiment. We captured birds during the incubation period from mid-May to mid-June in 2021 (n=20 individuals) and 2022 (n=30 individuals) and randomly assigned them to either the experimental (PHZ-treated) or control

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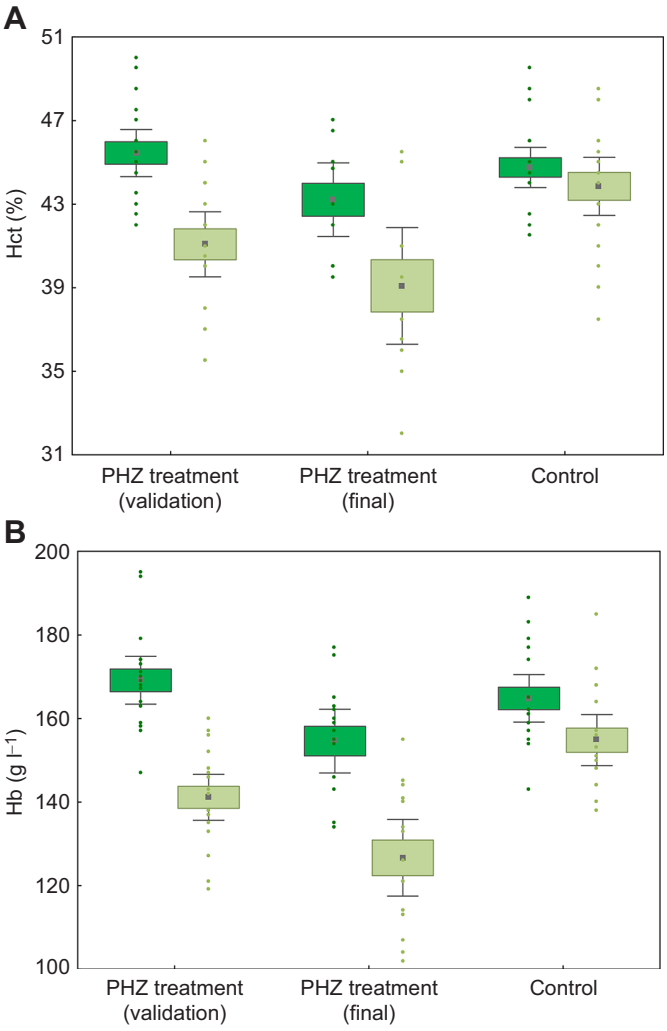

Fig. 2. Pre- and post-treatment levels of haematocrit and total blood haemoglobin concentration in experimental (PHZ-treated) and control (saline-treated) black-headed gulls. (A) Haematocrit (Hct) and (B) haemoglobin (Hb) concentration pre- (dark green) and post-treatment (light green). Experimental birds were divided into the groups used in experiment validation (no GPS loggers attached) and in the final experiment (GPS loggers attached). Data are shown for birds captured 2–7 days post-treatment (experiment validation: n=19) or 2–3 days post-treatment (final experiment: n=11; control: n=21). Box plots show means (central point), s.e.m. (box) and 95% confidence intervals (whiskers).

(saline-treated) group. On average, black-headed gulls incubate for 23 days (range 22–26 days) (Eising and Groothuis, 2003) and we avoided capturing birds during the first week after egg laying to reduce the risk of nest abandonment. We captured one parent per nest to avoid non-independency in foraging behaviour. All capture and marking procedures were identical to those described for the validation group. Pre-treatment body mass was measured and we also collected basic biometric measurements, including folded wing length (measured to the nearest 1 mm with a stopped ruler). We used the same procedures for blood sampling and Hct/Hb measurements as for the validation group, although we collected an additional 20 µl of blood into 96% ethanol for molecular sexing. We stored these samples at 4°C until analysis. We isolated DNA with a Bio-Trace DNA Purification Kit (EURx, Gdańsk, Poland) according to the kit protocol and we followed methodology developed by Griffiths et al. (1998) for molecular sex identification. Briefly, we amplified sex-specific fragments of chromodomain helicase DNA-binding region by PCR with a pair of P2/P8 primers and separated the products by 2% agarose gel electrophoresis, allowing us to distinguish females (two bands) and males (one band). Male and female black-headed gulls show similar parental investment and actively participate in incubation (Eising and Groothuis, 2003), so the two sexes should be roughly equally represented in the sample under random nest captures. Consistent with this expectation, sex ratio within our experimental and control samples did not significantly deviate from parity (G-test, G=2.72, d.f.=1, P=0.10). We subjected all birds from the experimental group to PHZ treatment (intramuscular injection of 1.25 µl of saline solution with 12.5 µg PHZ per 1 g body mass), while all birds from the control group were intramuscularly injected with 1.25 µl of saline per 1 g body mass. Experimental and control birds did not differ significantly in their pre-treatment levels of Hct (45.06±0.51% versus 44.50±0.47%; t-test: P=0.42) and Hb (160.9±2.6% versus 160.2 ±3.1%; t-test: P=0.86).

Foraging trips

We equipped all experimental and control birds with GPS loggers to collect data on their foraging behaviour. We used PinPoint-10 and PinPoint-40 store-on-board GPS loggers (Lotek Wireless Inc., St John's, NL, Canada), recording time and position. We attached all loggers to the central back feathers of each bird using two 3–4 mm wide strips of Tesa tape code 4965 (Tesa Tape Inc., Charlotte, NC, USA) applied crosswise. The standard logger mass including attachment was 1.6 g (PinPoint-10) and 2.3 g (PinPoint-40), being equivalent to an average of 0.6–0.9% of body mass of captured gulls, which was well under the 3–5% threshold recommended for external devices borne by flying animals (Portugal and White, 2018). While we acknowledge that logger attachment may possibly affect foraging behaviour (e.g. as a result of elevated energy expenditure associated with additional load) and that these effects should be proportional to device mass (Vandenabeele et al., 2012), we randomly assigned loggers of different types (mass) to control and experimental birds and, thus, we expected no directional bias between the two groups. We set the loggers to start recording position from the early morning after capture to avoid any direct effects of handling stress on bird behaviour. We set loggers to collect GPS position in 15 min intervals and after 2 days of tracking we started to recapture birds to retrieve loggers and conduct post-treatment measurement of Hct and Hb.

We successfully recaptured and retrieved high-quality GPS tracking data from 23 control and 22 experimental birds (n=18 individuals in 2021 and n=27 individuals in 2022). Birds were tracked for an average of 43.54±4.40 h (maximum 105.23 h), recording 176.11±17.93 GPS fixes per bird. In the downstream analyses, we retained only diurnal GPS fixes (80.1% of all recorded positions), as nocturnal flights probably represent roosting rather than foraging activity in gulls (Indykiewicz et al., 2021). We considered all diurnal flights with at least one GPS position recorded ≥1 km from the colony as foraging trips. We characterized each foraging trip with the following variables: (i) total distance covered (km) – the sum of the distances between all GPS positions recorded during the trip; (ii) maximum distance from the colony (km) – the distance from the colony to the most distal GPS position recorded during the trip; and (iii) trip duration (min) – the time interval between departure and return to the colony. Based on the time intervals and distances between consecutive GPS positions during foraging trips, we calculated average inter-position speed. Following Shaumoun-Baranes et al. (2011), we used these estimates to categorize inter-position intervals as primarily representing an active (flight with speed >4 km h^{-1}) or non-active (stationary or

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on-ground movement with speed ≤4 km h^{-1}) phase of a foraging trip. To estimate the total duration of an active and non-active phase, we summed the intervals of each category within each foraging trip. In total, we recorded 279 foraging trips (Fig. 1) and we also synthesized these data to characterize foraging behaviour at the individual level (i.e. for particular individuals) across the total tracking time. For this purpose, we quantified the following traits for each experimental and control bird: (i) total number of foraging trips; (ii) total distance covered during all trips; (ii) total time spent on foraging trips; and (iv) total time spent in active (flight) or non-active (stationary or on-ground movement) phases during all foraging trips. The data used in the study are available from the corresponding author upon request.

Statistical analyses

We conducted a two-step modelling with individuals and foraging trips used as a unit of analysis. In the first step (individual-level analysis) we focused on the following traits: total number of foraging trips, total distance covered during trips, total time spent on foraging trips and total time of active or non-active phases of foraging trips. In the second step (trip-level analysis), we analysed the total distance covered per foraging trip, maximum distance from the colony per trip, trip duration, duration of active or non-active phases of each trip, and an average inter-position speed for active flight intervals. We entered each of these traits as a response variable in separate models. Because of a strong right skewness (up to 2.5), we log-transformed all trip-level response variables (except for maximum distance) prior to analyses, which improved normality of their distributions (final skewness ≤1). We analysed the total number of foraging trips with a generalized linear mixed model with the Poisson distribution of the response variable and log-link function. All the other traits were analysed with general linear mixed models (Gaussian distribution). We entered the effects of PHZ treatment and sex as fixed factors, while pre-treatment Hb concentration, date and wing length were entered as covariates in each model. In the first step (individual-level analysis), we entered log total tracking time either as an offset (Poisson distribution model) or as an additional covariate (Gaussian distribution models), so that our results were standardized per unit time. We entered year as a random factor in all the models, while bird identity was included as another random factor in the second step (trip-level analysis) to avoid pseudoreplication resulting from repeated measurements (trips) of the same of individuals. Trip identity was also included as another random factor in the analysis of the average inter-position speed, as multiple active flight intervals were measured per trip. As birds were equipped with two different types of loggers, we also re-ran all the models with logger type included as a random factor, but they yielded qualitatively consistent results and, thus, are not shown. To test whether the effect of PHZ treatment is consistent across males and females, we included a treatment×sex interaction in all full models (removed if non-significant). To obtain more parsimonious reduced models, we also removed highly non-significant (P>0.10) predictors from the initial full models. We found no evidence for multicollinearity among predictors indicated by low values (<1.75) of the variance inflation factors, as calculated in the *car* package (Fox and Weisberg, 2019) developed for the R statistical environment (R Foundation for Statistical Computing, Vienna, Austria). We fitted all the models in the *lme4* R package (Bates et al., 2014) and calculated semi-partial R^2 estimates for all predictors using the Nakagawa and Schielzeth approach (Johnson, 2014) with the *r2beta* function implemented in the *r2glmm* R package (Jaeger et al., 2017).

RESULTS

Individual-level analysis

We found that birds from the experimental group performed significantly fewer foraging trips per unit time (on average 2.03 ±0.22 trips per 12 h) than control birds (2.88±0.27 trips per 12 h) (P=0.001; Table S1; Fig. 3A). The effect was consistent across males and females, as indicated by a non-significant (P=0.76) treatment×sex interaction. The total number of trips was not affected by pre-treatment Hb, sex or date (Table S1). Despite differences in the number of trips, we recorded no significant variation between experimental and control birds in the total distance covered during all trips per unit time (36.56±3.28 versus 35.43±3.73 km per 12 h, respectively; P=0.84) and in the total time spent on foraging trips (4.92±0.39 versus 5.17±0.29 h foraging per 12 h of GPS tracking, respectively; P=0.70) (Table S2). Similarly, the total time spent in active (flight) and non-active phases of the trips (across all trips per unit time) was similar for experimental and control birds (active phase: P=0.87; non-active phase: P=0.58; Table S3). Date was identified as the only significant predictor of the total time spent on foraging trips, as late breeding birds spent more time on trips than early breeders (P=0.018; Table S2). This association was explained by longer active and non-active phases of foraging trips in late breeders (Table S3). Interaction terms between treatment and sex were non-significant (all P>0.55) and removed from all the models.

Trip-level analysis

The distance covered per each foraging trip was significantly larger in experimental (24.51±1.64 km) than in control birds (17.40±1.33 km; P=0.020) (Table S4; Fig. 3B). Longer distances of foraging trips in experimental individuals were accompanied by longer trip duration (3.38±0.30 versus 2.54±0.20 h; P=0.033) (Table S4; Fig. 3C). This could be primarily attributed to a longer duration of active flight during each trip in experimental birds (1.37±0.08 h) compared with control individuals (1.09±0.06 h; P=0.041) (Table S5; Fig. 3D), as the duration of the non-active phase did not differ between the two groups (P=0.22; Table S5). All these effects were consistent across males and females, as indicated by non-significant treatment×sex interactions (all P>0.35). The maximum distance from the colony (per individual trip) and the average inter-position speed during active flight phases of a foraging trip did not differ significantly between experimental and control birds (maximum distance: P=0.09; inter-position speed: P=0.14; Table S6).

DISCUSSION

Our experimental study on black-headed gulls showed that reductions in blood oxygen-carrying capacity alter their foraging behaviour during the breeding season. Most importantly, experimental (PHZ-treated) birds showed longer, more distant and less frequent foraging trips than control (saline-treated) birds, and these longer trips were primarily driven by a longer duration of the active flight phase. All these effects were consistent between sexes and suggest a poorer foraging performance of experimental birds (lower capacity to find and collect food during foraging trips) or evasion of social competition. However, the similar total investment in foraging activities between experimental and control birds (e.g. total distance covered across all foraging trips) suggests that compensatory physiological responses to induced anaemia may be in operation.

Here, we formulated two *a priori* hypotheses on how birds may respond to experimental reductions in blood oxygen-carrying

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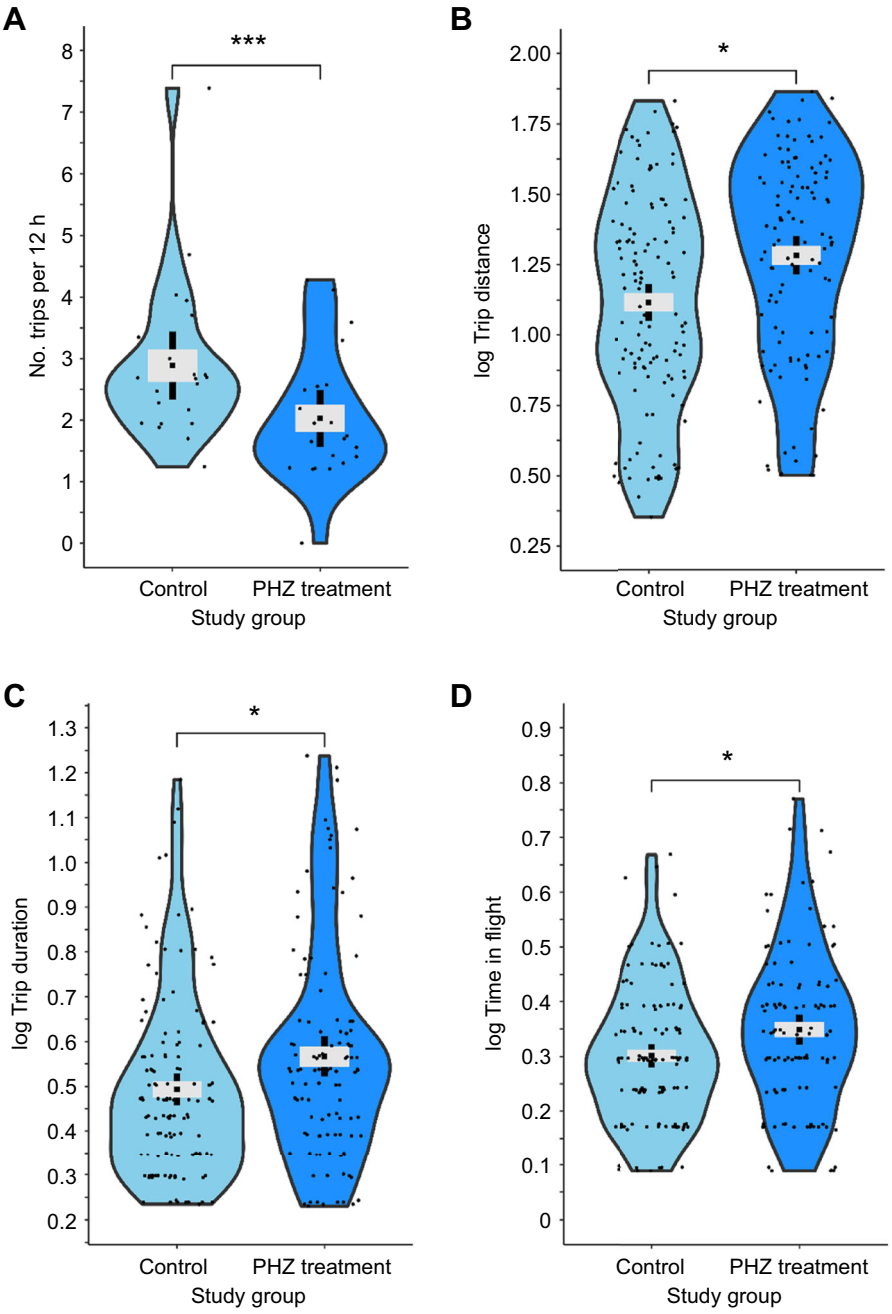

Fig. 3. Analysis of foraging trips in experimental and control black-headed gulls. Distribution of the number of foraging trips in 12 h (A), individual trip distance (km; B), individual trip duration (h; C), and duration of active flight per trip (h; D) is shown for control (light blue; *n*=23) and experimental (dark blue, *n*=22) black-headed gulls. Box plots show means (central point), s.e.m. (box) and 95% confidence intervals (whiskers); violin plot shows kernel density. Asterisks indicate significance: **P*<0.05, ****P*<0.001.

capacity. First, we expected that PHZ-treated birds may shorten the duration and distance of their foraging trips in order to reduce their energy expenditure under a handicapped aerobic performance and flight capacity (the activity reduction hypothesis). Although we are aware of no experimental studies in birds which have directly tested this mechanism, positive correlations between Hct and flight performance have been occasionally reported. For example, one of the early comparative analyses showed that high energy expenditure among birds using either flapping or gliding flight was associated with high Hct values (Viscor et al., 1985). Similar conclusions were drawn at a much broader phylogenetic scale, showing that species with a high general activity energy expenditure (energy spent specifically on costly activities) also showed significantly higher Hct (Yap et al., 2019). The same mechanism was found to operate at the intra-specific level, e.g. bar-tailed godwits, *Limosa lapponica*, showed peak levels in Hct and Hb just prior to departure on long-distance migratory flights, being an adaptation for an anticipated elevation of aerobic requirements (Landys-Ciannelli et al., 2002). All these findings not only indicate that evolutionary optima of haematological traits are shaped by energetic lifestyle but also show that haematology may be plastically modulated to meet current energy demands. As an experimental reduction in blood oxygen-carrying capacity is likely to cause an incompatibility between energy requirements and physiological abilities of an organism to satisfy them, we expected that birds may temporarily reduce their costly activities (e.g. long-distance flights) until they recover from the transient anaemia. The results of our study, however, provided no evidence for this kind of plastic reaction, as PHZ-treated gulls maintained their foraging activities and we recorded no significant differences in the total

distance covered during all foraging trips and the total time spent on foraging (per unit time) between experimental and control individuals.

Although the total investment in foraging activities was not affected by reductions in blood oxygen-carrying capacity, we found evidence for some fine-scale alterations in foraging behaviour of experimental birds, consistent with the compensation hypothesis. Specifically, foraging trips of PHZ-treated individuals were less frequent, but were of longer duration, which could reflect reduced foraging performance under lower physiological capacity. While we lack reliable data to unequivocally identify mechanisms that are functionally responsible for this pattern, we can provide three non-exclusive explanations for longer trip duration in PHZ-treated gulls: (i) reduced flight performance, (ii) reduced ability to find and collect food, and (iii) reduced competitive ability. Although experimental research on passerine birds revealed that flight performance at low altitudes may be negatively affected by haematological status (Yap et al., 2018), our data suggest that average flight speed of experimental and control birds was similar. While PHZ-treated individuals spent more time in active flight during foraging trips, it was primarily driven by longer distances covered rather than slower flight. Thus, it seems likely that birds with compromised aerobic capacity may have had lower feeding rates, possibly driven by reduced foraging capacity or competitive ability. Positive associations between haematological status and feeding rates have been reported for passerine birds, e.g. dark-eyed juncos, *Junco hyemalis* (Leary et al., 1999), although the functional nature of these correlative results remains undetermined. In contrast, PHZ treatment in the common starling had no effect on female provisioning rates during the chick rearing period (Fronstin et al., 2016). However, starlings either breed solitarily and search for food within their own territories of rather limited size or nest in social aggregations, but their foraging flight distances remain relatively minor (e.g. mean foraging distance of 200–250 m, up to a maximum of 2 km; Bruun and Smith, 2003; Heldbjerg et al., 2017). It is possible that the negative effects of reduced blood oxygen-carrying capacity on feeding rate may be much stronger in species with longer foraging trips, such as the black-headed gull, where searching for food requires much greater energy investment and higher oxygen consumption. Finally, haematology is known to correlate with social status and dominance in birds (Hammond et al., 2000; van Oort et al., 2007) and we suggest that PHZ-treated gulls may either avoid or reduce intra-specific competition by choosing less attractive and more distant forging areas, or they may be actively excluded from the most favourable foraging grounds by dominant non-handicapped conspecifics (consistent with ideal despotic distribution; Fretwell, 1972). Irrespective of the mechanism that drives these behavioural alterations, we speculate that longer trip durations of PHZ-treated birds may be unfavourable either for their partners, which may suffer from longer intervals between foraging bouts (if they wait to forage until the second parent returns), or for the brood when left unattended (if the incubating parent does not wait to forage until the second parent returns). Although these hypothetical scenarios require further testing, previous research on starlings provided clear support for reduced reproductive performance of birds that were PHZ treated during the incubation period (Fronstin et al., 2016).

As we had convincing evidence that our experimental treatment induced haemolytic anaemia in gulls, resulting in reduced Hct and Hb, we primarily interpreted the alterations of foraging behaviour in the light of physiological impairment. However, we need to explicitly acknowledge that our experimental birds may have shown some compensatory physiological responses to the treatment. In general, Hct and Hb constitute just a part of a complex oxygen transport cascade and any alterations in these parameters may be compensated for by parallel changes in other components of the Fick equation, e.g. by modification of heart rate and stroke volume (Milsom et al., 2021). Other compensatory mechanisms may include short-term alterations in Hb oxygen affinity mediated by erythrocytic effectors, such as inositol pentaphosphate (Weber, 2007). Finally, reduction in Hct decreases blood viscosity and increases blood flow rate, which may provide some compensation in terms of oxygen transport, but may also reduce heart beat rate and associated energy expenditure (Jenni et al., 2006). As we lack any quantitative data on the other components of blood oxygen-carrying capacity, it is difficult to unequivocally determine whether haemolytic anaemia induced by our experimental treatment caused significant physiological detriment to gulls, or whether we observed compensated physiological endurance at little biological cost. At the same time, we had no information on the biological costs of fine-scale alterations of foraging behaviour that we observed in PHZ-treated gulls. This could be examined in future research by testing for the effects of PHZ treatment and foraging behaviour on individual condition parameters.

So far, there is little evidence for associations between haematology and foraging behaviour in birds, and research has mainly focused on diving species. For example, Hct of female macaroni penguins, *Eudyptes chrysolophus*, showed strong positive correlation with the mean duration of foraging trips and the relative efficiency of foraging activity, i.e. the number of foraging behaviours recorded per dive (Crossin et al., 2015). Similar examples come from research on non-avian vertebrates, e.g. Galapagos sea lions, *Zalophus wollebaeki*, with low Hct and Hb predominantly adopted a shallow foraging strategy with lower maximum diving depths and lower dive duration, when compared with individuals with higher blood oxygen-carrying capacity (Villegas-Amtmann and Costa, 2010). Associations between aerobic capacity and foraging behaviour have also been investigated at the inter-specific level. In snakes, Hct values were associated with foraging mode, as species that use ambush foraging tactics had lower Hct compared with active foragers, reflecting their increased activity patterns and higher energy consumption (Lourdais et al., 2014). Inter-specific comparative analyses in birds revealed links between haematology and other types of non-foraging behaviour, e.g. risk-taking species with rapid escape behaviours had higher Hct than species with long flight initiation distances (Møller et al., 2013). The results of our experimental study on gulls add to the emerging but still limited evidence on the functional links between aerobic capacity and behaviour in wild avian populations.

In conclusion, we showed that black-headed gulls altered their foraging behaviour in response to experimental reductions in blood oxygen-carrying capacity, resulting in less frequent, but longer lasting and more distant foraging trips. While the proximate mechanism for this alterations could not be unequivocally determined, we suggest that it may primarily reflect lower foraging performance or lower competitive ability of physiologically handicapped individuals. At the same time, the lack of difference in the total investment in foraging activities between experimental and control birds suggests that detrimental costs of haemolytic anaemia could have been, at least partly, offset by compensatory physiological responses. Thus, whether the behavioural effects of PHZ-induced anaemia have any implications for reproductive performance of gulls, possibly mediated by non-optimal synchronization of daily schedules between parents or by reduced nest attentiveness, should be subject to further investigation. Despite these limitations, we are convinced that

our study contributes to a better understanding of the physio-ecological nexus in non-diving colonially breeding birds.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: P.M.; Methodology: P.M., M.K., P.I., D.J.; Validation: M.K., T.J., P.I., J.K.; Formal analysis: P.M., D.J.; Investigation: M.K., T.J., P.I., J.K.; Resources: P.M., D.J.; Data curation: M.K.; Writing - original draft: P.M.; Writing - review & editing: P.M., M.K., T.J., P.I., J.K., D.J.; Visualization: P.M., M.K., D.J.

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