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RESEARCH ARTICLE

Effects of experimental manipulation of hematocrit on avian flight performance in high- and low-altitude conditions

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ABSTRACT

Despite widely held assumptions that hematocrit (Hct) is a key determinant of aerobic capacity and exercise performance, this relationship has not often been tested rigorously in birds and results to date are mixed. Migration in birds involves high-intensity exercise for long durations at various altitudes. Therefore, it provides a good model system to examine the effect of Hct on flight performance and physiological responses of exercise at high altitude. We treated yellow-rumped warblers (*Setophaga coronata*) with avian erythropoietin (EPO) and anti-EPO to experimentally manipulate Hct and assessed flight performance at low and high altitudes using a hypobaric wind tunnel. We showed that anti-EPO-treated birds had lower Hct than vehicle- and EPO-treated birds post-treatment. Anti-EPO-treated birds also had marginally lower exercise performance at low altitude, committing a higher number of strikes (mistakes) in the first 30 min of flight. However, anti-EPO-treated birds performed significantly better at high altitude, attaining a higher altitude in a ramped altitude challenge to 3000 m equivalent altitude, and with a longer duration of flight at high altitude. Birds exercising at high altitude showed decreased Hct, increased glucose mobilization and decreased antioxidant capacity, regardless of treatment. In summary, we provide experimental evidence that the relationship between Hct and exercise performance is dependent on altitude. Future studies should investigate whether free-living birds adaptively modulate their Hct, potentially through a combination of erythropoiesis and plasma volume regulation (i.e. hemodilution), based on the altitude they fly at during migratory flight.

KEY WORDS: Hemoglobin, Aerobic capacity, Hemodilution, Wind tunnel, Exercise, Birds

INTRODUCTION

Hematocrit (Hct) and hemoglobin (Hb) are widely assumed to be key determinants of aerobic capacity and exercise performance (Böning et al., 2011; Calbet et al., 2006; Carpenter, 1975; Hammond et al., 2000; Piersma and van Gils, 2011); however, this functional relationship has not often been tested rigorously and results to date are mixed. For instance, studies in migratory birds show that birds up-regulate Hct and Hb in preparation for and during migration, presumably to sustain the high metabolic demand imposed by long migratory flights (Fair et al., 2007; Hahn et al., 2018; Krause et al., 2016). It has also been shown that Hct is positively correlated with exercise-induced maximal metabolic rate ($\dot{V}_{O_2,max}$) (Hammond et al., 2000) and cold-induced summit metabolic rate in birds (Petit and Vezina, 2014). However, although many studies on human athletes reported improved aerobic capacity as a result of increases in Hct and Hb due to treatment with recombinant human erythropoietin (EPO) or erythropoiesis-stimulating agents (Böning et al., 2011; Lundby et al., 2007; Thomsen et al., 2007), other studies have found minimal or no effects of EPO treatment on exercise performance in human athletes (Heuberger et al., 2017; Sgrò et al., 2018). In addition, unlike mammals such as horses and dogs, which store erythrocytes in the spleen and up-regulate Hct and Hb in response to exercise (Wu et al., 1996), birds do not have the ability to store erythrocytes in the spleen (John, 1994) and they decrease Hct during exercise as a result of hemodilution (Jenni et al., 2006). These findings suggest that the relationships between Hct, Hb, aerobic capacity and exercise performance may not be straightforward and might be taxon or activity specific. Therefore, experimental manipulations of Hct and Hb should be conducted to confirm their role in sustaining energetically demanding exercise (Petit and Vezina, 2014; Yap et al., 2017).

Migration in birds provides a good model system to examine the relationship between Hct and aerobic capacity (Yap et al., 2017). Bird migration often covers long distances and spans days to weeks of activity (Guglielmo, 2010; Piersma, 2011; Scott et al., 2015; Yap et al., 2017). Furthermore, migratory birds operate at 70–90% $\dot{V}_{O_2,max}$ during long migratory flights (Guglielmo, 2010; Piersma, 2011; Yap et al., 2017). Although most migratory passerines spend the majority of time flying at low altitude (<800 m), they do sometimes fly at higher altitudes (up to 4000 m) depending on wind and weather (Dokter et al., 2013; Kemp et al., 2013; Scott, 2011). However, most studies investigating the physiology of migration have been conducted at low altitude and have largely ignored this transient but potentially important altitude component. Outside of a few species adapted to high altitude (Barve et al., 2016; Fedde et al., 1989; Lague et al., 2016; Scott and Dawson, 2017; Scott et al., 2015), physiological responses to exercise at high altitude in many passerines are still poorly understood. Borras et al. (2010) found that citril finches (*Carduelis citronella*) increased Hct in response to increases in altitude, but these birds were transported passively to high elevation by the researchers. Therefore, it is unclear whether birds that are actively exercising would experience the same physiological response when exposed to high-altitude conditions. With regard to fuel metabolism, carbohydrate (glucose) has been thought to be the preferred fuel during exercise at high altitude (Hochachka et al., 1991), and at high exercise intensity (McClelland et al., 1998) in mammals. Given that migratory birds often fly at 70–90% $\dot{V}_{O_2,max}$ with fatty acids as the predominant fuel, at least at low altitude (Guglielmo, 2010), it is not known whether we would see similar changes in fuel selection (increased glucose metabolism)

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in birds exercising at high altitude. In addition, whereas it has been shown that endurance flight can generate a physiological cost, in terms of increased oxidative stress in migratory birds (Jenni-Eiermann et al., 2014), the synergistic effect of exercise and acute hypoxia exposure at high altitude on oxidative stress remains poorly understood at present.

Here, we investigated the effects of experimental manipulation of Hct and Hb on flight performance and the physiological response to exercise at low and high altitude in a migratory songbird. We treated yellow-rumped warblers (*Setophaga coronata*) with avian EPO, EPO antibody (anti-EPO) and vehicle and assessed flight performance using a hypobaric wind tunnel (Gerson and Guglielmo, 2011; Hedenström and Lindström, 2017). We predicted that (1) EPO-treated birds would have the highest Hct/Hb level, followed by vehicle-treated birds and anti-EPO-treated birds, (2) EPO-treated birds would have the best exercise performance (i.e. fewer strikes, longer flight duration and cost of transport) during wind tunnel flight at both low and high altitude, followed by vehicle-treated birds and anti-EPO-treated birds, and (3) regardless of treatment, all birds would increase Hct/Hb and glucose mobilization during flight at high altitude. We also predicted that (4) birds dosed with anti-EPO would have increased flight-induced oxidative stress compared with vehicle- and EPO-dosed birds, as a result of decreased endurance capacity and increased difficulty flying in the wind tunnel (Jenni-Eiermann et al., 2014).

MATERIALS AND METHODS

Animals and husbandry

Yellow-rumped warblers, *Setophaga coronata* (Linnaeus 1766) (n=53), were captured using mist nets at Long Point, ON, Canada, between 13 and 16 October 2015, and transported to the Advanced Facility for Avian Research (AFAR) at the University of Western Ontario, London, ON, Canada, by animal carriers in a vehicle. Birds were initially housed in cages (~914×508×508 mm W×H×L, 2–3 birds per cage) from 13 October 2015 to 22 February 2016. They were transferred to indoor aviaries (2.3×2.4×3.5 m W×H×L) on 23 February 2016 and were kept in aviaries until the end of the experiment, when all birds were released. Birds were kept at a constant room temperature (~20°C), with *ad libitum* access to a synthetic agar-based mash diet (60.2% carbohydrate, 13.4% protein and 10.7% lipid; Marshall et al., 2016). Temperature and humidity were similar for both the rooms with cages and the indoor aviaries. Light cycles were set to mimic seasonal overwintering and migratory conditions in the wild. Birds were housed on a 12 h:12 h light:dark cycle (to simulate autumn migration photoperiod) for 2 months upon being brought into captivity. They were switched to an 8 h:16 h light:dark cycle (to simulate overwintering photoperiod) from 22 December 2015 to 17 March 2016, prior to the beginning of the experiment. All birds were collected under a scientific collection permit from the Canadian Wildlife Service (CA-0256), and under Animal Ethics Protocol 2010-216 from the University of Western Ontario Animal Care Committee.

Dosing pilot: validation of the effects of EPO and anti-EPO on Hct and Hb

Birds were given 2 weeks to acclimate to captivity after being captured during their autumn migratory season, prior to the start of dosing pilot, and n=30 birds were randomly chosen for the dosing pilot. At the start of the pilot, a blood sample was taken from all birds to obtain baseline measurements of Hct and Hb. Birds were then given 7 days to recover from the initial blood sampling, at the end of which they were randomly assigned to three treatment groups (n=10 per treatment): EPO, anti-EPO and vehicle. Cross-use between mammalian and avian EPO is ineffective (Rosse and Waldmann, 1966). Therefore, EPO, anti-EPO and anti-EPO diluent were acquired from chicken EPO ELISA kits (MyBioSource Inc.). Birds in the EPO group were injected intramuscularly (i.m.; right pectoral muscle) with 100 µl of 8000 pg ml^{-1} purified chicken EPO; birds in the anti-EPO group were injected i.m. with 50 µl of diluted chicken anti-EPO; birds in the vehicle group were injected i.m. with 50 µl of anti-EPO diluent. A second blood sample was taken 3 days after injections to assess changes in Hct and Hb. These volumes and concentrations as well as the blood-sampling time points were chosen based on a previous dosing pilot conducted in captive zebra finches (*Taeniopygia guttata*) at Simon Fraser University, BC, Canada (Fig. S1; O. Kim et al., unpublished data). Anti-EPO diluent was chosen as the vehicle because neither EPO diluent nor anti-EPO diluent caused a significant change in Hct/Hb in a previous dosing pilot (O. R. Kim, K.N.Y. and T.D.W., unpublished data). Because of logistical constraints, the dosing pilot was conducted in the autumn while the flight experiment was conducted in the spring (the wind tunnel was occupied for another experiment in the autumn).

Hypobaric climatic wind tunnel and screening

The hypobaric climatic wind tunnel used in this experiment is housed in the AFAR. It is capable of simulating high-altitude conditions with low-turbulence air flow at various speeds. It has been used routinely to study flight in birds, and birds do exhibit natural flight behavior in the tunnel (Ma et al., 2018; Maggini et al., 2017). To familiarize birds with the wind tunnel and to screen for birds that would fly voluntarily and reliably for long periods in the wind tunnel, individuals were flown at 15°C, 70% relative humidity and at speeds between 7 and 8 m s^{-1} 2 weeks prior to the start of the experiment (i.e. 3 March). At the end of the screening process, 22 birds were selected to be included in the experiment based on their flight behavior.

Experimental time line and wind tunnel endurance flight

Starting on 17 March, birds were transferred to a separate indoor aviary with a long-day photoperiod (16 h:8 h light:dark) 3 weeks before the pre-treatment wind tunnel endurance flight (day −21), to be photo-stimulated into a spring migratory condition. To ensure that all birds were in a similar migratory state, we staggered our photo-stimulation treatment so that every bird had been exposed to a long-day photoperiod for exactly 21 days prior to pre-treatment flight. For instance, bird A was transferred to a long-day photoperiod on 17 March, flown on 7 April, injected on 14 April and flown again on 17 April; bird B was transferred to a long-day photoperiod on 18 March, flown on 8 April, injected on 15 April and flown again on 18 April. Birds were flown twice for 120 min in the wind tunnel, once on day 0 prior to any EPO-related treatment and once on day 10, 3 days after the EPO-related treatment was administered (on day 7). Immediately after each of these two flights, birds were bled for measures of Hct, Hb and oxidative stress, and body composition was estimated using a quantitative magnetic resonance body composition analyzer (QMR; Echo Medical Systems) (Guglielmo et al., 2011) (Fig. 1).

More specifically, on day 0, birds were fasted for 1 h before the start of pre-treatment flight, after which their body mass was measured (±0.01 g) while fat and lean mass were measured using QMR (Guglielmo et al., 2011). Birds were flown at 15°C, 70% relative humidity, ground level (low altitude: approximately 182 m a.s.l.) and 8 m s^{-1} equivalent wind speed for 120 min. If a bird failed to maintain continuous flight for 5 min after the initial 30 min

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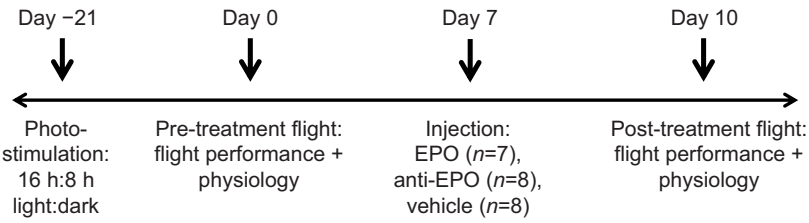

Fig. 1. Experimental time line. EPO, erythropoietin.

adjustment period, it was removed and the flight terminated. To determine pre-treatment (day 0) flight energetics and physiology (Hct, Hb, oxidative stress), all birds were weighed, scanned by QMR, and blood sampled immediately following flight. Birds were then returned to their respective aviaries and allowed to rest and recover for 7 days. On day 7, birds were randomly assigned to one of three treatment groups and injected with EPO (n=7), anti-EPO (n=8) or vehicle (n=7) by an experimenter blind to pre-treatment flight performance. Out of the 17 birds that successfully flew for 120 min during pre-treatment flight, six were assigned to the anti-EPO group, six were assigned to the EPO group and five were assigned to the vehicle group. Three days after treatment (day 10), prior to post-treatment flight, birds were fasted, weighed and scanned by QMR. During the first 105 min of post-treatment flight, birds were flown at low altitude and in the same wind speed and environmental conditions as the pre-treatment flight. We then decreased air pressure to simulate an increase in altitude at 5 m s^{-1} up to a maximum of 3000 m a.s.l. equivalent altitude, while maintaining temperature and humidity constant and the equivalent wind speed at 8 m s^{-1} (i.e. true wind speed was increased to maintain the same dynamic pressure and thus mechanical flight power; Pennycuick, 2008). Birds were kept flying in the wind tunnel at 3000 m for 5 min. Birds that failed to maintain continuous flight for 1 min during altitude challenge were taken out of the wind tunnel and the altitude attained was noted by the experimenter; 3000 m was chosen because it is at the upper limit of altitude at which most passerines migrate (Scott, 2011). In total, birds spent the same amount of time (120 min) flying in the wind tunnel during the post-treatment flight as the pre-treatment flight, with the last 15 min being an altitude challenge. However, because of logistical constraints, only 18 birds of the original 22 were subjected to altitude challenge, whereas the remaining four were flown at low altitude for the entire duration (i.e. 120 min). Of the 18 birds that were subjected to altitude challenge post-treatment, all six anti-EPO-treated birds, four out of six vehicle-treated birds and one out of six EPO-treated birds attained the 3000 m simulated altitude. To determine post-treatment flight energetics and physiology, all birds were weighed, scanned by QMR, and blood sampled immediately following flight. Blood samples were not collected before flight to avoid any effect of blood sampling on flight performance (i.e. puncture wound, dehydration due to blood loss, anemia, etc.). Although endurance flight could induce dehydration, it has been shown that birds actively regulate plasma osmolality and relative body water content to maintain water homeostasis (Gerson and Guglielmo, 2011). A summary of the experimental time line is provided in Fig. 1.

Behavioural observations and determination of flight performance

Behavioural observations of flying birds were conducted by an independent observer blind to dosing treatments. Four different metrics were used to assess flight performance during both pre-treatment and post-treatment flights: (1) number of strikes in the first 105 min of flight, (2) number of strikes in the first 30 min of flight, (3) energy expenditure and (4) total duration of flight. Strikes were defined as mistakes that individuals made during flight (i.e. when a bird landed or was blown to the back net of the wind tunnel; Maggini et al., 2017). Total energy expenditure was determined using fat and lean mass lost during flight as quantified by the QMR scans pre- and post-flight (E=Δfat mass×39.6 kJ g^{-1}+Δlean mass×5.3 kJ g^{-1}; Gerson and Guglielmo, 2011). We also calculated both cost of transport [COT=E/(wind speed×flight duration)] and flight power (E/flight duration). We chose to assess the number of strikes in the first 30 min of flight because it has been shown that the first 30 min are the most challenging time as birds adjust to conditions in the wind tunnel (Ma et al., 2018), possibly because birds make a suite of physiological adjustments during the onset of flight (Gerson and Guglielmo, 2013; Jenni et al., 2006). For post-treatment flights, in addition to the four performance metrics above, we also included three other performance metrics: (1) altitude (the maximum altitude attained by the bird at the end of the flight test period, or the altitude at which the bird failed to maintain continuous flight for 1 min), (2) altitude duration (the total duration of flight at 3000 m) and (3) altitude strikes (the number of strikes at 3000 m).

Physiological measurements and assays

Pre-treatment (day 0) and post-treatment (day 10) blood samples were obtained from the brachial vein following puncture with a 26G needle, and blood was collected using heparinized microhematocrit tubes. Hct (percent packed cell volume) was measured with digital calipers (±0.01 mm) following centrifugation of whole blood for 5 min at 10,000 ***g*** (Haematokrit 210, Hettich, Tuttlingen, Germany). Hb (g dl^{-1}) was measured using the cyanomethemoglobin method (Drabkin and Austin, 1932) modified for use with a microplate spectrophotometer (BioTek Powerwave 340, BioTek Instruments, Winooski, VT, USA), using 5 µl whole blood diluted in 1.25 ml Drabkin's reagent (D5941; Sigma-Aldrich Canada, Oakville, ON, Canada) with absorbance measured at 540 nm. Intra-assay coefficient was 2.51%. Blood glucose was also measured in individuals at the time of blood sampling using a glucose meter (Accu-Chek Aviva, Roche

Table 1. Pre-treatment and post-treatment values for hematocrit (Hct) and hemoglobin (Hb) during dosing pilot

	Pre-treatment			Post-treatment		
	Anti-EPO	EPO	Vehicle	Anti-EPO	EPO	Vehicle
Hct (%)	49.49±1.13	47.96±1.12	48.28±1.07	47.33±1.13	50.28±1.17	49.98±1.07
Hb (g dl^{-1})	13.14±0.77	12.78±0.76	14.32±0.73	11.93±0.76	13.01±0.77	14.54±0.73

Data are least-squares means±s.e.m. EPO, erythropoietin.

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Table 2. Pre-treatment and post-treatment values for the number of strikes, cost of transport, power and flight duration during wind tunnel endurance flight

	Pre-treatment			Post-treatment		
	Anti-EPO	EPO	Vehicle	Anti-EPO	EPO	Vehicle
$Strikes_{105}$	11.13±6.99	20.14±7.47	9.43±7.47	20.63±6.99	12.86±7.47	2.86±7.47
$Strikes_{30}$	6.25±4.21	11.43±4.50	2.28±4.50	11.38±4.21	2.28±4.51	2.14±4.50
COT (kJ km^{-1})	0.24±0.03	0.26±0.04	0.25±0.04	0.28±0.03	0.20±0.04	0.27±0.04
Power (W)	1.90±0.26	2.03±0.28	1.95±0.28	2.19±0.26	1.60±0.28	2.08±0.28
Duration (s)	4999.00±478.00	5572.85±511.00	6004.29±511.00	5655.75±478.00	6241.14±511.00	6267.86±511.00

Data shown are means±s.e.m. $Strikes_{105}$, number of strikes in the first 105 min of flight; $Strikes_{30}$, number of strikes in the first 30 min of flight; COT, cost of transport.

Diagnostics, Mannheim, Germany). Blood samples from both time points were also assayed for total antioxidant capacity (OXY, µmol HClO l^{-1}) and reactive oxygen metabolites (dROMs, mg H_2O_2 dl^{-1}). All plasma samples were analyzed using a microplate spectrophotometer (BioTek Powerwave 340) and 96-well microplates. Analyses of oxidative stress were carried out according to established protocols as described in Costantini et al. (2008, 2011), with slight modification. Specifically, we measured dROMs and OXY using the commercial kits dROMs and OXY-Adsorbent Test (Diacron International, Grosseto, Italy), respectively. Inter- (n=4) and intra- (n=3) assay coefficients for OXY were 5.95% and 5.41%, respectively. Inter- (n=2) and intra- (n=2) assay coefficients for dROMs were 0.27% and 3.56%, respectively.

Statistical analyses

Analyses were carried out using R version 0.99.467 (http://www.R-project.org/). Data were first examined for normality using the Shapiro–Wilk test and data were either transformed prior to analysis or analyzed using a non-parametric test (Kruskal–Wallis test). For the dosing pilot, we tested the effect of EPO, anti-EPO and vehicle on Hct and Hb using repeated measures with time and treatment as main effects, individual bird ID as a random factor and body mass as a covariate. We also report the least-squares means and s.e.m. of pre-treatment Hct and Hb and post-treatment Hct and Hb in a separate table (Table 1). F-statistics and P-values were generated using the lmerTest package (https://CRAN.R-project.org/package=lmerTest) and Tukey's HSD (package multcomp; Hothorn et al., 2008) was used to evaluate pairwise comparisons between treatments and time points following a significant mixed model.

For the wind tunnel flight experiment, physiological metrics (glucose, Hct, Hb, OXY, dROMs) were analyzed using repeated measures with time and treatment as main effects and individual bird ID as a random factor. Body mass was included in all models as a covariate. In addition, to investigate the effect of flight at high altitude on Hct and Hb, we analyzed Hct and Hb using repeated measures with flight and time as main effects and individual bird ID as a random factor. All treatment groups were pooled in this particular model because we did not detect any main effects of treatment, nor interactions between treatment and other variables (P>0.1 in all cases). We did not test for the effect of flight at high altitude on other physiological measures because we only had plasma samples from two birds (out of four) that were flown at low altitude for both pre- and post-treatment flight. F-statistics and P-values were generated using the lmerTest package (https://CRAN.R-project.org/package=lmerTest) and Tukey's HSD (package multcomp; Hothorn et al., 2008) was used to evaluate pairwise comparisons between treatments and time points following a significant mixed model.

To analyze the change in performance between pre-treatment and post-treatment flights, we subtracted pre-treatment values from their respective post-treatment values to obtain the change in the number of strikes in the first 105 min ($\Delta Strikes_{105}$) and first 30 min ($\Delta Strikes_{30}$), cost of transport (ΔCOT), flight power (ΔPower) and flight duration (ΔDuration). We then used a general linear model (GLM) to test for the effect of treatment on $\Delta Strikes_{105}$, $\Delta Strikes_{30}$, ΔCOT, ΔPower and ΔDuration. We also report the means and s.e.m. of the same performance metrics for both pre-treatment and post-treatment flights in a separate table (Table 2). Altitude attained, flight duration at altitude (altitude duration) and number of strikes at 3000 m (altitude strikes) were analyzed using the Kruskal–Wallis test, followed by multiple comparisons using the LSD test if the model was significant. We report the H-, F- and Z-statistics and the associated P-values. Final sample sizes for all behavioral and physiological metrics are listed in Table S1.

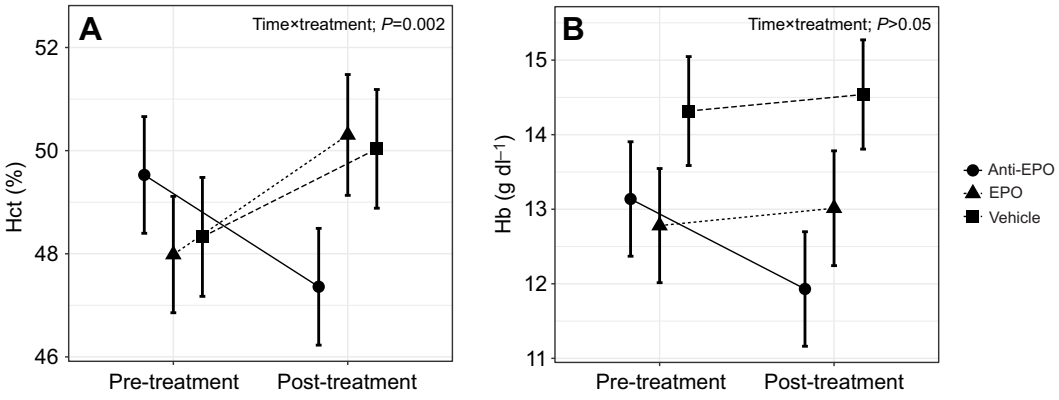

Fig. 2. Effects of avian EPO and anti-EPO on hematocrit (Hct) and hemoglobin (Hb). Hct (A) and Hb (B) data are least-squares means±s.e.m. Anti-EPO, n=10; EPO, n=10; vehicle, n=11. Linear mixed effect model (e.g. Hct~Treatment*Time+Body mass).

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RESULTS

Dosing pilot: effects of avian EPO and anti-EPO on Hct and Hb

There was a significant treatment×time interaction for Hct ($F_{2,26}$=8.51, P=0.002; Fig. 2A): birds dosed with anti-EPO decreased Hct from pre-treatment to post-treatment (t_{25}=2.6, P=0.01), birds dosed with EPO increased Hct from pre-treatment to post-treatment (t_{26}=−2.6, P=0.01) and vehicle-treated birds had a similar Hct from pre-treatment to post-treatment (t_{26}=−2.0, P=0.06). However, it should be noted that post-treatment Hct was not different between vehicle-treated and EPO-treated birds (t_{35}=−0.16, P=1.0). Hb was independent of the treatment×time interaction ($F_{2,26}$=1.18, P=0.32; Fig. 2B), and the main effects of treatment ($F_{2,27}$=1.06, P=0.36) and time ($F_{1,26}$=1.81, P=0.19). Body mass co-varied significantly with Hct (P=0.03) but not Hb (P=0.07).

Effects of EPO and anti-EPO on flight performance at low altitude

There was a marginally significant effect of treatment on the change in the number of strikes in the first 30 min of flight ($\Delta Strikes_{30}$; $F_{2,19}$=3.12, P=0.06; Fig. 3A). Anti-EPO-treated birds performed worse than EPO-treated birds during the post-treatment flight (pairwise contrast, Tukey HSD, Z=2.51, P=0.03). There was no significant difference between anti-EPO-treated birds and vehicle-treated birds (Z=1.01, P=0.57) and between EPO-treated birds and vehicle-treated birds (Z=−1.44, P=0.32) in terms of $\Delta Strikes_{30}$. There was no

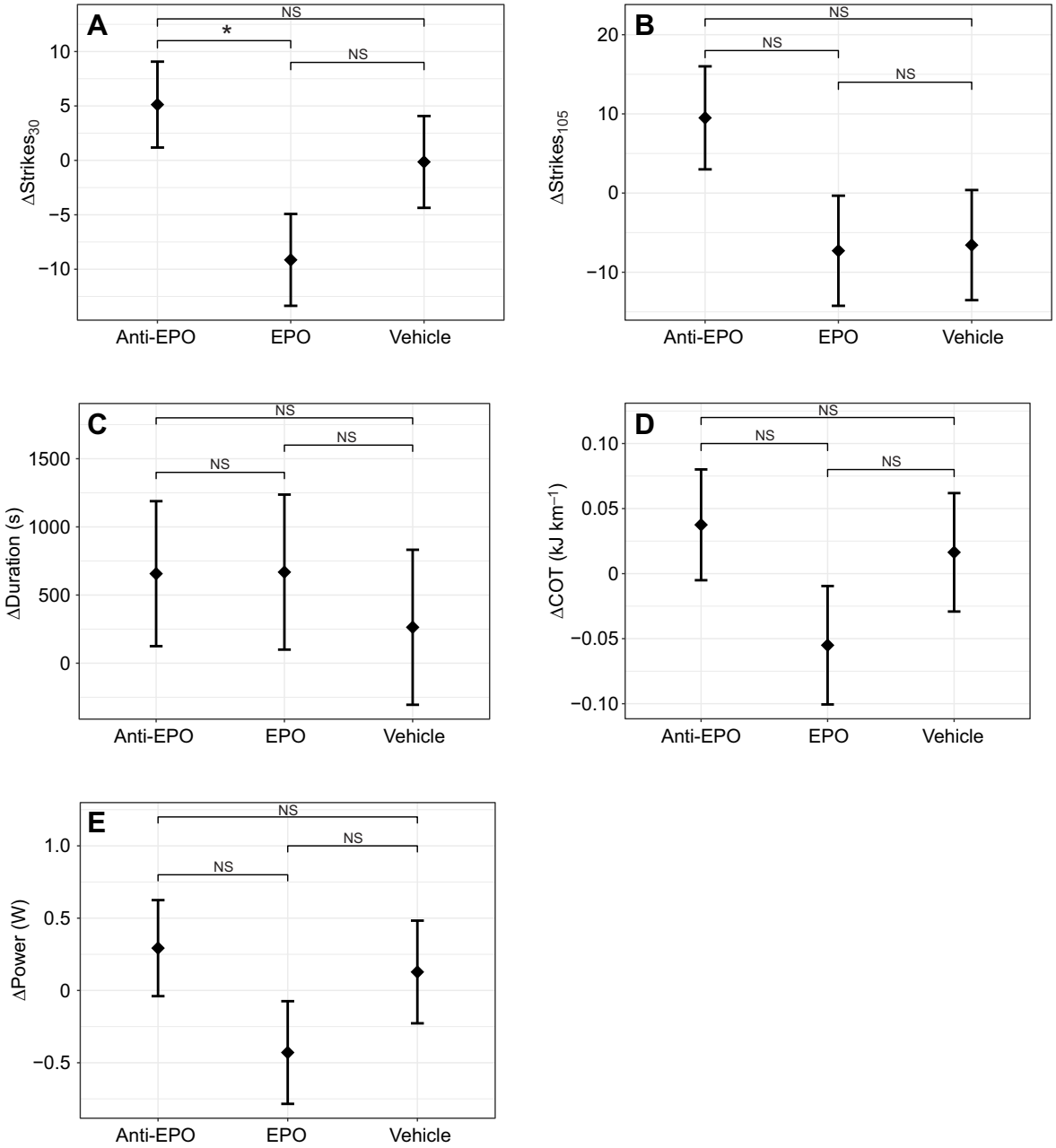

Fig. 3. Effects of EPO and anti-EPO on the number of strikes, flight duration, cost of transport and power. (A) Change in the number of strikes in the first 30 min of flight ($\Delta Strikes_{30}$). (B) Change in the number of strikes in the first 105 min of flight ($\Delta Strikes_{105}$). (C) Change in flight duration (ΔDuration). (D) Change in cost of transport (ΔCOT). (E) Change in power (ΔPower). Data are least-squares means±s.e.m. Anti-EPO, n=8; EPO, n=7; vehicle, n=7. General linear model (e.g. $\Delta Strikes_{30}$~Treatment). *P<0.05; NS, not significant.

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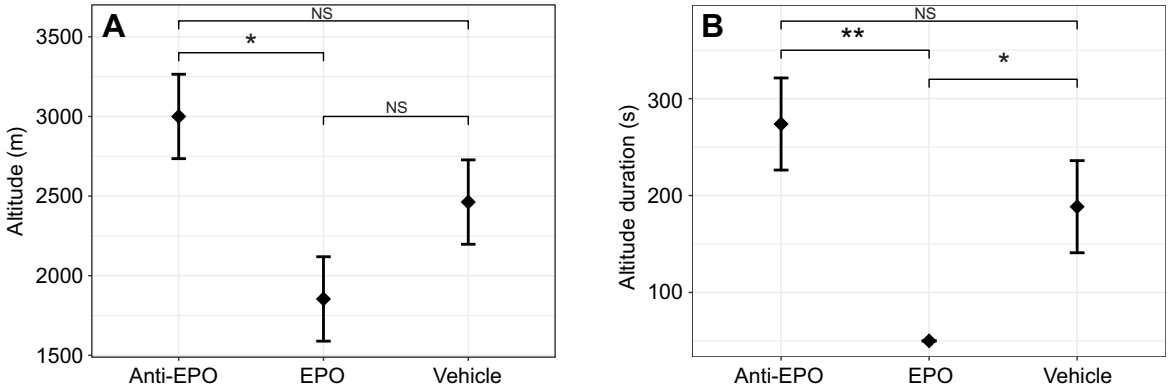

Fig. 4. Effects of EPO and anti-EPO on altitude attained and flight duration at altitude. Altitude (A) and altitude duration (B) data are least-squares means±s.e.m. For A: anti-EPO, *n*=6; EPO, *n*=6; vehicle, *n*=6; for B: anti-EPO, *n*=6; EPO, *n*=1; vehicle, *n*=4. Kruskal–Wallis test (e.g. Altitude~Treatment). Note: there are no error bars associated with the EPO group in B because only one EPO-dosed bird attained 3000 m, when altitude duration was quantified. **P*<0.05; ***P*<0.01; NS, not significant.

significant effect of treatment on the change in the number of strikes in the first 105 min of flight ($\Delta Strikes_{105}$; $F_{2,19}$=2.03, *P*=0.15; Fig. 3B), change in flight duration (ΔDuration; $F_{2,19}$=0.17, *P*=0.84; Fig. 3C), change in cost of transport (ΔCOT; $F_{2,19}$=1.18, *P*=0.33; Fig. 3D) or change in flight power (ΔPower; $F_{2,19}$=1.18, *P*=0.33; Fig. 3E).

Effects of EPO and anti-EPO on flight performance at high altitude

There was a significant effect of treatment on altitude attained (Altitude; *H*=7.37, d.f.=2, *P*=0.02; Fig. 4A): birds dosed with anti-EPO attained a significantly higher altitude than EPO-dosed birds (LSD test, *Z*=3.06, *P*<0.01), but a similar altitude to birds dosed with vehicle (*Z*=1.43, *P*=0.15). Altitude attained by EPO-dosed birds tended to be lower than that reached by vehicle-dosed birds but the difference was not significant (*Z*=−1.62, *P*=0.10). Similarly, there was a significant effect of treatment on flight duration at altitude for the birds that attained 3000 m (altitude duration; *H*=7.24, d.f.=2, *P*=0.02; Fig. 4B), where both anti-EPO-dosed birds and vehicle-dosed birds had significantly greater altitude duration than EPO-treated birds (*Z*=3.33, *P*<0.01 and *Z*=−2.06, *P*=0.03,

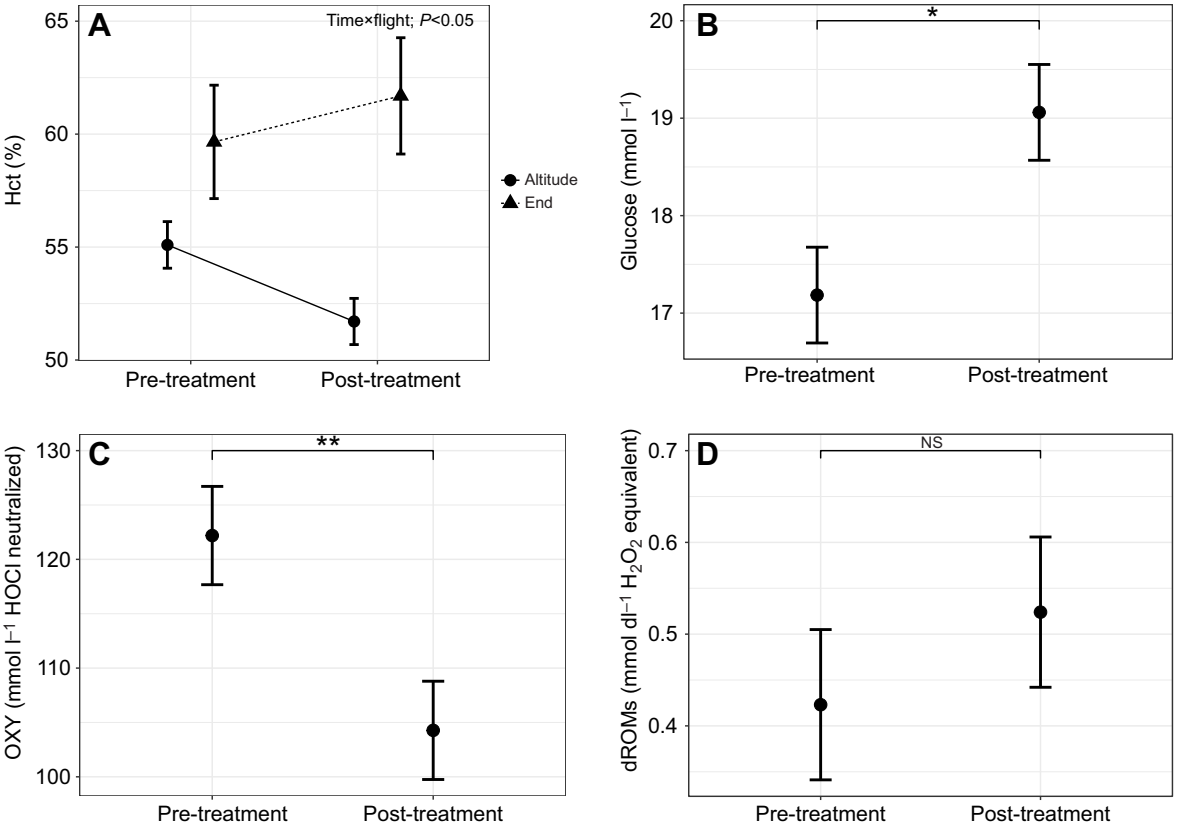

Fig. 5. Effects of exercise at high altitude on Hct, glucose, antioxidant capacity (OXY) and reactive oxygen metabolites (dROMs). Hct (A), glucose (B), OXY (C) and dROMs (D) data are least-squares means±s.e.m. Anti-EPO, *n*=6; EPO, *n*=6; vehicle, *n*=6. Treatments groups were pooled for A–C. In A, Altitude refers to birds that were flown at low altitude during pre-treatment and high altitude during post-treatment; End refers to birds that were flown at low altitude during both pre- and post-treatment. Linear mixed effect model (e.g. Hct~Treatment*Time+Body mass). **P*<0.05; ***P*<0.01; NS, not significant.

respectively). The anti-EPO group and vehicle group had similar altitude duration (Z=1.27, P=0.20). There was no significant difference in the number of strikes at 3000 m between the three different treatment groups (altitude strikes; H=4.30, d.f.=2, P=0.11) for birds that attained this altitude, probably because only a small subset of birds attained 3000 m when strikes were quantified.

Physiological responses to flight at high altitude

All three treatment groups were pooled for analyses of Hct, glucose and OXY as there was no significant treatment effect or treatment×time interaction (P>0.1 in all cases). There was a significant flight×time interaction for Hct ($F_{1,18}$=4.87, P=0.04; Fig. 5A). Regardless of treatment, birds that were flown at high altitude post-treatment had significantly lower Hct post-flight than when they were flown at low altitude pre-treatment (t_{18}=3.62, P<0.01), while birds that were flown at low altitude post-treatment had similar Hct post-flight to those at pre-treatment (t_{18}=−0.88, P=0.81). Hb was independent of the flight×time interaction ($F_{1,18}$=0.18, P=0.67). We did not examine the flight×time interaction for glucose, OXY and dROMs because we only had plasma samples from two birds (out of four) that were flown at low altitude for both pre- and post-treatment flight. Regardless of treatment, birds had higher blood glucose ($F_{1,16}$=7.40, P=0.01; Fig. 5B) and lower OXY ($F_{1,16}$=10.36, P<0.01; Fig. 5C) in response to flight at high altitude. Although we were not able to run formal statistics to test for a flight×time interaction for glucose and OXY because of the low sample size, raw numbers from the two birds that we did obtain data for suggested that birds that were flown at low altitude post-treatment had similar levels of blood glucose and OXY to those at pre-treatment. There was neither a main effect of treatment and time nor a treatment×time interaction for Hb ($F_{2,14}$=0.48, P=0.62) and dROMs ($F_{2,14}$=2.79, P=0.09; Fig. 5D).

DISCUSSION

We manipulated Hct and Hb of yellow-rumped warblers experimentally using EPO and anti-EPO and investigated flight performance at low and high altitude. Our experiment showed that compared with birds treated with EPO, birds treated with anti-EPO had significantly lower Hct and more difficulty flying in the wind tunnel (i.e. lower exercise performance) in the low-altitude condition. However, anti-EPO-treated birds performed significantly better than EPO-treated birds in high-altitude conditions. All birds appeared to experience similar physiological responses to exercise at high altitude regardless of treatment. When exercising in the high altitude condition, birds decreased Hct, increased glucose mobilization and decreased antioxidant capacity.

Migratory birds up-regulate the rate of red blood cell production and Hct prior to and during the migratory season to fuel energetically demanding migratory flights (Fudickar et al., 2016; Krause et al., 2016), potentially through action of the hypoxia-inducible factor (HIF) signaling pathway and downstream production of the hormone EPO (Fudickar et al., 2016; Jelkmann, 2011). We observed a significant decrease in Hct in birds treated with anti-EPO, as well as an increase in Hct in EPO-treated birds, largely consistent with findings from other taxa (Elliott et al., 2009; Schooley and Mahlmann, 1971; Thapliyal et al., 1982; Wolf et al., 2001). However, it should be noted that vehicle-treated birds and EPO-treated birds experienced a similar increase in Hct, suggesting that perhaps the observed increase in Hct is due to factors other than EPO alone. Anti-EPO and EPO did not produce the same anticipated effect on Hb in the dosing pilot, suggesting that Hb is regulated somewhat independently of Hct in birds (Wagner et al., 2008; Williams et al., 2012). It should also be noted that although we found an effect of anti-EPO and EPO on Hct in the dosing pilot, a treatment effect was not observed in the wind tunnel endurance flight experiment. However, the two findings are not directly comparable because post-treatment blood samples were collected under resting conditions in the dosing pilot whereas in the flight experiment, post-treatment blood samples were collected after flight at high altitude. Hemodilution exhibited by exercising birds (Jenni et al., 2006) could also wash out the effect of the anti-EPO treatment. Additionally, the values of Hct were higher in the wind tunnel endurance flight experiment than in the dosing pilot, perhaps because different individuals were used in the pilot and the flight experiment (screening of birds was only performed after the dosing pilot; see Materials and Methods for details), or because of differences in time of the year (autumn migration versus spring migration) or in water balance, which could affect plasma volume. Admittedly, the magnitude of the decrease in Hct in the anti-EPO group was rather small. However, some studies in human subjects have shown that a small change in Hct (3–5%) can have a significant effect on both submaximal and maximal exercise performance (Rasmussen et al., 2010; Thomsen et al., 2007).

Consistent with our initial prediction, as well as the physiological response observed in the dosing study, we found that birds dosed with anti-EPO had lower flight performance (i.e. higher number of strikes in the first 30 min of flight) in the wind tunnel in low-altitude conditions. However, it should be noted that anti-EPO birds were committing a higher number of strikes in the first 30 min in the pre-treatment flight relative to EPO- and vehicle-treated birds (Table 2), hence the ‘regression toward the mean’ phenomenon (Nesselroade et al., 1980) could not be ruled out completely. We also did not find a significant treatment effect for either measure of flight energy expenditure. In other words, the finding of low exercise performance in anti-EPO-treated birds was made based only on the number of strikes in the first 30 min of flight but this metric has been proven to be a valid measurement of flight performance in other wind tunnel studies (Ma et al., 2018; Maggini et al., 2017). As discussed before, migratory endurance flight is an energetically demanding activity (Piersma, 2011) and, thus, high oxygen carrying capacity is essential for maintenance of the intense exercise. The greater number of strikes in the first 30 min of post-treatment flight exhibited by anti-EPO-dosed birds could potentially be explained by impaired oxygen carrying capacity resulting from an anti-EPO-induced decrease in Hct (although Hb was not affected by anti-EPO). However, it should be noted that the total number of strikes throughout the endurance flight was not different between treatment groups, suggesting that perhaps anti-EPO-treated birds adopted other behavioral or physiological adjustments to compensate for low Hct or that lower Hct is adaptive as flight progresses. Our study suggests that birds with lower Hct, which we assume would result in lower aerobic capacity, may have lower migratory performance as a result of more erratic flight behavior (i.e. higher number of strikes). It should also be noted that plasma volume regulation could induce change in Hct by shifting water balance, without any modification of erythrocyte numbers through EPO/anti-EPO pathways (Jacob et al., 2012; Pichon et al., 2016). Therefore, an alternative explanation to the finding that birds dosed with anti-EPO had lower flight performance could be that birds that were able to modulate their plasma volume and, hence, Hct better during flight would have higher flight performance. Future studies should measure blood and plasma volume to validate this hypothesis. Additionally, it has also been shown that EPO can have a range of non-erythropoietic effects that could potentially enhance exercise performance (Schuler et al., 2012; Zhang et al., 2017), but we did not find any performance-enhancing effect of EPO in our study.

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Contrary to our initial prediction, we found that birds dosed with anti-EPO had better exercise performance at high altitude: they attained significantly higher altitude and had significantly longer flight duration at high altitude. We did not detect a treatment effect on the number of strikes at 3000 m (as at low altitude) but this was because most EPO-dosed birds did not even attain an altitude of 3000 m, when the number of strikes was quantified. As noted earlier, while all six anti-EPO-treated birds attained the 3000 m simulated altitude, only four out of six vehicle-treated birds and one out of six EPO-treated birds reached 3000 m. The average flight duration at 3000 m for the anti-EPO group, EPO group and control group was 273.83 s, 50 s (n=1 only) and 188.5 s, respectively). These findings contrast with findings from other studies that manipulated Hct and investigated performance (Fronstin et al., 2016; Schuler et al., 2010), which generally found impaired performance with reductions in Hct. However, it is widely accepted that the relationship between Hct and oxygen carrying capacity is not linear, but rather parabolic in shape (Birchard, 1997; Petit and Vezina, 2014; Schuler et al., 2010). An increase in Hct can lead to a linear increase in blood oxygen carrying capacity, but also an exponential increase in blood viscosity (Fig. 6), which would hinder transport of oxygen to active tissues (Birchard, 1997). This suggests that the higher performance of anti-EPO-treated birds at high altitude might be due to lower blood viscosity; to address this possibility, we need to first consider our data on the physiological response of exercise at high altitude.

Our study showed that yellow-rumped warblers decrease Hct when exercising at altitude, regardless of treatment, a result which is contrary to our initial prediction, as well as other studies of the effects of altitude on Hct (Borras et al., 2010; Jelkmann, 2003, 2011). It has been shown that birds decrease Hct as a result of hemodilution in response to endurance flight (Jenni et al., 2006). Jenni et al. (2006) suggested that hemodilution is an adaptive response to endurance exercise in birds because it lowers blood viscosity, thereby lowering heart energy expenditure, as well as increasing delivery of fuel in circulation. Given that hemodilution is an adaptive response to exercise in the low-altitude condition, and given that birds presumably have to work harder to extract oxygen from the environment in the high-altitude condition, perhaps it was not surprising to see further hemodilution with high-altitude exposure.

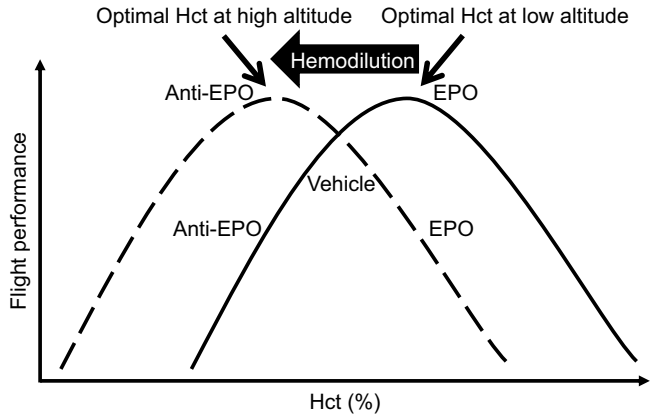

Fig. 6. Hypothetical *post hoc* rationale for the effects of anti-EPO on flight performance at low and high altitude. In the low-altitude condition, anti-EPO birds had lower performance as a result of the lower than optimal Hct values and consequently lower oxygen carrying capacity, but in the high-altitude condition they had higher flight performance because their initially low Hct was closer to a new 'optimal Hct' for exercise as a result of lower blood viscosity, at least over the short period measured in our study.

With further hemodilution, it seems intuitive that the optimal Hct for exercise at high altitude would be lower than the optimal Hct for exercise at low altitude; in other words, a left shift of the optimal Hct curve (Birchard, 1997) with increasing altitude (Fig. 6). If this is the case, we suggest a potential explanation for the seemingly paradoxical findings of flight performance in anti-EPO- versus EPO-dosed birds in low- and high-altitude conditions. In the low-altitude condition, anti-EPO birds would have lower performance as a result of the lower than optimal Hct values and consequently lower oxygen carrying capacity, but in the high-altitude condition, they would have higher flight performance because their initially low Hct would now be closer to a new 'optimal Hct' for exercise because of the lower blood viscosity (Fig. 6), at least over the short duration measured in our study. As noted earlier, changes in Hct could be due to changes in plasma volume and independent of changes in red blood cell numbers (Jacob et al., 2012; Pichon et al., 2016). It is also possible that a ceiling exists on how much hemodilution can occur and whether this can be influenced by EPO and/or anti-EPO. It seems plausible that anti-EPO-dosed birds were better able to regulate plasma volume at high altitude, perhaps via hemodilution, and consequently performed better during altitude challenge.

Plasma glucose and oxidative stress were measured to examine physiological responses to exercise at high altitude. Yellow-rumped warblers in our study had a higher glucose level during post-treatment flight, regardless of treatment, but this claim cannot be fully justified without a low-altitude control post-treatment. Mobilization of glucose is consistent with findings from mammalian studies (Hochachka, 1985; McClelland et al., 1998; Schippers et al., 2012). Although birds use predominantly fatty acids to fuel endurance flight, because lipid is the most energy dense metabolic fuel (Guglielmo, 2010), oxidizing lipid becomes increasingly challenging as altitude increases and partial pressure of oxygen decreases (Melzer, 2011). Therefore, birds likely altered their fuel selection and relied more on glucose as an oxygen-saving strategy during hypoxia (i.e. high-altitude) exposure (Melzer, 2011). Contrary to our initial prediction, we did not find a treatment effect of EPO and anti-EPO on OXY and dROM production. However, we did observe a lower total OXY during post-treatment flight at high altitude. This suggests that flying at high altitude for extended periods of time can be detrimental to the bird's physical condition, and could potentially explain why most birds spend the majority of time flying at low altitude during migratory flight (Scott, 2011). It should be noted that although we saw an overall increase in oxidative stress, similar to the general finding of Jenni-Eiermann et al. (2014), the increased oxidative stress observed in our study was mainly due to a decrease in OXY and not to an increase in dROM production, whereas Jenni-Eiermann et al. (2014) found increases in both antioxidant capacity and oxidative damage. This difference could be due to either inherent species differences or slight differences in migration strategy or behavior. Another possible explanation for the lower OXY observed in birds that were flown at high altitude is increased plasma volume due to hemodilution.

We acknowledge that there are a number of limitations in our study, some of which were related to logistical constraints, e.g. the complexity of training large numbers of birds and flying them in a wind tunnel. Our sample sizes were relatively small, which could be a contributing factor to the small effect sizes observed in our study, and a more rigorous pilot study to confirm optimal anti-EPO and EPO doses would have been valuable. Ideally, we would have blood sampled birds prior to the flight component of the experiment (day 7) but as blood sampling itself affects hematological traits, we

opted not to do this (relying on the pilot experiment). Other data on hematological responses, such as measurement of plasma volume (Jacob et al., 2012; Pichon et al., 2016), would have aided our interpretation. Although we are not aware of any studies measuring changes in plasma volume in relation to exercise in birds, Jenni et al. (2006) attributed the decreased Hct observed in response to endurance flight to an increase in plasma volume (i.e. hemodilution). We were unable to collect enough plasma samples from birds that were flown at low altitude only (i.e. not subjected to altitude challenge) during post-treatment flight, which limits our ability to firmly demonstrate physiological effects of altitude challenge on plasma glucose and oxidative stress. Finally, a longer altitude challenge (requiring separate flights or a follow-up experiment) would have helped us parse out the effects of time, training and altitude on some of the physiological measures.

In summary, our results suggest that the relationship between Hct and exercise performance is dependent on altitude, with low Hct being detrimental at low altitude, potentially due to low oxygen carrying capacity, but advantageous at high altitude, potentially as a result of low blood viscosity. Free-living birds may climb at slower speeds and thus adjust their physiology better to the changing altitude. Given that the exposure to high altitude in our experimental birds was rather short in duration, it is unclear whether birds exercising at high altitude for a longer duration would experience similar physiological adjustments. Therefore, determining whether free-living migratory birds adaptively modulate their Hct level based on the altitude they fly at during long-distance migratory flight requires further work (via either erythropoiesis or plasma volume regulation). Given the rapid advancement of technology associated with remote tracking of wild animals, such as biosensors and radio telemetry (Gumus et al., 2014, 2015; Killen et al., 2017), future studies should manipulate Hct in the field and investigate the migratory capacity of free-living migratory birds (Yap et al., 2017).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.N.Y., C.G.G., T.D.W.; Methodology: K.N.Y., C.G.G., T.D.W.; Validation: K.N.Y.; Formal analysis: K.N.Y.; Investigation: K.N.Y., M.D., C.G.G.; Resources: C.G.G., T.D.W.; Writing - original draft: K.N.Y.; Writing - review & editing: K.N.Y., M.D., C.G.G., T.D.W.; Visualization: K.N.Y.; Supervision: C.G.G., T.D.W.; Project administration: C.G.G., T.D.W.; Funding acquisition: C.G.G., T.D.W.

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Supplementary information
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