of red blood cells compared with the total blood volume) has dual but opposing effects on oxygen transport: an increase in Hct results in a linear increase in oxygen-carrying capacity of blood and an exponential increase in blood viscosity. Consequently, the relationship between oxygen transport and Hct is parabolic and in mammals there is evidence for an ÔoptimalÕ Hct that maximizes oxygen transport, with lower and higher Hct resulting in decreased oxygen transport due to reduced oxygen-carrying capacity and increased viscosity, respectively (Birchard 1997, Schuler et al. 2010).

Hct varies markedly among individuals and through the annual cycle in free-living birds (Morton 1994, H›rak et al. 1998, Davey et al. 2000). For example, in migratory White-crowned Sparrows Zonotrichia leucophrys orianth, the highest mean Hct was 60% at arrival on the breeding grounds and the lowest values (50%) occurred during post-nuptial moult (Morton 1994). During chick-rearing Hct can vary from 33 to 60% in provisioning adults (Ots et al. 1998, Burness et al. 2001). The functional, Þtness-related consequences of this variation are poorly understood. It has been suggested that putative ÔadaptiveÕ variation in Hct (between 5 and 10%) is associated with the varying demands for aerobic capacity and oxygen transport which occurs during migration (Bairlein & Totzke 1992, Piersma et al. 1996, Landys-Ciannelli et al. 2002), winter acclimatization (Swanson 1990), and with increasing altitude (Clemens 1990, Ruiz et al. 1995, Prats et al. 1996). It has also been proposed that decreased Hct during Ôreproductive anaemiaÕ might represent a Ôcost of reproductionÕ (Kalmbach et al. 2004, Williams et al. 2004). However, almost all studies to date have been either purely correlative or have involved manipulation of non-haematological traits to investigate correlated responses of Hct (e.g. Saino et al. 1997).

Here we describe a series of experiments that validate the use of phenylhydrazine hydrochloride (PHZ) for the transient, reversible experimental manipulation of Hct and plasma Hb in birds. PHZ causes oxidative denaturation or haemolysis of red blood cells and has been widely used in the study of erythropoiesis, haematological and cardiovascular responses in Þsh, anurans and mammals (Flores & Frieden 1968, McClelland et al. 2005, Simonot & Farrell 2007, Schuler et al. 2010) but rarely in birds, and then mainly in poultry species (Datta et al. 1990a, Riera et al. 1991). We suggest this is a widely applicable technique for use in experimental studies aiming to establish relationships among haematological status, aerobic capacity, workload (e.g. migration, parental care, thermoregulation), individual quality (of both adults and chicks) and trade-offs such as costs of reproduction.

METHODS

We conducted validation experiments on captive non-breeding and breeding Zebra Finches Taeniopygia guttata

and were given wet food (hard-boiled eggs, purŽed carrots, multi-vitamin powder) once a week. All animal husbandry and experiments were carried

we collected a maximum of 250 l L blood per 15-day period, or 3% of estimated blood volume. This is consistent with CCAC and AOU (Gaunt & Oring 2010) guidelines for blood sampling.

PHZ treatment and haematological measurements

We made up a PHZ stock solution by dissolving 100 mg phenylhydrazine hydrochloride (Sigma-Aldrich Canada, Oakville, Ontario, Canada) in 10 mL saline (10 mg ml mL, or 1 mg ml 100 l L). European Starlings received injection volumes of 100 l L of this stock solution, equivalent to 12.5 l g PHZ kg BW for an average 80-g bird. For Zebra Finches we diluted the PHZ stock solution 1 : 1 with saline (0.5 mg ml 100 l L) and birds received an injection volume of 30 or 40 l L equivalent to 10.0 and 12.5 l g PHZ kg BW, respectively, for an average 16-g bird. This procedure for PHZ treatment was approved by Simon Fraser University Animal Care Committee permits (901B-94 and 829B-96) and followed guidelines of the Canadian Committee on Animal Care.

Hct (% packed cell volume) was measured with digital callipers (±0.01 mm) following centrifugation of whole blood for 3 min at 13 000 g (Microspin 24; Vulcon Technologies, Grandview, MO, USA). Hb (g dL whole blood) was measured using the cyanomethaemoglobin method (Drabkin & Austin 1932) modiÞed for use with a microplate spectrophotometer (BioTek Powerwave 340; BioTek Instruments, Winooski, VT, USA), using 5 l L whole blood diluted in 1.25 mL DrabkinÕs reagent (Sigma-Aldrich Canada, Oakville, Ontario, D5941) with absorbance measured at 540 nm. Intra- and inter-assay coefÞcients were 1.1% (n = 12) and 2.2% (n = 6), respectively, for Hb assays. The proportion of reticulocytes (number of immature red blood cells total red blood cells · 100) was estimated from whole blood smears after supravital staining with new Methylene Blue (R4132; Sigma Aldrich Canada). A total of 1000 red blood cells were counted per slide, and reticulocytes were distinguished from mature erythrocytes by their relatively larger size and less condensed chromatin (Campbell & Ellis 2007). Red blood cells were classiÞed as reticulocytes if at least Þve reticulum (RNA) aggregations were visible in the cytoplasm or if there was a distinct ring of reticulum surrounding the nucleus (Fernandez & Grindem 2006).

All statistical analyses were carried out using SAS software version 9.2 (SAS Institute 2008). Unless otherwise stated, data were analysed using repeated-measures mixed linear models (MIXED procedure) with treatment, day or reproductive stage as Þxed effects and individual (band number) as a random effect. We used a constant injection volume which was too small to adjust accurately to variation in body mass (Zebra Finches, 15.5 ± 1.4 g; 5 and 95% quantiles, 13.4–18.2 g; European Starlings, 76.9 ± 4.3, 69.5–84.7 g); thus, the actual PHZ dose varied with body mass. Therefore, we initially included body mass as a covariate in all analyses. However, this term was not signiÞcant in any model ($P > 0.10$ in all cases) so we subsequently omitted this and report results of the reduced models. P-values for post-hoc multiple comparison tests of differences between means were Bonferroni-corrected. Values are least-squares means ± se unless otherwise stated.

RESULTS

Dose-dependent effects of PHZ in non-breeding Zebra Finches

PHZ treatment at 10 and 12.5 l g kg BW caused a signiÞcant decrease in Hct at days 4, 7, 9 and 11 post-injection (Fig. 1a). Hct was signiÞcantly lower at day 9 with 12.5 l g kg BW treatment (45.6 ± 1.8%), compared with day 8 and 10 l g kg BW treatment (49.9 ± 0.9%; $F_{1,42} = 4.29$, $P < 0.05$), conÞrming a dose-dependent effect. PHZ treatment at 12.5 l g kg BW caused a decrease in plasma Hb at days 7, 9 and 11 post-injection (Fig. 1b). Body mass changed with 10 l g kg BW treatment ($F_{4,112} = 13.10$, $P < 0.001$): mass decreased by 9.3% from day 0 to day 8 but then remained constant through day 16. Similarly, body mass changed with 12.5 l g kg BW treatment ($F_{4,34} = 13.4$, $P < 0.001$): mass decreased by 4.3% from day 0 to day 8 but then remained constant through day 11 and returned to pre-injection values by day 14.

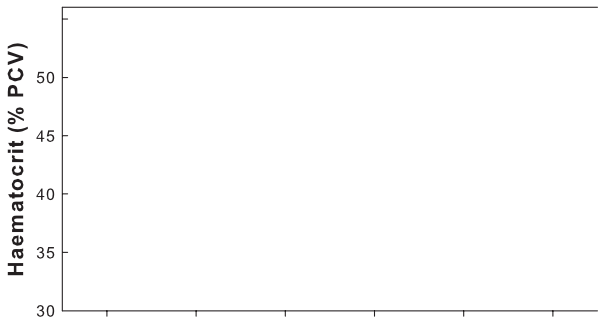
In the second experiment comparing effects of 12.5

Body mass decreased with time in this experiment () 4.5%; $F_{3,54}$ = 4.76, P < 0.01) but there was no effect of treatment (P > 0.8) and no treatment · time interaction (P > 0.6).

We pooled data for PHZ-treated birds from trials 1 and 3 to investigate factors that might affect the initial decrease in Hct between day 0 and days 4‡5. The change (decrease) in Hct was independent of initial body mass (P > 0.50) but was negatively correlated with initial pre-injection Hct (r_{34} =) 0.37, P < 0.05; Fig. 2), i.e. females with higher initial Hct had a greater decrease in Hct in response to PHZ treatment.

Rapid effects of PHZ over 24 h

There was a signiÞcant decrease in Hct over 24 h post-injecs with



Effect of PHZ in photostimulated European Starlings

There was a signiÞcant treatment time interaction for PHZ- and saline-treated female Starlings for Hct ($F_{4,44.9}$ = 14.22, P < 0.001; Fig. 4a) and plasma Hb ($F_{4,44.9}$ = 5.40, P < 0.01; Fig. 4b). Hct and Hb varied signiÞcantly with time in PHZ-treated females (P < 0.001) but not in saline-treated control females (P > 0.15). Hct was signiÞcantly lower in PHZ-treated females than in saline-females at 1 day post-injection, and plasma Hb was signiÞcantly lower at days 1 and 5 (Fig. 4). Both Hct and plasma Hb were signiÞcantly lower 3 days after PHZ treatment compared with pre-injection values (P < 0.05 in both cases; Fig. 4). Body mass decreased with time, from 78.8 to 76.9 g () 2.5%; $F_{4,45}$ = 4.22, P < 0.01) but there was no effect of treatment or a treatment · time interaction (P > 0.25).

DISCUSSION

We have shown that the drug PHZ can be used effectively and consistently for the transient, reversible experimental manipulation of Hct and Hb in two species of passerine bird, Zebra Finch

and European Starling. We saw no evidence of negative effects of PHZ treatment other than on the targeted haematological traits: no birds were observed with lethargy, ßuffed-up feathers or other general signs of ill-health, and there was no mortality. In several experiments there was signiÞcant mass loss (2Ð9%). However, this was a generic response to frequent handling and blood sampling (every 4Ð5 days) rather than a speciÞc response to PHZ, as we found no treatment by time interaction for mass loss in our experiments. This is unlikely to be a concern in Þeld studies where one-time capture and treatment will be the norm (see below). However, for other laboratory-based studies this problem could be minimized by obtaining baseline, pretreatment Hct values 2Ð3 weeks in advance of PHZ treatment, with a single blood sample at day 5 post-treatment to assess variation in PHZ response.

PHZ treatment caused a rapid decrease in Hct

We suggest that PHZ treatment represents a widely applicable technique for experimental manipulation of Hct and plasma Hb in ecological and evolutionary research in studies of both free-living and captive birds. Clearly PHZ can only be used to reduce (not increase) Hct; we have tried using human recombinant erythropoietin to increase Hct, but with inconsistent results so far. It is also clear that oxygen transport involves a complex, highly integrated, multi-functional physiological system and that Hct and plasma Hb are

Flores, G. & Frieden, E. 1968. Induction and survival of hemoglobin-less and erythrocyte-less tadpoles and young bullfrogs. Science 159: 101–103.

Gaunt, T.S. & Oring, L.W. 2010. Guidelines to the Use of Wild Birds in Research. Washington, DC: The Ornithological Council.

Hõrak, P., Jenni-Eiermann, S., Ots, I. & Tegelmann, L. 1998. Health and reproduction: the sex-speci c clinical pro- le of Great Tits (Parus major) in relation to breeding. Can. J. Zool. 76: 2235–2244.

Itano, H.A., Hosokawa, K. & Hirota, K. 1976. Induction of haemolytic anaemia by substituted phenylhydrazines. Br. J. Haematol. 32: 99–104.

Jenni, L., Muller, S., Spina, F., Kvist, A. & Lindstro ¨m, A. 2006. Effect of endurance ight on haematocrit in migrating birds. J. Ornithol. 147: 531–542.

Jones, J.H. 1998. Optimization of the mammalian respiratory system: symmorphosis versus single species adaptation. Comp. Biochem. Physiol. B 120: 125–138.

Kalmbach, E., Grif ths, R., Crane, J.E. & Furness, R.W. 2004. Effects of experimentally increased egg production on female body condition and laying dates in the Great Skua