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Validation of the use of phenylhydrazine hydrochloride (PHZ) for experimental manipulation of haematocrit and plasma haemoglobin in birds

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The levels of haematocrit (Hct) and plasma haemoglobin (Hb) vary markedly through the annual cycle of birds, as well as among individuals at all life-stages (embryos, chicks, adults). It is thus surprising that the functional, fitness-related consequences of this variation are poorly understood. Putative 'adaptive' variation in these haematological traits has been associated with varying demands for aerobic capacity and oxygen transport, for example during migration, winter acclimatization, with increasing altitude, or during parental care. It has also been proposed that changes in Hct might reflect 'costs' of parental investment, for example during 'reproductive anaemia'. However, almost all studies to date have been correlative. Here we describe a series of experiments that validate the use of phenylhydrazine hydrochloride (PHZ) for the transient, reversible experimental manipulation of Hct and Hb in birds. A single bolus injection (12.5 μg PHZ/g body weight delivered via intra-muscular injection) caused a rapid decrease in Hct and plasma Hb within 24 h, from pretreatment values of 50–54% to 40–44% in non-breeding Zebra Finches *Taenipoygia guttata* and European Starlings *Sturnus vulgaris*, and to 35% in breeding female Zebra Finches, changes within the normal physiological range. Hct and Hb returned to pre-injection levels within 5–10 days of treatment. Changes in plasma Hb paralleled those for Hct. We suggest that PHZ treatment provides a widely applicable technique for use in experimental work to establish relationships between 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.

Keywords: European Starlings, experimental manipulation, haemolytic anaemia, individual variation, Zebra Finches.

Whole-organism aerobic capacity is one of the main predictors of endurance or the ability to sustain a high workload, and this in turn depends on the oxygen-carrying capacity of the blood (Wagner 1996, Calbet *et al.* 2006). Oxygen transport involves a complex, highly integrated, multi-functional physiological system and all components of this system, from oxygen uptake in the lungs to oxygen utilization in muscle tissue, contribute to aerobic capacity and maximum oxygen consumption (VO_{2max}; e.g. Jones 1998). However, two easily measured haematological traits, haematocrit (Hct) and haemoglobin (Hb), are important determinants of aerobic capacity and VO_{2max} even when the circulating blood volume is maintained at a constant level, via changes in the oxygen-carrying capacity of the blood (Calbet *et al.* 2006). Hb is the major respiratory pigment for oxygen transport in vertebrates and is also thought to play a role in oxygen off-loading and transfer from capillaries to mitochondria in muscle tissue (Wagner 1996). Hct (packed cell volume, the relative volume

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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 'optimal' 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, Hõrak *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, fitness-related consequences of this variation are poorly understood. It has been suggested that putative 'adaptive' 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 'reproductive anaemia' might represent a 'cost of reproduction' (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 fish, 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 captive non-breeding (but photosensitive and long-day photostimulated) European Starlings *Sturnus vulgaris*. Zebra Finches were housed under controlled environmental conditions (temperature 19–23 °C, humidity 35–55%, constant light schedule of 14L : 10D, lights on at 07:00 h). During the experiments, six to 10 non-breeding birds were held in single-sex double cages (105 × 42 × 44 cm), and were not visually or acoustically isolated from other birds. Breeding birds were housed as single, randomly assigned pairs in single cages (52.5 × 42 × 44 cm) each equipped with an external nestbox (15 × 14.5 × 20 cm). All birds received a mixed seed diet (Panicum and White Millet 1 : 1; approximately 11.7% protein, 0.6% lipid and 84.3% carbohydrate), water, grit and cuttlefish bone (calcium) *ad libitum*, and a multi-vitamin supplement in the drinking water once a week. Breeding pairs were provided with 6 g/pair/day of a high-quality egg food supplement (one whole boiled Domestic Hen *Gallus gallus domesticus* egg; 13 g cornmeal; 13 g breadcrumbs; 20.3% protein, 6.6% lipid) during egg-laying and chick-rearing. European Starlings were removed from nestboxes as chicks (April 2010, at our long-term study site in Langley, British Columbia, 49°10′N, 122°50′W) and were hand-reared from 14 days of age (as part of a different experiment). Birds were transferred to, and held on, short day photoperiods (8L : 16D) between 21 May and 18 August, after they had completed growth and were self-feeding. They were then exposed to a gradually increasing photoperiodic cycle that mimicked natural changes in day length between January and April at 49°N (the latitude of our study site). At the time of the PHZ experiment (December 2010) the photoperiod was 14L : 10D and birds were therefore photosensitive and photostimulated. Starlings received Whole Earth 26% poultry and game starter crumbles (26% protein, 4% fat) and water *ad libitum*

and were given wet food (hard-boiled eggs, puréed carrots, multi-vitamin powder) once a week. All animal husbandry and experiments were carried out under Simon Fraser University Animal Care Committee permits (901B-94 and 829B-96) following guidelines of the Canadian Committee on Animal Care (see below).

Experimental protocols

To our knowledge, PHZ had only been used previously in birds in studies of the Domestic Hen. Thus, in a preliminary experiment (Experiment 1) we obtained a pre-breeding blood sample just prior to PHZ treatment (day 0) from non-breeding female Zebra Finches (n = 34); birds were then injected in muscle tissue (intra-muscular; i.m.) with PHZ at 10 μg PHZ/g body weight (BW). PHZ was dissolved in saline as an administration vehicle and injection volume was 30 μL. Birds were blood sampled at 4, 8, 12 and 16 days post-injection (no control birds were included in this initial experiment and only 24 females were blood-sampled on day 4). Our preliminary dose was based on Datta *et al.* (1990a) and Riera *et al.* (1991). Given the magnitude of the PHZ effect and the relatively rapid recovery in this first experiment (see Results) we repeated this experiment with a higher dose of 12.5 μg PHZ/g BW (40 μL injection volume, n = 10 females) and blood was sampled at day 0 and then at 7, 9, 11 and 14 days post-i.m. injection to better characterize the recovery phase. For the second of these trials we also measured plasma Hb (see below). To confirm these results, we then repeated this experimental treatment in a third trial, using 12.5 μg PHZ/g BW (n = 10 females), including a control group (30 μL saline i.m., n = 10) and standardized blood sampling at day 0 and days 5, 10 and 15 post-injection.

To determine short-term or immediate effects of PHZ treatment we obtained pre-injection blood samples from non-breeding female Zebra Finches, injected them i.m. with PHZ (12.5 μg PHZ/g BW) and obtained a second blood sample 24 h post-injection (Experiment 2). We repeated this experiment twice, obtaining pre-injection blood samples either 6 days (n = 12 females) or 1 day (n = 8 females) before PHZ treatment, and in the second trial included a control group which received a single i.m. injection with saline (n = 8 females).

PHZ treatment of breeding females would potentially augment the endogenous reproductive anaemia associated with egg production (e.g. Willie *et al.* 2010) such that breeding females might have different sensitivity to PHZ compared with non-breeders. Therefore, in Experiment 3, we determined the effect of PHZ-treatment during egg production on Hct and plasma Hb in breeding female Zebra Finches. Breeding females were given a single i.m. injection of either 12.5 μg PHZ/g BW (n = 9) or saline (n = 9) on day 3 after pairing, so that PHZ-induced anaemia coincided with egg production (average time from pairing to laying is 6 days, and average clutch size is six). Blood samples were then obtained at the one-egg stage (4.3 days post-injection), clutch completion (10.6 days), hatching (19.6 days), mid-chick rearing (29 days) and fledging (38.4 days post-injection). For all samples we measured Hct, plasma Hb and the proportion of immature red blood cells (reticulocytes) as a measure of regenerative erythropoiesis (see Discussion).

In Experiment 4, a pre-injection blood sample (day 0) was obtained from non-breeding female European Starlings and birds were then given a single i.m. injection of either 12.5 μg PHZ/g BW (n = 7) or saline (n = 6). Subsequently, females were blood-sampled at day 1, 5, 10 and 15 post-injection for measurement of Hct and plasma Hb. Preliminary data suggested that Starlings might have a different time-course of PHZ response compared with Zebra Finches (see Results and Discussion). Therefore, 7 days after the final blood sample in trial one, a further blood sample was obtained from saline-treated birds; these were then treated with 12.5 μg PHZ/g BW i.m. (n = 6) and blood-sampled 3 days after PHZ treatment.

All birds were blood-sampled from the brachial vein following puncture with a 26G needle and blood was collected using a 75-μL micro-haematocrit tube. Different birds were used in each experiment but some non-breeding birds were blood-sampled up to five times over a 15-day period in a single experiment (breeding Zebra Finches were sampled five times over a 40-day period, and a maximum of three times in 15 days). Therefore, for Zebra Finches we adjusted the blood volume that we collected depending on the frequency of blood sampling, e.g. for birds repeat-sampled five times, we took 15–30 μL blood per sampling event for Hct measurement. Thus, we attempted to remove no more than 15 μL blood over a 15-day period, or 10% of estimated blood volume for Zebra Finches. For European Starlings

we collected a maximum of 250 μL 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, or 1 mg/100 μL). European Starlings received injection volumes of 100 μL of this stock solution, equivalent to 12.5 μg PHZ/g BW for an average 80-g bird. For Zebra Finches we diluted the PHZ stock solution 1 : 1 with saline (0.5 mg/100 μL) and birds received an injection volume of 30 or 40 μL equivalent to 10.0 and 12.5 μg PHZ/g 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) modified for use with a microplate spectrophotometer (BioTek Powerwave 340; Bio-Tek Instruments, Winooski, VT, USA), using 5 μL whole blood diluted in 1.25 mL Drabkin's reagent (Sigma-Aldrich Canada, Oakville, Ontario, D5941) with absorbance measured at 540 nm. Intra- and inter-assay coefficients 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 classified as reticulocytes if at least five 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 fixed 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 significant 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 μg/g BW caused a significant decrease in Hct at days 4, 7, 9 and 11 post-injection (Fig. 1a). Hct was significantly lower at day 9 with 12.5 μg/g BW treatment (45.6 ± 1.8%), compared with day 8 and 10 μg/g BW treatment (49.9 ± 0.9%; $F_{1,42} = 4.29$, $P < 0.05$), confirming a dose-dependent effect. PHZ treatment at 12.5 μg/g BW caused a decrease in plasma Hb at days 7, 9 and 11 post-injection (Fig. 1b). Body mass changed with 10 μg/g 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 μg/g 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 μg PHZ/g BW with saline controls there was a significant treatment × time interaction ($F_{3,54} = 13.3$, $P < 0.001$; Fig. 1c). In PHZ-treated females Hct was significantly decreased at days 5 and 10 but not day 15. In saline-treated females, Hct did not change between day 0 and day 5 but was significantly lower at days 10 and 15 (Fig. 1c).

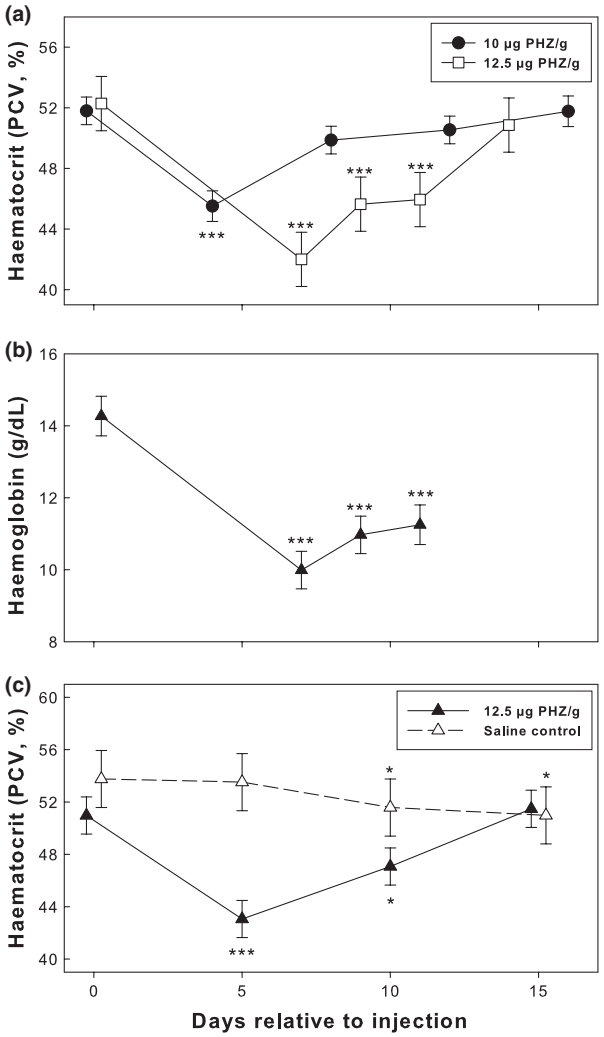

Figure 1. (a) Change in haematocrit with a single treatment of 10 or 12.5 µg PHZ/g BW on day 0; (b) change in plasma haemoglobin with single treatment of 12.5 µg PHZ/g BW, and (c) change in haematocrit in PHZ- (12.5 µg PHZ/g BW) and saline-treated controls. Values are means ± se and asterisks indicate values significantly different from pretreatment values at day 0 (*$P < 0.05$; ***$P < 0.001$).

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.

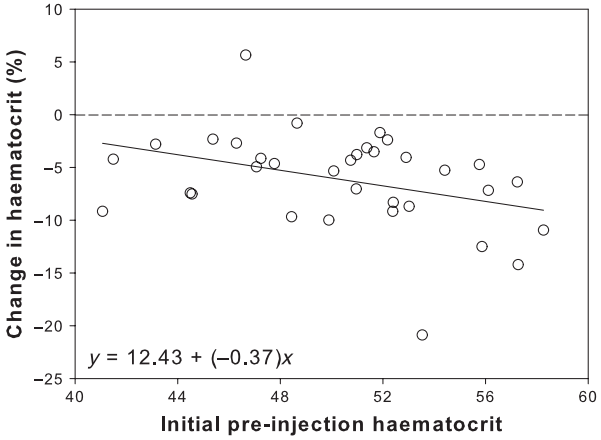

Figure 2. Relationship between change in haematocrit (pre-injection to 4/5 days post-injection) and initial, pre-injection haematocrit in PHZ-treated, non-breeding, female Zebra Finches.

Rapid effects of PHZ over 24 h

There was a significant decrease in Hct over 24 h post-injection in PHZ-treated females in trial 1 (−4.1%; $F_{1,7} = 12.31$, $P < 0.01$) and trial 2 (−3.9%; $F_{1,11} = 25.31$, $P < 0.001$), and the change in Hct was not significantly different among PHZ-treated females sampled either 6 days or 1 day before injection ($F_{1,26} = 0.08$, $P > 0.70$). Hct decreased in saline-treated females over 24 h (−2.2%) but this was not significant ($P > 0.05$).

Effect of PHZ in breeding Zebra Finches

There was a significant treatment × breeding stage interaction for PHZ- and saline-treated females for Hct ($F_{5,54.7} = 5.38$, $P < 0.001$; Fig. 3a), plasma Hb ($F_{5,54.2} = 4.94$, $P < 0.001$; Fig. 3b), and reticulocytes ($F_{5,58.1} = 2.74$, $P < 0.05$; Fig. 3c). For all three traits, changes with time were significant in both PHZ- and saline-treated females ($P < 0.05$, except for Hb in saline females, $P < 0.07$). For Hct and plasma Hb, values at the one-egg stage were significantly lower in PHZ-treated females compared with saline-treated females ($P < 0.001$ in both cases, no difference at other breeding stages; Fig. 3a,b). In contrast, reticulocytes were significantly more abundant in PHZ-treated females at the one-egg stage ($P < 0.001$) and at clutch completion ($P < 0.05$; no difference for other stages, Fig. 3c).

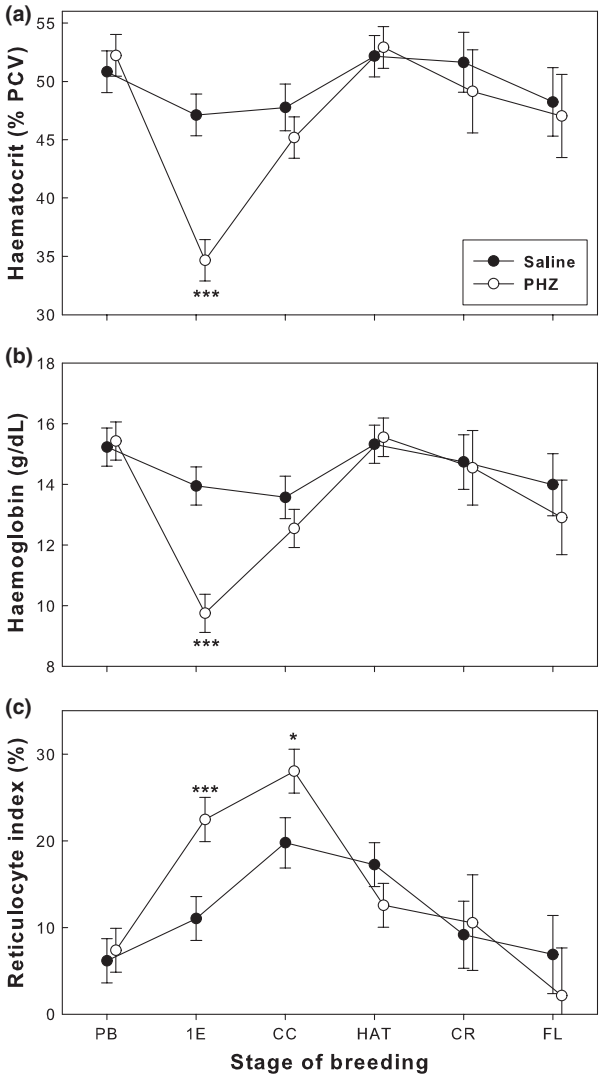

Figure 3. Changes in (a) haematocrit, (b) plasma haemoglobin and (c) reticulocytes in relation to PHZ- or saline- (control) treatment and breeding stage in female Zebra Finches. PB, pre-breeding; 1E, one-egg stage of laying; CC, clutch completion; HAT, hatching; CR, day 10 of chick-rearing; FL, fledging (21-day chick age). Values are means ± se and asterisks indicate significant differences between PHZ- and saline-treated females at each breeding stage (*$P < 0.05$; ***$P < 0.001$).

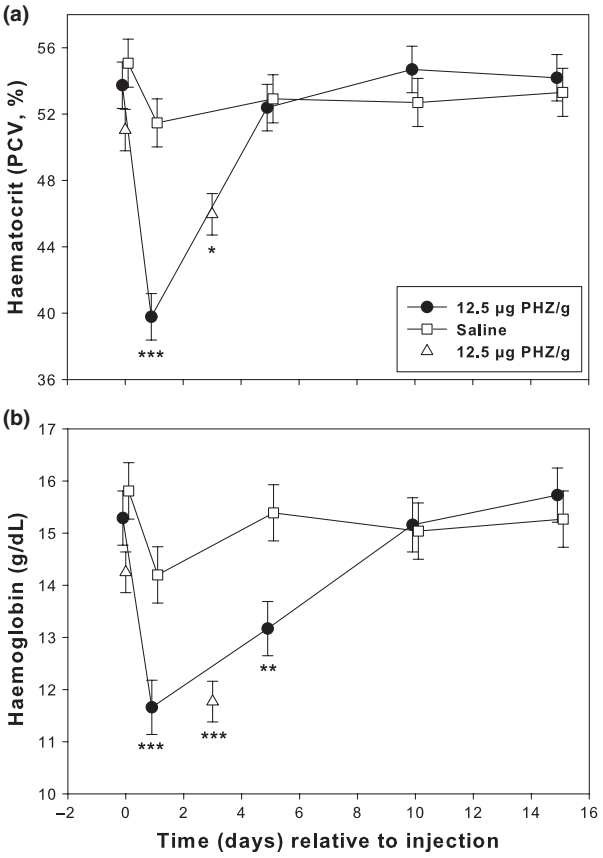

Figure 4. Changes in (a) haematocrit, and (b) plasma haemoglobin in relation to PHZ- or saline- (control) treatment in non-breeding female European Starlings. Values are means ± se and asterisks indicate values significantly different from pretreatment values at day 0 (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Effect of PHZ in photostimulated European Starlings

There was a significant 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 significantly with time in PHZ-treated females ($P < 0.001$) but not in saline-treated control females ($P > 0.15$). Hct was significantly lower in PHZ-treated females than in saline-females at 1 day post-injection, and plasma Hb was significantly lower at days 1 and 5 (Fig. 4). Both Hct and plasma Hb were significantly 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, fluffed-up feathers or other general signs of ill-health, and there was no mortality. In several experiments there was significant mass loss (2–9%). However, this was a generic response to frequent handling and blood sampling (every 4–5 days) rather than a specific 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 field 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 2–3 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 and plasma Hb within 24 h in both species, and Hct and Hb returned to pre-injection levels within 5–10 days of treatment (depending on dose). This time-course is entirely consistent with the known mode of action of PHZ. PHZ causes oxidative denaturation or haemolysis of red blood cells, with the formation of denatured, oxidized Hb (Heinz bodies) and destabilization and disruption of the mechanical integrity of the erythrocyte cell membrane. This leads to cellular alterations that in turn cause direct cellular haemolysis or selective removal of damaged erythrocytes from the circulation, ultimately leading to haemolytic anaemia (Itano *et al.* 1976, Vilsen & Nielsen 1984). In Domestic Hens treated with PHZ (6 mg/100 g BW) 'dumb-bell-shaped' erythrocytes with shrunken cytoplasm and Heinz bodies are seen only 1 day after treatment and peak at 3 days post-injection (Datta *et al.* 1990b). However, PHZ is readily biodegraded by the liver and kidney and haematological parameters therefore return to normal levels within 1–2 weeks of exposure. In Domestic Hens most red blood cells have normal morphology 7 days after PHZ treatment (Datta *et al.* 1990b) and in the Rock Dove *Columbia livia* and Domestic Hen Hct and Hb return to pre-treatment levels between 7 and 12 days after PHZ treatment (Ramis & Planas 1982, Datta *et al.* 1990a), with no long-term impairment of erythropoietic capacity (Byrne & Houston 1988).

The change in Hct in response to PHZ treatment was independent of initial, pretreatment body mass. However, we found some evidence that individual variation in the response to PHZ treatment was negatively correlated with initial, pre-injection Hct: females with higher pretreatment Hct showed a greater decrease in Hct in response to PHZ treatment. The same pattern is seen with changes in Hct during endogenous reproductive anaemia associated with egg production: the magnitude of reproductive anaemia is inversely correlated with pre-breeding Hct (Wagner *et al.* 2008a). This suggests that individual sensitivity to, or the mechanism of, PHZ-induced haemolytic anaemia might be similar to endogenous reproductive anaemia.

Although Hct and Hb returned to pretreatment levels between 5 and 10 days of PHZ treatment in Starlings and Zebra Finches there was some evidence for a more prolonged physiological recovery via regenerative erythropoiesis, as indicated by the presence and abundance of reticulocytes (see Fig. 3c). Increased levels of reticulocytes are observed within 5 days of PHZ treatment (Datta *et al.* 1990b) but peak levels (25–30%) are often seen at the time, or slightly after, Hct and Hb return to normal values (Riera *et al.* 1991). More prolonged reticulocytosis is also observed after 'endogenous' reproductive anaemia associated with egg production in birds (Wagner *et al.* 2008b).

Using a dose of 12.5 μg PHZ/g BW we decreased Hct on average from pretreatment values of 50–54% to 40–44% in non-breeding birds and to as low as 35% in breeding female Zebra Finches, i.e. a 20–33% decrease. These PHZ-induced Hct levels are within the normal physiological range for Hct in free-living birds, which is highly individually variable. For example, Hct varied between 33 and 60% in chick-rearing adult birds (Ots *et al.* 1998, Burness *et al.* 2001). In our captive Zebra Finches, Hct varies from 44 to 62% in apparently healthy, non-breeding birds, and from 31 to 55% in females sampled during egg-laying (Wagner *et al.* 2008a, Willie *et al.* 2010). In free-living European Starlings Hct varies from 36 to 58% in egg-laying females and from 43 to 59% in chick-rearing adults (T. D. Williams unpubl. data). We found some evidence for a sensitive dose–response to PHZ in that treatment with 10 μg PHZ/g BW caused a smaller reduction in Hct and led to a more rapid recovery (experiment 1). This dose–response effect of PHZ could potentially be exploited depending on the aims of specific experimental studies.

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 probably only two of a suite of characters involved in phenotypic adjustments. However, these specific traits are easily measurable, they do appear to play a key role in oxygen transport (see Introduction), and numerous studies have proposed that there are fitness consequences of variation in Hct (see below). Although we only used adult females in our experiments (due to our interest in reproductive anaemia and costs of egg production; Williams *et al.* 2004, Willie *et al.* 2010) the non-specific mode of action of PHZ suggests this technique can be applied equally to male birds as well as embryos and chicks. The fact that a single bolus injection of PHZ is sufficient to decrease Hct for 5–10 days means that this technique is ideal for field studies where most individual birds will only be caught once. In captive studies, longer term, chronic anaemia could be maintained with repeated PHZ treatment every 5 days (although further research could examine whether there are less invasive routes of PHZ administration, e.g. osmotic minipumps). PHZ treatment could therefore be used to confirm and further explore hypothesized relationships between haematological traits, aerobic capacity, workload and individual variation in adult birds in relation to: (i) reproductive investment (Dufva 1996, Norte *et al.* 2010), costs of reproduction (Kalmbach *et al.* 2004 Williams *et al.* 2004) and the physiological basis of variation in parental care, such as chick provisioning; (ii) migration, in both free-living birds, using telemetry (Wikelski *et al.* 2003), and in wind-tunnel experiments with captive birds (Jenni *et al.* 2006); (iii) metabolic costs of thermoregulation and winter acclimatization (Swanson 1990); and (iv) adaptation to hypoxia at high altitude (Ruiz *et al.* 1995, Prats *et al.* 1996). Hct and plasma Hb increase rapidly during embryo and chick development but, again, there is marked individual variation in both traits at hatching, e.g. 21–52% in 13-day old Tree Swallow *Tachycineta bicolor* chicks (Morrison *et al.* 2009), and 27–52% in 17-day-old European Starling chicks (R.B. Fronstin unpubl. data). Experimental studies using PHZ treatment could therefore help elucidate the functional significance of this individual variation in Hct in the context of developmental or rearing conditions, for determining offspring phenotype, quality and post-fledging survival.

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