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### A HARD LOOK AT BLOOD SAMPLING OF BIRDS

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RECENTLY, MARY AND Charles Brown (2009) published an eye-opening study on adult Cliff Swallows (*Petrochelidon pyr-rhonota*) wherein they estimated that blood sampling led to a 21–33% decrease in survival. This is a staggering estimate that few would have anticipated. Moreover, it promises to provoke a thorough and critical reevaluation of the consequences of blood sampling, which we welcome. Blood sampling is well established as a standard tool in ornithological research; a recent Google Scholar search produced 149,000 references for the term “avian blood samples.” Sheldon et al. (2008) reviewed numerous uses of blood sampling, including (1) its necessity for understanding fundamentals of avian physiology such as endocrinology (Wingfield et al. 2008), metabolism (Schekkerman and Visser 2001), and parasitology (Dawson and Bortolotti 2000); (2) its value as a source of DNA for population genetics or evolutionary studies (e.g., Irwin et al. 2005, Hellgren et al. 2007); (3) the stable-isotope record it provides for connecting migrant breeding populations with their wintering sites and for describing diet (e.g., Rubenstein and Hobson 2004); and (4) its use in tracking infectious diseases such as avian influenza, malaria, and West Nile virus (e.g., Gancz et al. 2006). A curtailment of blood sampling would severely hinder—and, in many cases, completely impede—important lines of inquiry in myriad areas of ornithology, including behavior, conservation, ecology and evolution, and physiology. It is therefore important that the Browns' recent findings be put into perspective while we reexamine accepted blood-sampling protocols. Here, we remind readers of the potential consequences of blood sampling, suggest ways to mitigate some of these consequences, and advocate additional research to further refine our field sampling techniques. We hope that this will provide some perspectives on the Browns' (2009) findings and stimulate further discussion.

#### INTERPRETING THE BROWNS' FINDINGS

We anticipate that many reactions to Brown and Brown's (2009) findings will be published, and we urge everyone to read the

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Browns' responses to them. At the outset, we take it as a given that the Browns' results are genuine, although the cause(s) and generality of their results require rigorous evaluation and scrutiny. First, as they point out, blood sampling may induce greater dispersal by bled than by nonbled birds, which would be incorrectly interpreted as mortality in survival analyses. Even though the Browns may not have data on long-distance dispersal outside of their study population, it may be worth the effort for them to compare dispersal distances of bled and nonbled birds within their population (see Shutler and Clark 2003). If they find that blood sampling increases dispersal even over short distances, this would suggest that they may need to reduce the estimated difference in survival between bled and nonbled birds. However, dispersal is not always without costs, so there may be other unmeasured effects of blood sampling; for example, birds that moved farther may have reduced reproductive success. This may also be worth testing.

Second, birds that have been bled may be warier and less frequently recaptured, which would also contribute to higher mortality. Unfortunately, because there is no easy way to quantify undetected dispersal or capture averseness and distinguish them from mortality, for ethical reasons alone we feel it prudent to assume that blood sampling is a significant cause of mortality for Cliff Swallows and address this in our own research.

Perhaps the most important issue is the generality of the Browns' (2009) findings. Already, several researchers will be considering whether they have sufficient data to test whether survival is affected by blood sampling in their study populations, whereas others will initiate data collection to test for possible negative effects of drawing blood. Given the serious ethical and scientific importance of this, and assuming that the Browns' results can be generalized, these initiatives will hopefully identify environmental variables that contribute to or negate the effects. First, are consequences of blood sampling reduced in species that live in environments that are not as arid as the Browns' Nebraska study area? Birds in less arid environments may be better able to recover from fluid loss. Second, does diet (seeds vs. insects or other kinds

of meat, protein-poor vs. protein-rich foods, etc.) affect the ability to recover from the loss of blood or other fluids? Third, another, apparently unique, aspect of the Browns' study system is the extreme intensities of ectoparasitism, especially by Swallow Bugs (*Oeciacus vicarius*), which may aggravate the consequences of blood sampling, so that researchers working on species with low-intensity ectoparasitism may have less to worry about. We note, however, that the Browns found that birds whose nests had been fumigated and were free of swallow bugs also had lower survival as a consequence of blood sampling. Fourth, an obvious predictor of potential effects of blood sampling will be body size. This will be small comfort to researchers who work on birds that weigh less than 25 g, which encompasses a substantial number of us. Fifth, birds that are less aerial may be able to get by long enough to survive the consequences of blood sampling (including, but not limited to, hematomas that may make flight difficult or impossible; see below). But again, many of us work on very aerial species.

There is a noticeable gap in the literature on blood sampling of aerial-feeding species. Furthermore, it is unclear how applicable our current understanding of avian physiology is to these species. Periods of gliding may allow aerial-feeding species to achieve a lower mass-specific metabolic rate during flight than birds of similar size (Hails 1979); however, Bryant (1997) showed that aerial-feeding species still have the highest overall daily energy expenditure (4.4× basal metabolic rate) compared with other foraging modes (e.g., hovering, gleaning, etc.). Although variability in energy expenditure may exist between aerial-feeding species, much of what we know of avian hematology and respiratory function is based on the physiology of larger-bodied birds with more sedentary modes of foraging (e.g., *Anas* spp. and domestic chickens [*Gallus gallus domesticus*]). It is possible that bird species with higher mass-specific metabolic rates, and therefore higher demand for both oxygen and glucose, may be more susceptible to negative effects of both low blood volume and a subsequent reduction in oxygen capacity from a decrease in circulating red blood cells. The Browns' (2009) study is the first to document effects of blood sampling on any aerial-feeding species.

Brown and Brown (2009) found that the lower survival associated with blood sampling was detectable only between the year of sampling and the next year. This suggests that the possible negative effects of blood sampling on survival manifest in the short term (within days), which is consistent with proximate mechanisms such as circulatory shock from low blood pressure (hypovolemia) and reduced oxygen-carrying capacity, rather than, for example, infections that arise from puncture of the skin. It should be noted, however, that recent immunocompetence studies of Tree Swallows (*Tachycineta bicolor*) have not reported significant short-term mortality (e.g., within 18 days; Ardia 2005, 2007). Environmental differences between sampling sites may play a role in this contradiction. Birds sampled in cool mesic environments may adequately compensate for acute physiological side effects from reduced blood volume, whereas species sampled in more arid conditions or at extreme temperatures may have limited capacity to compensate. Thus, where possible, close monitoring of birds after sampling may provide valuable data about the causes of mortality and suggest ways to mitigate those causes. If mortality occurs shortly after blood sampling, it suggests that mitigation should be de rigueur as soon as possible.

As the Browns (2009) pointed out, proper evaluation of survival requires control birds that were not bled within the same year, and, preferably, controls should come from the same nest as bled birds (adults or nestlings). Moreover, modern mark–recapture analyses of survival need to be applied to such evaluations (although, as indicated above, even modern methods do not distinguish differential dispersal caused by manipulation).

#### AVIAN PHYSIOLOGY AND PAST EVALUATIONS OF CONSEQUENCES OF BLOOD SAMPLING

Birds exhibit an exceptional ability to mobilize extravascular fluids; this occurs twice as rapidly in domestic chickens as in Norway Rats (*Rattus norvegicus*; Ploucha and Fink 1986). In addition, the avian renal portal system (an arterial network) is extremely effective at redirecting blood from the peripheral vascular system to offset drops in systemic blood pressure (Raidal and Raidal 2006). This ability to rapidly offset drops in systemic blood pressure is one reason why large birds (e.g., domestic chickens and anadid ducks) rarely experience lactic acidosis as a result of blood loss (Sturkie 1986). On the basis of these findings, the avian research community has long considered birds, in general, to be highly resistant to negative effects of decreased blood pressure and, therefore, hypovolemic shock. Furthermore, early work on avian cellular metabolism suggested that birds were extremely resistant to lactic acidosis (compared with mammals) because of their red-blood-cell chemistry (Barron and Harrop 1928). As a result, anaerobiosis from reduced oxygen-carrying capacity has never been considered problematic in birds. Data accumulated over the past 40 years seem to support this view; a recent review that included data for 49 avian species from seven orders found no evidence to implicate blood sampling in any short- or long-term detrimental effects (Sheldon et al. 2008). This also suggests that the Browns' (2009) study cannot be generalized to all systems.

#### MITIGATING CONSEQUENCES OF BLOOD SAMPLING

It should now be clear that the physiological consequences of blood sampling can result in two related, but potentially different, outcomes. Reductions in blood volume (1) necessarily reduce the number of circulating red blood cells, hemoglobin volume, oxygen- and glucose-carrying capacity; and (2) may result in stress and, ultimately, systemic shock from low blood pressure. Although we noted above that birds are likely to be more resistant to lactic acidosis from low numbers of red blood cells, we encourage readers to consider this mechanism a potential problem. Symptoms of acidosis include fatigue and a reduced capacity for appropriate fight-or-flight responses. Renewal rates for avian red blood cells range between 30 and 42 days, depending on species: for example, 30 days in domestic chicken, 35 days in domestic Rock Pigeon (*Columba livia*, Carneau strain), and 45 days in domestic Mallards (*Anas platyrhynchos domestica*) (Rodnan et al. 1957). The only practical mitigation for a reduction in hemoglobin volume and oxygen-carrying capacity as a function of low blood volume will be to reduce sample volume or find alternatives to blood sampling. Current best practice suggests that blood samples equivalent to 1% body mass should be within safe limits (Gaunt and Oring 1999, Fair et al. 2010). However, this estimate cannot account for

seasonal and intraspecific variation in lean body mass. When sample volumes are determined by average body mass with little consideration for body composition (e.g., percent body fat), the possibility of removing unintentionally high volumes of blood increases. A more conservative method for determining safe blood-sample volumes would involve calculating average blood volume for species-specific lean body mass and limiting sample volume to less than 10% of total blood volume. We acknowledge that 1% of body mass is an easily adopted metric for field use, but with additional research, similar metrics could be developed that are more conservative and appropriate to account for variation in seasonal and individual body composition. We urge readers to be sensitive to this problem and strongly encourage additional research into this potential cause of sampling-induced mortality.

There are options that compensate for physiological problems associated with low blood volume, so here we focus on what can be done to mitigate this specific problem. Brown and Brown (2009) followed blood-sampling recommendations set forth by Gaunt and Oring (1999). According to this protocol, the volumes of blood taken should not have had lasting adverse effects. Animals that weigh less than a few hundred grams can, however, have their total blood volume significantly altered with removal of even small volumes (Samour 2008). For example, a 100-g juvenile Japanese Quail (*Coturnix japonica*) has a total blood volume of ~7 mL (Nirmalan and Robinson 1972), and Gaunt and Oring's (1999) recommendation that a blood sample could be drawn up to 1% of body mass would allow a 1-mL sample of blood to be taken (0.23 mL for a Cliff Swallow). If an investigator used 100 g as the average body mass to calculate sample volume for a population of young Japanese Quail, there would be a risk of drawing 1 mL from individuals that weighed less than the average and, thus, oversampling (e.g., a 1-mL sample from an 85-g bird would be 1.2% instead of the intended 1%). However, this concern is mitigated by the fact that most research, with the exception of hormone studies or studies on several separate blood variables, generally does not require samples of more than 50  $\mu$ L (0.05 mL). Another consideration is that subcutaneous hematomas may compound the effects of blood loss associated with blood sampling to cause hypovolemia. One may remove less than 1% of body mass of blood; however, additional blood lost into the extravascular space as a result of inadequate hemostasis may result in a critical loss of fluid. When compounded with an increased metabolic demand, even a short duration of hypovolemia can have negative effects on the animal's ability to compensate. Finally, there can be significant differences in blood volumes among animals of the same species (McGuill and Rowan 1989); as we pointed out above, these differences can be an effect of seasonal differences in body composition, or even of genetic differences among strains or subpopulations. It is, therefore, extremely important that investigators apply pressure to venipuncture sites long enough to ensure adequate hemostasis and that they be cognizant of the effects of repeated or large sample volumes. And once again, a more accurate method to prevent overestimation of blood-sample volume would be to develop new metrics based on blood volume and body composition, rather than body mass.

Although the probability of hypovolemic shock can be reduced by modifying sample volume to reflect individual (as opposed to the species' average) lean body mass, it is still possible for

negative effects to become apparent before reaching lower critical limits for total blood volume. If low blood pressure impairs flight and reduces a bird's ability to escape predators, the risk of mortality may increase even though birds appear to be asymptomatic. Early studies on avian physiology linked low blood pressure with an inability to withstand physical exertion (e.g., domestic chickens; Sturkie and Textor 1961) and increased mortality (Hollands and Merritt 1973); these effects may be further intensified by sex (estrogen depresses blood pressure so that females may be more susceptible; Sturkie and Ringer 1955) and temperature extremes (Whittow et al. 1965, Sturkie 1967). The Browns (2009) identified several additional environmental factors (e.g., ectoparasite load, sampling time with respect to reproductive cycle, and food availability) that may increase the probability of a negative outcome from blood sampling. Investigators should be mindful of these potentially negative and synergistic influences when they design blood-sampling protocols.

The effect of blood sampling on Cliff Swallow populations led the Browns (2009), and us, to specifically question whether blood loss in a small-bodied aerial-feeding species with a relatively high metabolic rate might be more problematic than similar blood loss in other, larger species. Removal of small volumes of blood in smaller species may be sufficient to induce early (compensatory) phases of hypovolemic shock in some individuals. Note that the resulting systemic circulatory shock would be a function of both the loss of critical blood volume and the loss of oxygen-carrying capacity. In this scenario, even a slight drop in blood pressure would result in a baroreceptor-mediated release of catecholamines (e.g., epinephrine). The body would attempt to compensate for the decrease in blood volume by increasing blood pressure through increased cardiac output and peripheral vascular resistance (Lichtenberger 2004). A simultaneous increase in circulating epinephrine would induce a hypermetabolic state (fight-or-flight response), resulting in release of glucagon and stress hormones and a subsequent rise in blood sugar. Similar symptoms have been observed in birds stressed by handling (Siegel 1980). Handling-induced metabolic acidosis has been clearly documented in birds that show little outward sign of distress (Le Maho et al. 1992) and can result in fatigue.

Animals in shock can present many different clinical signs. The combination of blood loss and increased metabolic response may not always lead to death, given the compensatory mechanisms mentioned above. However, associated shock may weaken birds, making them less able to escape predators or to acquire food on the wing. It is, therefore, conceivable that removal of small volumes of blood, in conjunction with handling-induced stress, could severely impair normal function in small bird species with high metabolic rates. Like possible reductions in oxygen-carrying capacity, possible blood-sampling-induced hypovolemia amplified by handling stress requires further investigation. Until we have a better understanding of this mechanism, however, it may be possible to offset effects of blood collection in small species by (1) prophylactic fluid replacement following blood sampling and (2) reduction of the volume of blood drawn from smaller individuals.

The superior ability of birds to mobilize extravascular fluids should make subcutaneous fluid therapy a viable mitigation of sampling-induced low blood pressure. In laboratory rats, fluid replacement has been used successfully to offset experimental side

effects on prolactin levels of removing relatively small volumes of blood (~6% total blood volume; Lawson and Gala 1974); when the lost volume of blood was replaced with saline, there was no procedure-induced change in prolactin levels. Thus, we propose that if researchers have reason to suspect, or have determined, that drawing blood from birds reduces return rates, they attempt a subcutaneous injection of a small volume of a crystalloid fluid solution (e.g., 0.9% saline solution or a lactated Ringers solution) to offset effects of decreased blood volume in small birds. They should then test whether this reverses the negative effect of drawing blood on return rates. Many researchers who draw blood are already familiar with injection techniques (e.g., PHA, testosterone). A small amount (3 mL per 100 g body weight; Lichtenberger 2007) of either saline solution or lactated Ringers solution injected beneath the skin with a tuberculin syringe and 28-gauge needle should more than compensate for sampling-induced drops in blood volume in most small bird species. Adding a 10% solution of electrolytes, vitamins, amino acids, and dextrose (e.g., Duphalyte; Solvay Duphar Veterinary, Weesp, The Netherlands) to injection fluids might further offset stress responses (Stocker 2005). In addition, it is possible to add small quantities of Oxyglobin, a hemoglobin-based oxygen carrier, to fluid treatments to offset the consequences of low levels of blood hemoglobin (Lichtenberger 2007). Avian skin is relatively inelastic, so only small boluses of fluid should be administered in this way, and care should be taken to not administer fluids into a bird's air sacs or overstretch the skin. The wing web or the inguinal region just above the inner thigh would probably be the best routes of injection (Stocker 2005). Although administering fluids is no more invasive than sampling blood or other forms of injection, training from a licensed veterinarian would be advisable. Moreover, we recommend these interventions only when it is clear that there has been a blood-sampling-induced increase in mortality or reduction in return rate.

The recommendation to compensate for decreases in blood volume from sampling is based on evidence that birds in early compensatory stages of hypovolemic shock respond well to fluid replacement (Lichtenberger 2004). If, however, the compensatory phase of shock is allowed to progress to the next phase (early decompensatory), uneven distribution of blood flow and acidosis can occur, even in resistant avian species. Thus, as the Browns (2009) pointed out, it is also imperative that investigators be aware that some individuals may not be able to withstand even extremely conservative levels of blood loss. Recommended sample volumes (for examples, see Gaunt and Oring 1999, Canadian Council on Animal Care 2003, Fair et al. 2010) should be substantially decreased during energetically demanding seasons or life stages, when birds would be exposed to temperatures outside their thermoneutral zone, in species or individuals with low body mass, or in species with high metabolic rates (e.g., hummingbirds and swallows). Although blood collection is now ubiquitous in ornithology, the procedure may still produce unexpected and substantial physiological reactions, especially when species-specific physiology is not well understood. Even when physiological responses to sampling appear to be minimal, internal homeostasis is altered and the autonomic nervous system will correct the imbalance; thus, sampling-induced stress may confound experimental results (Adams 1989). We should therefore take care to minimize confounding effects from stress-induced handling as well as the

potential to decrease survivorship. We have provided some thoughts on how it might be possible to decrease potential complications associated with blood sampling, but the Browns (2009) pointed out that there are also less invasive techniques that can provide endocrine, DNA, and viral data. The need to draw blood should always be carefully considered, and we again refer readers to the Browns' paper for a brief review of alternatives.

Brown and Brown's (2009) study demonstrates that we have too infrequently evaluated the effects of blood collection in many bird species. More work is required to evaluate those effects in a greater diversity of bird species. Clearly, we do not advocate the cessation of blood sampling; much progress in ornithology depends on information that can be obtained only through blood sampling. We believe that ornithologists should be proactive in understanding the implications of our sampling techniques. We have the means and the knowledge to truly evaluate potential problems associated with field practices. Diligence in assessing our methods and making modifications as needed should prevent regulatory bodies (e.g., institutional animal care and use committees, conservation groups, and government agencies) from unnecessarily limiting or removing useful methods from our research toolkit. This will, however, require additional research into the unique physiologies of a wide range of bird species. Until that time, we are all well advised to consider alternative methods, use conservative sampling plans, and employ preemptive methods to alleviate stress associated with blood sampling.

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