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REVIEW

The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists

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Summary

1. A growing number of ecologists are turning to the enumeration of white blood cells from blood smears (leukocyte profiles) to assess stress in animals. There has been some inconsistency and controversy in the ecological literature, however, regarding their interpretation. The inconsistencies may stem partly from a lack of information regarding how stress affects leukocytes in different taxa, and partly from a failure on the part of researchers in one discipline to consult potentially informative literature from another.

2. Here, we seek to address both issues by reviewing the literature on the leukocyte response to stress, spanning the taxa of mammals (including humans), birds, amphibians, reptiles and fish.

3. We show that much of the early literature points to a close link between leukocyte profiles and glucocorticoid levels. Specifically, these hormones act to increase the number and percentage of neutrophils (heterophils in birds and reptiles), while decreasing the number and percentage of lymphocytes. This phenomenon is seen in all five vertebrate taxa in response to either natural stressors or exogenous administration of stress hormones. For the ecologist, therefore, high ratios of heterophils or neutrophils to lymphocytes ('H : L' or 'N : L' ratios) in blood samples reliably indicate high glucocorticoid levels. Furthermore, this close relationship between stress hormones and N : L or H : L ratios needs to be highlighted more prominently in haematological assessments of stress, as it aids the interpretation of results.

4. As with hormone assays, there are challenges to overcome in the use of leukocytes profiles to assess levels of stress; however, there are also advantages to this approach, and we outline each. Given the universal and consistent nature of the haematological response to stress, plus the overwhelming evidence from the veterinary, biomedical and ecological literature reviewed here, we conclude that this method can provide a reliable assessment of stress in all vertebrate taxa.

Key-words: glucocorticoids, heterophils, leukocytes, lymphocytes, neutrophils, stress, vertebrates

Introduction

At one time confined to veterinary practice, methods and techniques that advance the understanding of physiological responses to the environment are now becoming a common place in ecological studies of wild animals. This development has led to the emergence of a new discipline, coined 'conservation physiology' by Stevenson *et al.* (2005) and explored further by Wikelski & Cooke (2006), who eloquently outlined the questions in conservation that can be addressed using a variety of physiological approaches, especially those that focus on stress. Indeed, stress in animals is clearly an important factor to consider when assessing their welfare in both captive and wild settings. In recent reviews, Wikelski & Cooke (2006) and Romero (2004) outlined a popular method of assessing physiological stress: the measurement of levels of adrenal glucocorticoid hormones, such as corticosterone, in plasma. Measuring these hormones clearly has many applications in ecology and has proven to be an invaluable tool. As with all methods, however, there are drawbacks associated with it. For example, levels of plasma corticosterone rise quickly immediately following capture of wild animals (Romero & Reed 2005), thus making it difficult to obtain baseline measurements in field situations. We review here a complementary method of physiological stress assessment that is becoming

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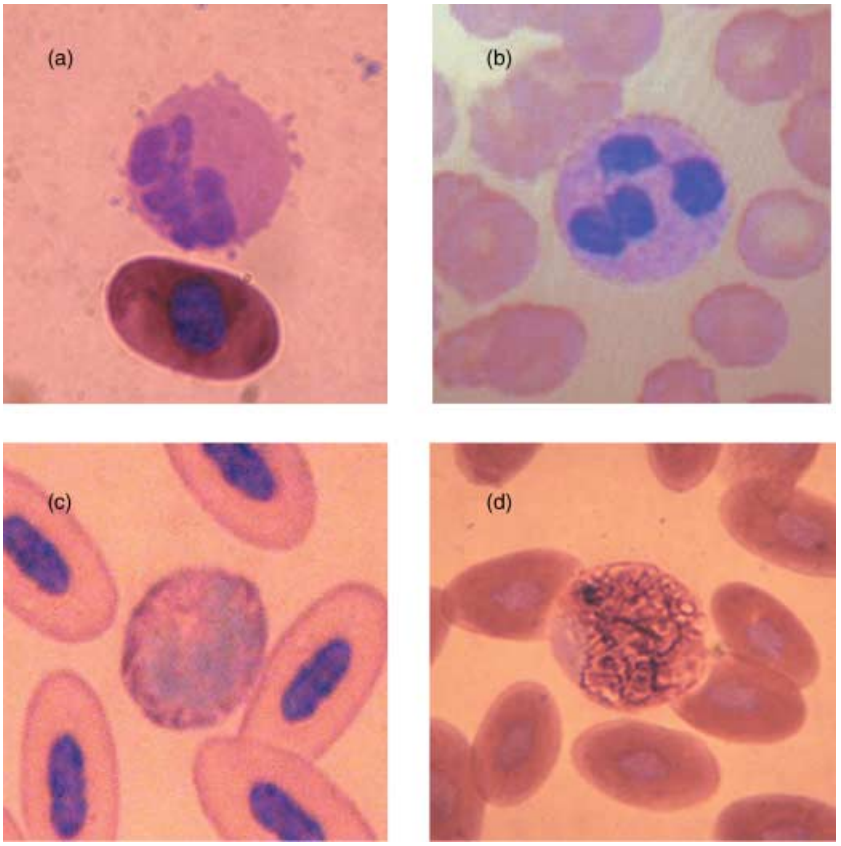

Fig. 1. Photomicrographs of amphibian (a) and mammalian (b) neutrophils, and avian (c) and reptilian (d) heterophils.

popular with ecologists, especially those studying birds: the use of haematological parameters such as relative white blood cell (WBC) counts made from blood smears. This approach may represent an alternate method for measuring corticosterone because, as reviewed below, increase in glucocorticoid hormones cause characteristic changes in the leukocyte component of the vertebrate immune system that can be quantified and related to hormone levels. Moreover, the leukocyte approach offers certain advantages over direct glucocorticoid measurement in that it does not require prohibitively rapid sampling and is relatively inexpensive. Finally, the haematological response to stress is conserved across taxonomic groups, ensuring that this approach to measuring stress can be applied to most vertebrates, and that results obtained from one taxonomic group should be useful for making predictions in others.

THE WHITE BLOOD CELL DIFFERENTIAL IN VERTEBRATES

We begin by outlining basic information about WBCs across vertebrate taxa. Most vertebrates have five types of WBCs: lymphocytes, neutrophils, eosinophils, basophils and monocytes. The morphology of each cell type appears to be conserved across taxa, except in the case of the neutrophil. In birds and reptiles, the neutrophil is replaced with the heterophil, which performs the same immunological function (Hawkey & Dennett 1989; Jain 1993). In amphibians, this cell type is occasionally referred to as a heterophil (e.g. Cabagna *et al*. 2005; Forbes, McRuer & Shutler 2006); however it appears more similar to a neutrophil (Fig. 1) (Thrall 2004) and the majority of authors use that term (e.g. Bennett & Harbottle 1968; Rouf 1969; Wojtaszek & Adamowicz 2003). Reptiles appear to have a sixth cell type, called an azurophil, which most researchers group with monocytes (Hawkey & Dennett 1989; LeBlanc, Heatley & Mack 2000).

The immunological function of each of the WBC types has been reviewed extensively elsewhere (Ellis 1977; Jain 1986; Maxwell 1987; Jain 1993; Maxwell & Robertson 1998; Thrall 2004). Briefly, neutrophils/heterophils and lymphocytes make up the majority (i.e. nearly 80% combined) of WBCs in mammals (Jain 1993), birds (Rupley 1997), amphibians (Bennett *et al*. 1972; Cathers *et al*. 1997; Thrall 2004) and reptiles (Eliman 1997; Fisse *et al*. 2004; Werner 2007) (see also Table 1). Neutrophils/heterophils are the primary phagocytic leukocyte, and proliferate in circulation in response to infections, inflammation and stress (Jain 1993; Campbell 1995; Rupley 1997; Harmon 1998; Thrall 2004). Lymphocytes are involved in a variety of immunological functions such as immunoglobulin production and modulation of immune defence (Campbell 1996). The remaining 20% of the leukocytes represent a combination of eosinophils, which play a role in the inflammation process (Jain 1993) and are associated with defence against parasites (Maxwell 1987; Rupley 1997; Kiesecker 2002), monocytes, which are long-lived phagocytic cells associated with defence against infections and bacteria (Campbell 1995; Davis, Cook & Altizer 2004) and basophils, the function of which is not clearly understood (Rupley 1997) but is thought to involve inflammation (Campbell 1995).

Table 1. Comparison of leukocyte profiles (percentage of total leukocytes) across vertebrate taxa. For reptile species, azurophils, if reported, were pooled with monocytes. Parameters from wild or control animals used if reported

Taxon	Species	Lymphocyte	Neutro/ Heterophil	Eosinophil	Basophil	Monocyte	Source
Mammals	Dog (*Canis lupus familiaris*)	23·1	66·4	6·3	0·1	4·0	(Jain 1986)
	Human (*Homo sapien*)	34·0	59·0	2-7	0·5	4·0	(Albritton 1952)
	Horse (*Equus caballus*)	38·7	52·6	3·4	0·5	4·3	(Jain 1986)
Birds	Chicken (*Gallus gallus*)	63·0	30·1	2·5	1·3	3·1	(Branton *et al*. 1997)
	Great tit (*Parus major*)	68·5	19·6	5·6	5·6	1·0	(Hauptmanova, Literak & Bartova 2002)
	Glaucous-winged gull (*Larus glaucescens*)	43·0	53·0	3·0	0·0	1·0	(Newman, Piatt & White 1997)
Amphibians	Red-spotted newt (*Notopthalmus viridescens*)	63·5	24·3	6·2	3·2	2·8	(Bennett & Daigle 1983)
	Bullfrog (*Rana catesbeiana*)	62·9	22·0	8·9	2·5	0·6	(Cathers *et al*. 1997)
	American toad (*Bufo americanus*)	20·0	68·0	3·3	7·4	1·5	(Forbes *et al*. 2006)
Fish	North African catfish (*Clarias gariepinus*)	58·8	39·4	0·0	0·0	2·6	(Gabriel, Ezeri & Opabunmi 2004)
	Channel catfish (*Ictalurus punctatus*)	43·0	3·5	0·0	0·0	1·6	(Ellsaesser & Clem 1986)
	Tilapia (*Oreochromis mossambicus*)	69·5	7·8	1·3	0·0	21·5	(Nussey *et al*. 1995)
Reptiles	Diamondback terrapin (*Malaclemys terrapin*)	17·7	74·6	1·1	1·6	6·1	(Werner 2007)
	Russian tortoise (*Agrionemys horsfieldi*)	46·7	37·2	4·8	5·0	6·3	(Knotkova *et al*. 2002)
	Inland bearded dragon (*Pogona vitticeps*)	59·0	27·0	0·0	9·0	5·0	(Eliman 1997)

The relative proportions of each WBC type, usually obtained by light microscope examination of 100 leukocytes in a stained blood smear, are the components of the leukocyte 'profile' (also called 'complete blood count', 'leukocyte differential' or 'haemogram') for any animal. The baseline leukocyte profile varies considerably among the vertebrate taxa. In mammals, for example, the most abundant WBC is the neutrophil, whereas in birds, lymphocytes are usually the most common (see Table 1). Furthermore, within taxa there is much variation among species.

STRESS-INDUCED CHANGES IN LEUKOCYTE PROFILES

Leukocyte profiles are particularly useful in the field of conservation physiology because they are altered by stress and can be directly related to stress hormone levels. As Dhabhar *et al*. (1996) pointed out, leukocyte profiles that deviated from normal parameters were routinely used to indicate mammalian hormonal stress responses in the 1940s, before methods were available to directly assess plasma glucocorticoids. Specifically, the changes brought on by stress or glucocorticoid treatment are increases in numbers of neutrophils (neutrophilia) and decreases in lymphocyte numbers (lymphopenia or lymphocytopenia). Moreover, since numbers of neutrophils and lymphocytes are affected by stress in opposite directions, researchers have often considered the ratio of one to the other, that is, the relative proportion of neutrophils to lymphocytes (hereafter, 'N : L' ratio) in mammals and amphibians, and heterophils to lymphocytes (hereafter, 'H : L' ratio) in birds and reptiles, as a composite measure of the stress response. This ratio, as read from standard blood smears made before and after a stressful event, is positively related to the magnitude of the stressor and to the circulating glucocorticoids (reviewed below). There is also evidence that this ratio is influenced by diseases and infections (or the stress hormones produced as a result of the infection), and we review this evidence later in this article.

A long history of research on mammals indicates that exogenous treatment with stress hormones (i.e. mimicking a physiological stress response) results in a marked alteration in leukocyte counts within 1–2 h (e.g. Dougherty & White 1944; Gordon 1955; Jain 1986; Dhabhar *et al*. 1995). This research, which has been conducted primarily in humans, livestock and mammalian laboratory animals, has involved treatment with cortisol or synthetic glucocorticoids, such as hydrocortisone, dexamethasone or prednisolone. These agents produce neutrophilia or lymphopenia, or both, in cattle (Anderson, Watson & Colditz 1999), sows (Kranendonk *et al*. 2005), horses (Burguez *et al*. 1983), bottlenose dolphins (Reidarson & McBain 1999), rats (Cox & Ford 1982), mice (Van Dijk *et al*. 1979), guinea pigs (Fauci 1975) and humans (e.g. Fauci & Dale 1974; Dale *et al*. 1975). Similar research has been conducted using adrenocorticotrophic hormone (ACTH), which stimulates release of glucocorticoids from the adrenal glands. Treatment with ACTH increases N : L ratio in boars (Bilandžič *et al*. 2006) and horses (Rossdale, Burguez & Cash 1982), probably via its effects on glucocorticoid secretion.

Long-term effects of glucocorticoid hormones on leukocyte populations are seen in humans with chronic medical disorders; for example, Cushing's syndrome is a disorder characterized by chronically elevated levels of plasma cortisol. Not long after the link between stress hormones and leukocytes was discovered in the early-1940s, medical researchers found that patients with this disorder had chronically elevated neutrophil counts and lower lymphocyte counts compared to healthy individuals (de la Balze, Reifenstein & Albright 1946). Similarly, neutrophils are elevated and lymphocytes depressed in humans with psychological disorders such as depression and schizophrenia, which are characterized by chronically elevated plasma cortisol levels (Kronfol *et al*. 1984). Chronically elevated glucocorticoids may therefore cause long-term elevations in N : L ratios.

The mechanism underlying the effect of stress hormones on leukocyte profiles has been well-documented in biomedical studies of mammals (reviewed in Ottaway & Husband 1994; Brenner *et al*. 1998). Stress-induced reductions in circulating lymphocyte numbers are not due to large-scale destruction of cells, but rather to glucocorticoid-induced alterations in the 'trafficking', or redistribution, of lymphocytes from the blood to other body compartments (Dhabhar 2002). In response to glucocorticoids, circulating lymphocytes adhere to the endothelial cells that line the walls of blood vessels, and subsequently undergo transmigration from circulation into other tissues, for example lymph nodes, spleen, bone marrow and skin, where they are sequestered (Cohen 1972; Fauci 1975; Dhabhar 2002). This exodus of lymphocytes from the blood causes a significant reduction in their circulating numbers. In contrast, glucocorticoids also stimulate an influx of neutrophils into the blood from bone marrow and attenuate the egress of neutrophils from the blood to other compartments (Bishop *et al*. 1968). These changes are thought to ensure that the different types of cells are routed to where they are needed during the stress response (Dhabhar *et al*. 1994; Dhabhar *et al*. 1996). For the ecologist seeking a tool to assess stress, they result in an increase in N : L or H : L ratio that is proportional to the level of glucocorticoid release.

Given the very clear effect of stress hormones on leukocyte profiles, it is not surprising that increases in N : L ratios are observed in response to stressors. For example, this phenomenon has been well-studied in the context of transport stress in mammals, which has broad ramifications across many fields, including veterinary medicine, biomedical research, agriculture and wildlife management (reviewed in Obernier & Baldwin 2006). Transporting animals from, for example, a vendor to a research laboratory, or from a farm to a slaughter house, causes a hormonal stress response as well as changes in leukocyte profiles. The N : L ratio has been shown to increase after the transportation in a variety of mammals including horses, goats, swine and cattle (reviewed in Obernier & Baldwin 2006) as well as beagles (Frank, Gauthier & Bergeron 2006), dolphins (Noda *et al*. 2007) and rhinos (Kock *et al*. 1999). Other types of stressors also affect haematological parameters of mammals; for example, N : L ratios increase after strenuous exercise in horses (Cardinet, Littrell & Schalm 1964; Rossdale *et al*. 1982) and humans (reviewed in Brenner *et al*. 1998), after restraint stress in rhesus monkeys (Morrow-Tesch *et al*. 1993) and southern chamois (López-Olvera *et al*. 2007), and after transfer from the wild to captivity in brushtail possums (Baker, Gemmell & Gemmell 1998). These examples represent only a fraction of the large literature on the effects of a variety of stressors on leukocyte profiles in a wide range of mammalian species, although we point out that much of this literature has been published in biomedical or veterinary journals and may not be readily available to ecologists.

Although the early studies of stress-induced neutrophilia and lymphopenia were conducted in mammals (e.g. Dougherty & White 1944; Gordon 1955), the phenomenon appears to be universal across vertebrates, having been demonstrated not only in mammals but also in birds, amphibians, reptiles and fish. The utility of the avian H : L ratio was first realized by poultry researchers (Gross & Siegel 1983) and is now commonly used to assess the welfare of chickens under different rearing conditions (Altan *et al*. 2000; Davis, Anderson & Carroll 2000; Elston *et al*. 2000; Onbasilar & Aksoy 2005; Nicol *et al*. 2006). Much of the early literature on this subject has been reviewed by Maxwell (1993). Throughout this body of work, there are numerous cases where the relationship between leukocytes and stress hormones is highlighted. For example, Davis *et al*. (2000) showed how reduced feed leads to increases in both corticosterone and H : L ratios in domestic chickens (*Gallus gallus*). Importantly, poultry researchers have also discovered that natural variation in the H : L ratios of newly-hatched chicks can be used to assess future susceptibility to diseases, with more susceptible individuals identifiable as chicks with high ratios (Al-Murrani, Al-Rawi & Raof 2002; Al-Murrani *et al*. 2006). This research, which has had a measurable impact on the poultry husbandry industry, has laid the groundwork for using leukocyte parameters to infer physiological stress in other avian species.

In recent years, ornithologists have capitalized on the findings in poultry and have increasingly quantified H : L ratios in wild birds across a number of ecological settings. For example, in passerines, H : L ratios increase in response to a wide variety of stressors, including long-distance migration (Owen & Moore 2006), transport (Parga, Pendl & Forbes 2001; Groombridge *et al*. 2004), parasitic infection (Davis *et al*. 2004; Lobato *et al*. 2005) and radioactive contamination (Camplani, Saino & Moller 1999). H : L ratios have also been associated with other measures of individual health and quality; for example, high ratios (indicating high stress) are associated in pied flycatcher (*Ficedula hypoleuca*) nestlings with reduced growth (Moreno *et al*. 2002), and low ratios are associated with large song repertoires in song sparrows (*Melospiza melodia*) (Pfaff *et al*. 2007). Low ratios in nestling pied flycatchers also positively predict their recruitment into the adult population (Lobato *et al*. 2005). These are but a few examples of a growing body of literature from the ornithological community, and because ornithologists report on leukocyte parameters more often than researchers in other fields, the body of relevant literature on avian species is growing faster than any other.

A lesser-known, earlier body of literature clearly demonstrates that the stress-induced leukocyte responses are also

exhibited by amphibians. In a series of carefully conducted laboratory experiments spanning three decades, Bennett and colleagues showed that the proportion of neutrophils increases while the proportion of lymphocytes decreases in newts and frogs after injection with hydrocortisone (Bennett & Alspaugh 1964; Bennett & Harbottle 1968; Bennett *et al*. 1972), limb amputation (Bennett 1986), osmotic stress (Bennett & Johnson 1973) and exposure to constant light (Bennett & Reap 1978). Increases in neutrophil numbers in amphibians exposed to agricultural pesticides have also recently been recorded (Cabagna *et al*. 2005), while the authors of this paper recently found that increased N : L ratios are associated with the onset of reproduction (Davis & Maerz 2008a) and with captivity (Davis & Maerz 2008b) in wild salamanders.

There is some evidence that the haematological response seen in mammals, birds and amphibians also occurs in reptiles. Saad & Elridi (1988) reported that 3–4 weeks of hydrocortisone treatment caused a pronounced lymphopenia in ocellated skinks (*Chalcides ocellatus*). Similarly, Morici, Elsey & Lance (1997) found that long-term treatment with corticosterone elevated H : L ratios in juvenile alligators (*Alligator mississippiensis*). In the same species of alligator, holding juveniles out of water with their jaws held closed caused a rapid rise in plasma corticosterone, followed by a dramatic increase in H : L ratio (Lance & Elsey 1999). In a recent study of box turtles (*Terrapene c. carolina*), H : L ratios were used to determine how individuals responded to captivity in low- vs. high-quality housing conditions (Case, Lewbart & Doerr 2005). In this case, individuals housed in high-quality conditions (i.e. with mulched floors, shredded paper and a hide box) had lower H : L ratios than those housed in low-quality conditions (i.e. only newspaper). Finally, Chen, Niu & Pu (2007) recently showed that increases in rearing density caused increases in H : L ratios in farm-raised soft-shelled turtles (*Pelodiscus sinensis*). These studies suggest that H : L ratios can be used to assess glucocorticoid levels and stress in reptiles; however, we point out that less work has been conducted in this taxon than in others.

The leukocyte response to stress has been well-studied in fish despite some confusion in the early literature over cell identification and nomenclature (Ellis 1977). Furthermore, thrombocytes (the equivalent to mammalian platelets) have sometimes been included in early investigations of 'leukocyte differentials' of fish (Saunders 1968), which makes comparison with modern counts difficult because most authors do not routinely report these non-leukocytic cells in differential counts (Thrall 2004). This trend has apparently continued in the ichthyology community, with authors of recent haematological studies also reporting thrombocytes in differential counts (e.g. Silveira-Coffigny *et al*. 2004). Nevertheless, neutrophils and lymphocytes appear to be readily quantifiable in fish, and the same leukocyte responses to stress and to exogenous glucocorticoid treatment (neutrophilia and lymphopenia) can be measured. Ellsaesser & Clem (1986), Bly, Miller & Clem (1990) and Harris & Bird (2000) provide excellent reviews on these responses. In general, acute stress induces both neutrophilia and lymphopenia in fish (e.g. Pulsford *et al*. 1994), although sometimes only lymphopenia is reported (Larsson, Lehtinen & Haux 1980), and these stress-induced changes have been shown repeatedly to be related to elevated glucocorticoids. Neutrophilia, lymphopenia and increased N : L ratios are apparent after treatment with either cortisol or hydrocortisone (Ellsaesser & Clem 1986; Wojtaszek *et al*. 2002). Treatment with ACTH also induces neutrophilia and lymphopenia in fish (McLeay 1973). These responses in fish are therefore identical to those seen in other vertebrate taxa.

Many ichthyologists have capitalized on the leukocyte stress response in fish as a tool for understanding the physiological effects of exposure to heavy metals and other contaminants in a wide range of species (e.g. Mishra & Srivastava 1979; Dick & Dixon 1985; Murad & Houston 1988; Dethloff *et al*. 1999; Witeska 2005). In fact, ichthyologists consider changes in the differential leukocyte count to be one of the most sensitive indicators of acute stress in fish (Wedemeyer, Barton & McLeay 1990). Thus, the size of the literature set on leukocyte responses to environmental contaminants in fish is indeed large, and although it is too extensive to review adequately here, we do highlight a selected subset of examples. A number of laboratory studies have exposed fish to heavy metals such as lead (Witeska 2005), zinc (Mishra & Srivastava 1979), copper (Dick & Dixon 1985; Dethloff *et al*. 1999) or cadmium (Murad & Houston 1988), and in each of these studies lymphopenia was reported, with neutrophilia reported in most (neutrophils were not always counted). The exception is a study of Mozambique tilapia (*Oreochromis mossambicus*) where exposure to copper resulted in the opposite pattern (Nussey *et al*. 1995). Experimental exposure to polluted water (i.e. industrial effluent) resulted in lymphopenia in flounders (*Platichthys flesus*) (Larsson *et al*. 1980). Finally, although not necessarily related to environmental contaminants, fish have also been exposed other stressors, including immersion in near-freezing water (Bennett & Gaudio Neville 1975), 24 h light (Valenzuela, Silva & Klempau 2008) and transport (Ellsaesser & Clem 1986). In all cases, lymphopenia and neutrophilia ensued.

ADVANTAGES OF LEUKOCYTE STRESS INDICATORS

The researcher using plasma glucocorticoid levels to assess stress is generally interested in two different measures: the baseline level prior to capture and handling, and the peak level during the stress response. Both of these measures provide information relevant to the stress physiology of the animal. Whereas obtaining a blood sample during the peak phase of the stress response is usually not a problem, quantification of baseline levels is more difficult because it requires the collection of a blood sample within a few minutes of capture. The exact timing of the rise is not known precisely in most species; however, the existing literature demonstrates that it typically occurs within 5 min and can begin in as soon as 2 min after capture in birds (Romero & Romero 2002; Romero & Reed 2005). Examples of two responses are compared in Fig. 2, which shows the time course of the adrenal response to stress in skinks (taken from Langkilde & Shine 2006) and in rats (taken from Muir & Pfister 1987). This rapid hormonal

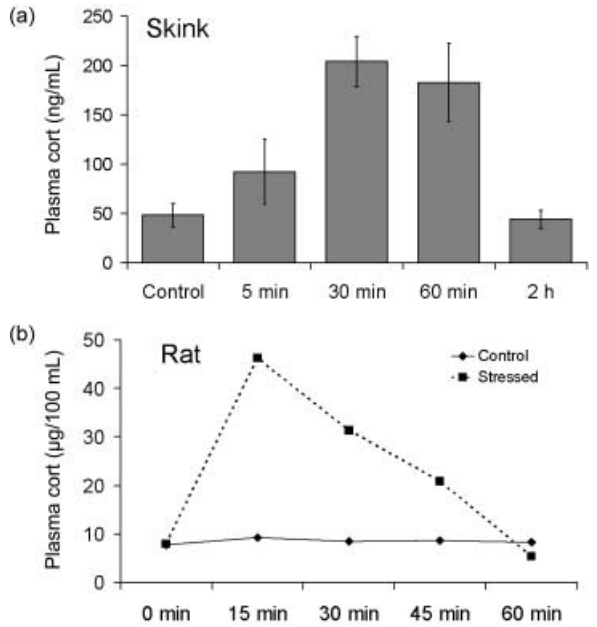

Fig. 2. Plasma corticosterone levels in (a) female water skinks (*Eulamprus heatwolei*) reproduced from Langkilde & Shine (2006); and (b) rats (reproduced from Muir & Pfister 1987). Skinks were stressed by chasing for 30 s. Rats were subjected to restraint stress and compared with a control group (no restraint).

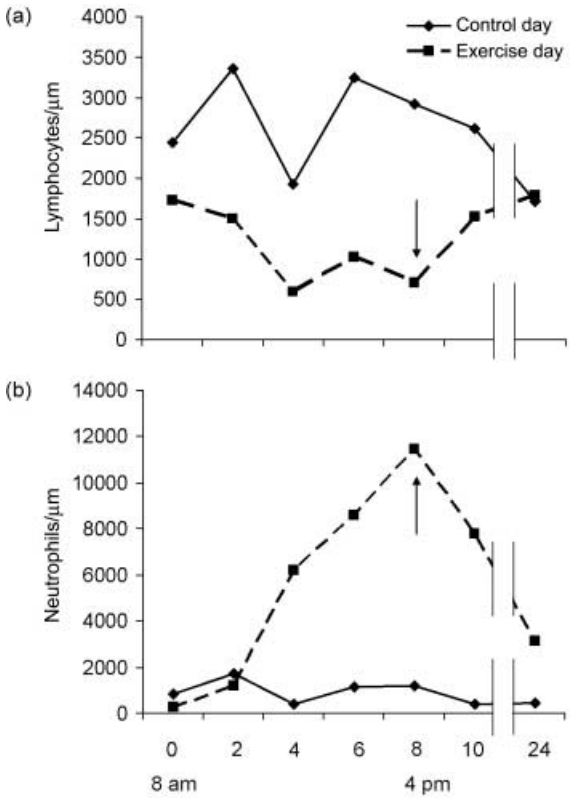

Fig. 3. Lymphocyte (a) and neutrophil (b) responses of a horse to exercise (Exercise day: a 32-mile (51·5-km) track traversed over 8 h; control day: no exercise). Blood samples were collected at 2-h intervals throughout. Graphs reproduced from Cardinet *et al.* (1964). Arrows indicate end of exercise.

response means that a field investigator wishing to take a baseline hormone sample from any vertebrate needs to monitor traps closely and must be very skilled at rapid blood collection. In fact, a recent study of house sparrows (*Passer domesticus*) emphasizes this point (Lynn & Porter 2008). In this project, the corticosterone stress response was found to begin as soon as individuals entered traps, even though they did not exhibit prolonged escape behaviour. For the researcher interested in glucocorticoid hormones, this study indicates that traps cannot be left unattended, even for brief periods.

Unlike the hormonal response to stress, the initial leukocyte response begins over a time span of hours to days, depending on the taxon. To demonstrate this point for mammals, we have reproduced two figures from an early study by Cardinet *et al.* (1964), who examined the short-term effect of strenuous exercise on leukocytes of the horse (Fig. 3). In this simple but effective study, a horse was taken across a 32-mile course over 8 h and its blood was sampled every 2 h for leukocyte examination. The resulting counts were compared to those made during a day with no exercise. Figure 3 shows how lymphocytes (A) remained lower than normal throughout the exercise, and then took 14 h after the end of the exercise to return to normal levels. Meanwhile, neutrophil numbers (B) increased throughout the exercise, then gradually declined upon termination, but had not yet returned to the baseline level 14 h later. It is important here to point out that neutrophil counts were not appreciably elevated until 4 h after the start of the exercise period. Similarly, Burguez *et al.* (1983) showed that injections of cortisol did not significantly elevate N : L ratios until 2 h after injection in foals and 4 h later in adult horses. In contrast to these studies on domestic mammals, more recent work shows that the leukocyte response may occur on a more rapid time scale in wild mammals. In a study of wild ungulates, neutrophil numbers were found to double, and lymphocytes reduced by half, within 1 h of capture (López-Olvera *et al.* 2007). Even this time course, however, is not so rapid that sampling becomes problematic.

Research with birds also demonstrates a relatively slow leukocyte response time that makes obtaining baseline samples convenient. Davis (2005) found that H : L ratios do not increase significantly within 1 h of capture in house finches (*Carpodacus mexicanus*). To demonstrate this idea graphically, we have reproduced a figure from that study as well as one from another study (Lindström *et al.* 2005) in which corticosterone was measured after capture in house finches (Fig. 4). In both studies, individuals were captured and sampled three times over a period of 1 h. Lindström *et al.* measured plasma corticosterone (Fig. 4a), whereas Davis quantified H : L ratios using blood smears (Fig. 4b). Plasma corticosterone increased dramatically within 30 min (Lindström *et al.* 2005), whereas H : L ratios did not increase significantly throughout the hour (Davis 2005). In a separate set of individuals held for 1 h and sampled only once at 60 min, H : L ratios did not increase at all, indicating that the slight trend of increasing H : L ratios seen in Fig. 4b may have been caused by repeated sampling, not the handling time *per se*.

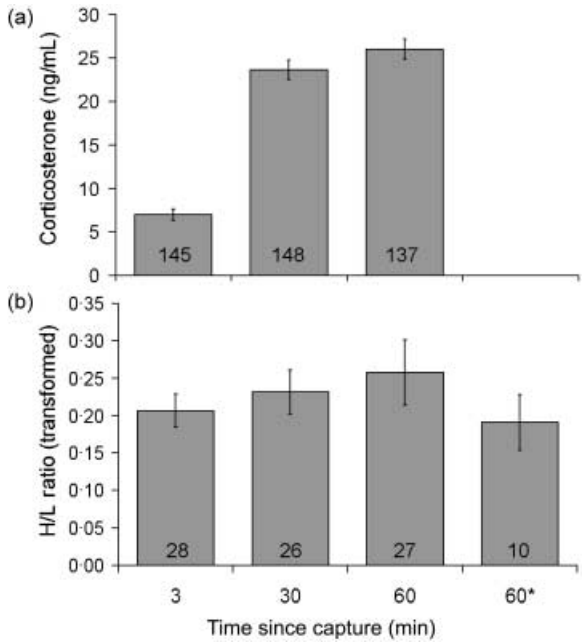

Fig. 4. Plasma corticosterone levels (a) and heterophil-lymphocyte ratios (b) in house finches (*Carpodacus mexicanus*) held for 1 h and sampled at 3, 30 and 60 min after capture. Number of birds sampled is indicated in each column. (a) modified from Lindström *et al.* (2005) and (b) from Davis (2005). Fourth column in lower chart (60*) indicates average H : L ratios of birds held for 1 h and sampled once at time 60.

The time lag associated with the leukocyte response to stress may be the longest in ectothermic animals, perhaps owing to their temperature-dependent metabolism, which is slow at low temperatures (Pough 1980). Bennett *et al.* (1972) found that experimental injections with hydrocortisone did not alter leukocyte differentials in newts (i.e. no neutrophilia or lymphopenia was observed) until 3 days later, whereas similar experiments with frogs showed that some individuals responded within 12 h, but others took 144 h (Bennett & Harbottle 1968). If this slow response is due to a slow metabolism, we might expect equally slow leukocyte responses to stress in reptiles since their metabolism is also slow at low temperatures (Pough 1980). Similarly, the time course of the leukocyte response in fish is lengthy. The effects of transport stress on peripheral blood neutrophil counts in channel catfish (*Ictalurus punctatus*) were not detectable until 12 h post-stress and peaked at 24 h (Bly *et al.* 1990). Thus, among ectotherms, the leukocyte response time appears to be much slower than that of endothermic birds and mammals.

Few researchers have directly quantified the endogenous hormonal and leukocyte responses with the intent of comparing their relative utility, with the exception of scientists studying poultry. McFarlane *et al.* (1989) measured the leukocyte (H : L ratio) and adrenal hormone responses in chickens that were chronically exposed to a variety of stressors, including beak trimming, air fouling, coccidiosis, heat stress, continuous noise and intermittent electric shocks. These treatments initially caused the typical rises in both plasma corticosterone and H : L ratios. After 7 days, however, corticosterone was no longer elevated, suggesting acclimation to the stressors (see Romero 2004). Interestingly, H : L ratios remained elevated, and this led the investigators to conclude that the leukocyte response to stress was more enduring and perhaps a more reliable indicator of long-term stress than corticosterone sampling. Whether acclimation to stressors occurs in two dissociable phases, one characterized by a reduction in the adrenal response followed by another involving the leukocyte response, is a question that should be explored by future research.

Besides allowing more time for researchers to obtain baseline samples, the use of leukocyte profiles for assessing stress in vertebrates offers additional advantages. First, the technique is relatively inexpensive, requiring only microscope slides, stain and a microscope. Second, the blood sample required for a blood smear is very small. Corticosterone assays require 30–60 μL of blood, which is not easily taken (if to be done so non-destructively, at least) from animals less than 15 g in weight (Washburn *et al.* 2002). In contrast, leukocyte profiles can be obtained from blood samples as small as 5–10 μL (A. K. Davis, personal observation), which in theory means that smaller animals with lower blood volumes can be studied.

PREDICTIVE VALUE OF LEUKOCYTE PROFILES

One of the reasons for assessing stress in ecological settings is to detect early warning signals of potential problems in vertebrate populations. For example, corticosterone levels have been used to predict survival in populations of Galápagos marine iguanas (*Amblyyrhynchus cristatus*) (Romero & Wikelski 2001). Leukocyte profiles also have predictive power, and some of the best examples of this power come from human medical studies in which patients can be followed for years after routine blood smears are made. Multiple studies report that low lymphocyte counts (which the authors of these studies attribute to elevated glucocorticoids) reliably predict the risk of mortality associated with a range of ailments, such as coronary artery disease (Ommen *et al.* 1997), haemodialysis (Reddan *et al.* 2003), heart failure or myocardial infarction (Thomson *et al.* 1995; Ommen *et al.* 1998; Acanfora *et al.* 2001) and surgical implantation of defibrillators (Ommen, Hammill & Gibbons 2002). The N : L ratio was found to be a good predictor of cardiovascular risk, better than high neutrophil counts or low lymphocyte counts (Horne *et al.* 2005). Other examples of the predictive capacity of leukocyte stress parameters come from avian research. High H : L ratios have been associated in birds with susceptibility to infection (Al-Murrani *et al.* 2002), slow growth rates (Moreno *et al.* 2002) and survival to the next breeding season (Lobato *et al.* 2005; Kilgas, Tilgar & Mand 2006). These associations make H : L ratios valuable for predicting future problems in both populations and in individuals.

INTERPRETING CHANGES IN LEUKOCYTE PROFILES

Controversy surrounding the use of leukocyte profiles in ecology seems to centre around the perception that data collected on

leukocyte parameters are difficult to interpret. As with any tool, it is important to understand what leukocyte profiles can and cannot tell us. Although they can give information about whether an individual may be subjected to more or less stress relative to other individuals, the information obtained from one blood smear tells us little about the *ability* of that individual to mount an immune response. Researchers who attempt to tie leukocyte profiles to immune function or fitness are faced with a dilemma: does heterophilia indicate stress and illness (and therefore low fitness), or does it indicate a superior ability to respond to infection (high fitness)? Does lymphopenia indicate an active stress response, or a lack of parasites? Or does it indicate immunosuppression? This troubling paradox is illustrated in the literature on plumage brightness and colouration in passerine birds. Saks, Ots & Horak (2003) reported a negative correlation between the brightness of the yellow feathers of greenfinches (*Carduelis chloris*) and heterophil count, and interpreted this result to mean that the brighter birds were in better health because their heterophil counts were low. In contrast, Dufva & Allander (1995) found the opposite relationship between yellow colouration and H : L ratios in great tits (*Parus major*), and argued that heterophilia (high heterophil count), which in this case was associated with bright coloration, indicated an 'efficient immune response' and 'superior immunity to parasites.' Similarly, Figuerola *et al*. (1999) reported that yellow coloration correlated positively to H : L ratio in cirl buntings (*Emberiza cirlus*), and suggested that the high ratios indicated an 'absence of parasites and infectious diseases and correspondingly better overall health.' Although Dufva & Allander (1995) and Figuerola *et al*. (1999) considered alternative explanations, they concluded that brighter colouration signals better health. If, however, H : L ratios are related to stress, as we have reviewed here, the brighter birds in their studies were actually experiencing more stress, and possibly greater rates of infection, than the duller ones. We recently found that in northern cardinals (*Cardinalis cardinalis*), the more deeply saturated, redder birds had higher H : L ratios than less colourful birds (Maney *et al*. 2008). Our result is consistent with Dufva & Allander (1995) and Figuerola *et al*. (1999); however, we believe that the saturated colouration is associated with greater stress, perhaps due to greater energy expenditure on mate seeking and/or territory defence. This result is consistent with that reported by (Mazerolle & Hobson 2002), who found that birds defending high-quality territories (and presumed to be most fit) had higher H : L ratios than those in lower quality territories. Note that none of these H : L data give any information regarding 'immunocompetence' or 'immunosuppression' because the immune systems of these birds were not systematically challenged by the researchers in the course of these studies. Leukocyte profiles inform only on the relative proportions of WBC types that are currently circulating in blood. They do not indicate the number of heterophils or lymphocytes that are available in reserve in other body compartments, or (without doing another smear after a stress paradigm) how many would be released or redistributed in response to a stressor or infectious agent. Assessing leukocyte profiles as described here is therefore not the same as measuring immune responses *per se*, which can be more directly quantified using other methods.

A second important challenge in the interpretation of leukocyte profiles is that the 'normal' parameters for many species of wildlife are not known. Thus, ecologists are often faced with the problem of trying to determine if the leukocyte differentials they obtained from blood smears (i.e. the proportions of lymphocytes, neutrophils, etc.) differ from what normally occurs in their study species. In the field of wildlife medicine, investigators attempt to address this issue by reporting 'reference' haematological parameters (i.e. means and minimum and maximums) for species that have not yet been examined haematologically, and these can include leukocyte profiles, plasma chemistry values (Eliman 1997; Hrubec, Cardinale & Smith 2000; Werner 2007) and sometimes even red blood cell and WBC morphology (Martinez-Silvestre *et al*. 2005; Sacchi *et al*. 2007). If the ecologist is fortunate enough to study an animal that has had leukocyte reference values established, their task of interpreting the leukocyte profiles at hand is certainly made easier. However, we point out that any set of haematological 'reference' values must be viewed with one caveat in mind, which is that the animals examined in such studies usually come from captive collections, or if wild-caught, from a single population, and as such their haematological parameters may not necessarily be representative of the species as a whole. Therefore they should be treated more as a starting place for future work than a set of absolutes. Furthermore, reference values can vary among investigators because of differences in handling procedures before sampling, or captive housing conditions (see Case *et al*. 2005), leading to cases where two or more groups of investigators report vastly different 'reference' haematological parameters for the same species. In one such case, Cathers *et al*. (1997) reported leukocyte profiles of American bullfrogs (*Rana catesbeiana*) with lymphocyte and neutrophil proportions of 63% and 22%, respectively. Meanwhile, Coppo *et al*. (2005) reported values of 27% and 61% for lymphocytes and neutrophils, respectively, for the same species. This example highlights the inherent difficulty in making comparisons of haematological parameters between investigators; however, we point out that this problem can be overcome, at least in experimental scenarios, by comparing leukocyte parameters from experimental groups of animals with those from non-manipulated controls (e.g. Bennett & Johnson 1973; Davis & Maerz 2008b; Valenzuela *et al*. 2008).

A third challenge in the interpretation of leukocyte profiles is distinguishing stress responses from those of inflammation or disease. Given that leukocytes make up the primary line of defence in the innate immune system of vertebrates, it is not surprising that infection and diseases cause alterations in their numbers. In fact, the effect of disease on leukocyte profiles is similar to that of stress in that neutrophilia/heterophilia and lymphopenia are commonly observed in all taxa. Indeed, it is well-established that neutrophils/heterophils, being phagocytic, proliferate in circulation to combat infections (Jain 1986; Latimer *et al*. 1988; Campbell 1996), and the increase in this

cell type alone can cause increases in N : L or H : L ratios during infections (e.g. Davis *et al*. 2004). Therefore, interpreting changes in leukocyte profiles (i.e. attributing them to stress vs. infection) can be problematic, especially if infection status is not known. Indeed, the two factors are closely related: stress is known to lead to susceptibility to diseases (e.g. Al-Murrani *et al*. 2002), and infections or diseases can cause increases in stress (e.g. Lindström *et al*. 2005). The two may be dissociated, however, by looking at other haematological parameters. In addition to causing relative neutrophilia and lymphopenia, infections commonly cause general increases in monocytes, which also phagocytize foreign particles and infections (e.g. Jain 1986; Campbell 1996; Davis *et al*. 2004), and general increases in total WBC count (Jain 1986; Latimer *et al*. 1988; Jain 1993; Thrall 2004). In addition, Jain (1986) reports that a reduction in relative eosinophil numbers is more often a stress reaction than a response to disease (discussed in detail later). Thus, by considering the relative number of both monocytes and eosinophils, as well as the total leukocyte count (and see below), it may be possible to dissociate the effects of infection from those of stressors. The identification of parameters associated solely with one or the other that could be incorporated into standard haematological screenings represents an area where more work is needed.

OTHER LEUKOCYTE STRESS INDICATORS

Leukocytosis, or general increases in total WBC numbers, has been occasionally used as a measure of stress (e.g. Ots, Murumagi & Horak 1998), although using this parameter to infer stress is problematic. First, WBC counts are inherently widely variable among individuals (Jain 1986). Second, as is the case for H : L ratios in the plumage brightness literature, some authors have attempted to connect this measure to immunocompetence, but there appears an inconsistency in the ecological literature regarding the definition of immunocompetency in terms of total WBC counts. For example, Nunn, Gittleman & Antonovics (2000) argued that higher 'baseline' counts indicate stronger immune systems in animals, whereas Pap (2002) suggested in contrast. Forson & Storfer (2006) found lower numbers of leukocytes in salamanders exposed to a herbicide (compared to a control group), and interpreted this result as a suppression of the innate immune system (implying that higher counts are the norm). Murad & Houston (1988) observed lowered leukocyte counts in fish exposed to cadmium, and Wedemeyer *et al*. (1990) state that lowered WBC counts are an indication of acute stress in all fishes. In fact, the effect of stress on WBC counts in other animals seems only to add to the confusion. In house finches, capture followed by 1 h of handling leads to decreases in total leukocytes (Davis 2005); transport of wild and domestic birds also causes reductions in total leukocytes over 1–3 h (Parga *et al*. 2001; Scope *et al*. 2002). In the example of the exercised horse shown in Fig. 2, total leukocyte numbers *increased* until the end of the exercise period (Cardinet *et al*. 1964). Early work by Dougherty & White (1944) demonstrated that total leukocyte numbers dropped in mice and rats after injection with ACTH, reaching a low at 9 h post-injection and returning to normal levels at 24 h. Similar trends were seen in rats subjected to restraint for 2 h (Dhabhar *et al*. 1996). Injections of steroid hormones in horses, however, led to a doubling of total leukocytes within 2 h (Jain 1986).

Part of the problem with interpreting total leukocyte counts, especially in little-studied species, is that prior information regarding the 'normal' range of leukocyte numbers in such species, like information on leukocyte profiles (discussed previously), is rarely available. Without this information one cannot know whether the counts obtained are 'high' or 'low'. If the normal range *is* known, as it is for humans and some domesticated animals, then higher than normal WBC numbers are most commonly interpreted as a sign of inflammation (Jain 1986; Latimer *et al*. 1988; Ford 2002), which in itself is informative because of its prognostic value. In fact, in the medical literature there is a growing body of evidence demonstrating that high WBC counts are an important predictor of patient mortality from a variety of ailments, including heart disease, stroke and diabetes (Do Lee *et al*. 2001; Ford 2002, Ha Jee *et al*. 2005; Núñez *et al*. 2006). Interestingly, however, other medical researchers found that the neutrophil : lymphocyte ratio was even more predictive of patient mortality than their total leukocyte count (Horne *et al*. 2005)!

A final leukocyte parameter to consider as a measure of stress is the eosinophil count. Although not commonly cited in contemporary work, historic research on humans and mammals demonstrated that glucocorticoid-induced stress leads to a reduction in eosinophil numbers (Hills, Forsham & Finch 1948; Gordon 1955; Cardinet *et al*. 1964). This phenomenon is also listed as an indicator of stress in veterinary haematology texts (Jain 1986, 1993). Oddly, this parameter is not routinely reported in modern stress research, even in studies involving mammals (Dhabhar *et al*. 1995, 1996; López-Olvera *et al*. 2007), although it has been reported in fishes that were administered cortisol (Wojtaszek *et al*. 2002). Within the amphibians, there is also some experimental evidence that stress hormones reduce eosinophil numbers in amphibians (Belden & Kiesecker 2005), and that this reduction leads to reduced resistance to parasites. Since eosinophil numbers may help distinguish leukocyte responses to stress from those caused by infection (Jain 1986), this variable should be incorporated more often into haematological measurements.

Conclusions

Research conducted over the past several decades indicates that the quantification of haematological parameters such as neutrophilia/heterophilia, lymphopenia, or H : L ratio (but not total WBC counts), may be measured as a compliment to, or even in lieu of, the measurement of adrenal hormones in the study of vertebrate stress responses. A large body of evidence demonstrates that the adrenal and leukocyte responses to stress are tightly linked, and are similar across vertebrate taxa. The leukocyte approach offers several advantages, such as a longer period of time within which to obtain initial blood samples (especially in ectotherms), low cost and feasibility when working with small animals. Moreover, work currently

being conducted in humans and birds shows that leukocyte profiles can help predict an individual's future performance and viability. Regardless of the taxon under study, an understanding of any animal's leukocyte parameters and what they mean ultimately comes from immunology, a field in which the majority of research is conducted in a biomedical context and published in biomedical journals. As is the case with any physiological measure, ecologists must be aware of and consider this body of research when designing their studies and interpreting their data (Romero 2004). With that effort, along with an increasing understanding of how to apply biomedical knowledge to novel species, haematological measures will no doubt become more established in ecology. Considering all of the issues discussed here, we conclude that counts of leukocytes can provide a reliable method in ecological research to study vertebrate responses to stress, and this approach can be used to help ascertain the current and future welfare of study subjects.

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