Amphibian Immunity—Stress, Disease, and Climate Change

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Abbreviations: ACTH, adrenocorticotropic hormone; AMP, antimicrobial peptide; CRF, corticotropin-releasing factor; HPI axis, hypothalamic-pituitary-interrenal axis; *Bd*, *Batrachochytrium dendrobatidis*; *Bsal*, *Batrachochytrium salamandrivorans*; MALDI-TOF, matrix-assisted laser-desorption ionization time-of-flight

Abstract

Like all other vertebrate groups, amphibian responses to the environment are mediated through the brain (hypothalamic)-pituitary-adrenal/interrenal (HPA/I) axis and the sympathetic nervous system. Amphibians are facing historically unprecedented environmental stress due to climate change that will involve unpredictable temperature and rainfall regimes and possible nutritional deficits due to extremes of temperature and drought. At the same time, amphibians in all parts of the world are experiencing unprecedented declines due to the emerging diseases, chytridiomycosis (caused by *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*) and ranavirus diseases due to viruses of the genus Ranavirus in the family Iridoviridae. Other pathogens and parasites also afflict amphibians, but here I will limit myself to a review of recent literature linking stress and these emerging diseases (chytridiomycosis and ranavirus disease) in order to better predict how environmental stressors and disease will affect global amphibian populations.

Keywords: amphibian, *Batrachochytrium*, chytrid, corticosterone, HPI axis, ranavirus, stress,

1. Introduction

In view of recent world-wide declines of amphibian species due to fungal and viral infections, a review on the subject of the possible role of stress/neuroendocrine-immune system interactions in amphibian immune responses to these pathogens is needed. From a broad perspective, stress affecting amphibians comes in many forms including habitat destruction, overuse by humans, environmental chemicals, introduced species, climate change, and infectious diseases (Collins, 2010). For this article, I will limit my subject matter to natural stressors that

may activate the HPI axis or the sympathetic nervous system and the effects of disease alone or disease in the face of unpredictable climate changes. For a somewhat longer and broader review of amphibian declines, disease, amphibian immunity, and stress in the context of the field of "ecoimmunology", please see Rollins-Smith and Woodhams (2012). For more detailed reviews of neural-immune system interactions with an evolutionary perspective, see Cohen and Kinney (2007) and Kinney and Cohen (2009). For an excellent and updated overview of ranaviruses and ranavirus diseases, see "Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates (M.J. Gray and V.G. Chinchar, eds., 2015). Throughout the text, I have indicated amphibian species by their common names and their binomial (genus and species) names. These names conform to the listings in AmphibiaWeb, the online amphibian information system developed and maintained by the University of California, Berkeley.

2. Ontogeny of Immunity in Amphibians

Because amphibians undergo metamorphosis, the gradual development of immune functions in tadpoles is interrupted during the metamorphic period. Lymphocyte-mediated adaptive immune responses of tadpoles are characterized by having a limited B and T cell recognition repertoire resulting in poorer allorecognition and less diverse antibody responses than adults (rev. in Rollins-Smith, 1998; Robert and Ohta, 2009). Following metamorphosis, lymphocyte numbers expand rapidly and immune defenses mature. In the best-studied model amphibian, the South African clawed frog *Xenopus laevis*, lymphocytes in the spleen at the end of metamorphosis number about 1-2 x 10^5. By one to two months post-metamorphosis, the numbers have increased to about 10^6, and they level off at about 10^7 between six and 12 months of age (Du Pasquier and Weiss, 1973; Rollins-Smith *et al.*, 1984). Thus, for the rapidly

developing *X. laevis*, an adult-type immune response capacity has developed within two months of metamorphosis and expands during the first year of life. However, the early juvenile period is a time of possible increased vulnerability to disease for most anurans (frogs and toads) because the adaptive immune defenses are not yet mature.

Many amphibians also have well developed granular glands in the skin capable of producing antimicrobial peptides (AMPs) (rev in Rollins-Smith and Conlon, 2005; Rollins-Smith, 2009; König *et al.*, 2015). AMPs are thought to be an important first line defense against pathogenic bacteria, fungi, viruses, and other pathogens that would enter by way of the skin (Rollins-Smith, 2009). The ontogeny of granular gland development has been studied in only a few species. In northern leopard frogs [*Rana (Lithobates) pipiens*] and *X. laevis*, granular gland development occurs in response to thyroid hormones and is complete at the end of metamorphic climax (Bovbjerg, 1963; Heady and Kollros, 1964; Seki *et al.*, 1989). A study of the synthesis of mRNA for one AMP (brevinin-1SY) in developing wood frogs (*Rana sylvatica*) showed that transcription was at very low levels until prometamorphic stages (Gosner 36-41) (Gosner, 1960) and then increased significantly at subsequent stages through metamorphosis (Katzenback *et al.*, 2014). Juvenile *R. pipiens* have also been shown to have a full complement of AMPs at 3-6 months post-metamorphosis detected by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Pask *et al.*, 2013). A study of five species sampled for AMPs at metamorphosis [*R. pipiens*, *Rana catesbeiana (Lithobates catesbeianus)*, *Rana sierrae*, *Litoria serrata*, and *Alytes obstetricans*] showed that the repertoire of skin peptides expressed in newly metamorphosed frogs (sampled within a few weeks after tail resorption) was the same as that of more mature adults detected by MALDI-TOF mass spectrometry (Woodhams *et al.*, 2016). Thus, we hypothesized that newly metamorphosed juveniles of the southern leopard frog

[*Rana (Lithobates sphenocephalus) sphenocephala*] would have a full complement of antimicrobial peptides characteristic of adults as soon as they resorbed their tails. However, analysis of the induced skin peptides in metamorphs by MALDI-TOF mass spectrometry showed that the full complement and adult levels of AMPs were not present until 12 weeks after metamorphosis (Holden *et al.*, 2015). Because the southern leopard frogs in this study were reared in outdoor mesocosms, it is possible that they were not developing under optimal conditions or metamorphosed rapidly because tank conditions were warming rapidly. This finding suggests that both innate AMP defenses and adaptive lymphocyte-mediated defenses may be compromised in young juveniles for several months after metamorphosis if growth conditions are not ideal, and thus, they may be more vulnerable to disease in this developmental period.

3. The Brain-Stress Axis in Amphibians

3.1. The hypothalamo-pituitary-interrenal axis

Amphibians share with other vertebrate groups a neuroendocrine stress axis (the hypothalamo-pituitary-interrenal axis, HPI) that mediates the response of the animal to its environment (Fig. 1). Neurosecretory neurons in the hypothalamus produce corticotrophin-releasing factor (CRF) that stimulates cells of the pituitary gland to release corticotropin (homologous to adrenocorticotropic hormone, ACTH) (rev. in Dores and Lecaude, 2005). In amphibians, the interrenal glands serve the same function as the adrenal cortex in mammals and synthesize and release corticosteroid hormones (rev. in Denver, 2009). The main corticosteroid hormone released is corticosterone, and the main mineralocorticoid is aldosterone (Leboulenger *et al.*, 1986; Feuilloley *et al.*, 1990; Kloas and Hanke, 1990). The corticosteroid hormones have

important roles in normal development, energy mobilization, and osmoregulation (rev in Denver, 2009). Although baseline levels of corticosteroids remain quite low through premetamorphic and prometamorphic stages of development, the HPI axis is responsive to natural stressors or to injection of ACTH (Glennemeier and Denver, 2002b). Thus, the HPI axis appears to be responsive to stress even in young tadpoles. Several natural stressors have been shown to increase corticosterone levels in larval amphibians in laboratory, mesocosm, and field settings. These stressors that elevate corticosterone are food restriction (Crespi and Denver 2005; Crespi and Warne 2013; Reeve *et al.*, 2013), crowding (Glennemeier and Denver, 2002a), chronic exposure to predation cues (Middlemis Maher *et al.*, 2013), low pH (Chambers *et al.*, 2013), and pond drying (Denver, 1998, Crespi and Warne, 2013; rev. in Denver, 2013). Ranavirus infection itself may be viewed as a stressor, as corticosterone levels were shown to increase within four days of exposure of wood frog (*R. sylvatica*) tadpoles to ranaviruses (Warne *et al.*, 2011). Within a population of *R. sylvatica* tadpoles, there was heterogeneity in baseline corticosterone levels, with those growing more slowly having higher corticosterone levels (Warne *et al.*, 2013, Reeve *et al.*, 2013). Prior to metamorphosis, HPI activity increases and is important in stimulating thyroid hormone secretion needed for morphological conversion from the aquatic tadpole form and physiology to one adapted for terrestrial or semi-terrestrial life (rev. in Denver, 2013).

Interspersed within the interrenals and adjacent to steroidogenic tissues are also clusters of chromaffin cells that synthesize catecholamines (epinephrine and norepinephrine). There are few studies of the regulation of catecholamine release, but it is generally thought to be a central component of the ACTH-induced stress response in amphibians (rev. in Perry and Capaldo, 2011). Stress, such as induced activity to escape a predator, was shown to elevate catecholamines (Tufts *et al.*, 1987; Withers *et al.*, 1988; Hillman *et al.*, 1998) and resulted in

increased release of defensive skin peptides (Ramsey *et al.*, 2010; Pask *et al.*, 2012). The stress of dehydration also resulted in the release of catecholamines in the marine toad (*Bufo marinus, now Rhinella marina*) (Withers *et al.*, 1988). Norepinephrine elevated glucose in bullfrogs (*R. catesbeiana*) and the common frog (*Rana temporaria*), suggesting that this acute stress response is important for rapid mobilization of energy reserves (Harri, 1981; MbangKollo and de Roos, 1983).

3.2 The sympathetic nervous system, catecholamines, and acute stress

There is a rich literature describing the role of the sympathetic nervous system on immune system function in mammals (reviewed in Madden and Felten, 1995). Pioneering studies of the connections of the sympathetic nervous system with the immune system of amphibians were conducted in the 1990s. As reviewed by Cohen and Kinney (2007), the main features of neural-immune system interactions shared by mammals and amphibians are as follows. Lymphoid tissues are richly innervated and express receptors for neuropeptides, neurotransmitters, and hormones (Felten et al., 1987; Kinney et al., 1994). When the receptors are engaged, the functions of the immune cells are changed. When the sympathetic nervous system is incapacitated by chemicals, lymphocytes become more easily activated in mammals (reviewed in Madden and Felten, 1995) and in *X. laevis* (Kinney and Cohen, 2005). As shown in mammals, cells of the immune system produce neuropeptides and hormones (Blalock, 2005; Smith, 2003). Cytokines such as IL-1, IL-6, and TNF produced by immune cells are recognized by receptors on cells of the nervous system (Maier, 2003). Thus, there is bi-directional communication between the immune system and the nervous system of amphibians as well as mammals.

One component of the amphibian immune defense system that is especially sensitive to catecholamines is the network of granular glands in skin (also called poison glands) of many amphibian species. The granular glands produce and store defensive peptides including antimicrobial peptides that inhibit *Bd* and ranaviruses (reviewed in Rollins-Smith and Conlon, 2005; Rollins-Smith 2009; and Rollins-Smith et al., 2011), and they are innervated by sympathetic nerves (Sjoberg and Flock, 1976). Following alarm or injury, the sympathetic nervous system is activated, adrenergic receptors are stimulated (Benson and Hadley, 1969; Holmes and Balls, 1978), and the contents of the glands are released to the surface of the skin. Even a mild simulated "alarm stress" due to forced activity for 5 to 10 minutes induces release of significant amounts of defensive peptides in *X. laevis* and *R. pipiens* (Ramsey et al., 2010; Pask et al., 2012). Thus, acute and temporary stress that activates release of catecholamines would be protective against skin pathogens introduced by an injury.

4. Corticosteroids and the Immune System in Amphibians

Amphibian lymphocytes are very sensitive to the effects of corticosteroids. In previous studies, supraphysiological concentrations of corticosteroids inhibited antibody responses and reduced thymus size and numbers of circulating lymphocytes in adult frogs (Garrido *et al.,* 1987, Plytycz *et al.,* 1993). Tumor allograft rejection was impaired (Rollins and McKinnell, 1980), and the immune response to *Mycobacterium marinum* was reduced in leopard frogs (Ramakrishnan *et al.,* 1997). Other studies suggested that elevated corticosteroids change the pattern of lymphocytes, neutrophils, and eosinophils detected in circulating blood (Falso *et al.,* 2015; de Assis *et al.,* 2015; Kaiser *et al.,* 2015). A frequent observation is that the ratio of neutrophils to lymphocytes changes when amphibians are stressed and corticosteroids are involved (Peterson et al. 2010; Narayan and Hero, 2011; Falso *et al.,* 2015; de Assis *et al.* 2015).

This pattern is not replicated in other studies (Kaiser, *et al.*, 2015). It has been suggested that simply monitoring leukocyte changes is an adequate measure of physiological stress in amphibians (Davis *et al.*, 2008). However, given the complexity of leukocyte movements into and out of the blood and tissues, assessing stress using leukocyte counts alone without monitoring corticosteroids is a highly imperfect measure.

In vitro, proliferation of frog lymphocytes in response to the classical T cell mitogen, phytohemagglutinin (PHA), was inhibited, and viability of both thymocytes and splenocytes was reduced by physiologically relevant concentrations (1-10 ng/ml = 3-30 nM) of corticosterone (Rollins-Smith & Blair, 1993). Both corticosterone and aldosterone at concentrations of 1-10 nM inhibited lymphocyte proliferation and induced apoptosis of lymphocytes from tadpoles or adults (Rollins-Smith *et al.*, 1997). The loss of lymphocytes *in vitro* and in tadpoles at metamorphosis was reversed by the corticosteroid receptor antagonist RU486 (Rollins-Smith *et al.*, 1997; Barker *et al.*, 1997).Thus, physiologically relevant increases in corticosteroids alter lymphocyte activity *in vitro* and *in vivo*.

5. Amphibian Declines Due to Disease

In recent years, three emerging pathogens have been linked to global amphibian declines. They are ranaviruses and two species of chytrid fungi in the genus *Batrachochytrium.*

Viruses in the genus *Ranavirus*, family *Iridoviridae*, are aquatic DNA viruses that infect salamanders and frogs, as well as fish and reptiles. They target a number of cell types (e.g., liver, kidney, interrenal gland) in susceptible animals causing necrosis and hemorrhaging (rev. in Gray *et al.*, 2009; Miller *et al.*, 2011). Symptoms include lethargy, anorexia, and buoyancy problems. Death occurs due to tissue death in multiple organs (Gray *et al.*, 2009). Outbreaks of

ranavirus disease in which entire populations of larvae succumb to disease have occurred in North America, South America, Australia, Asia, and Europe. Thus, ranaviruses present serious risks for many amphibian populations around the world (rev. in Miller *et al.,* 2011). Susceptibility varies by species. Among North American species, those with a short larval period, limited species range, or semi-permanent breeding sites are more susceptible than slowly developing and more widespread species (Hoverman *et al.,* 2011). Ranavirus infections appear to cause mortality at larval stages while adults are more resistant and are known to carry infections that are asymptomatic (De Jusús Andino *et al.,* 2012; Robert *et al.,* 2014; Crespi *et al.,* 2015; rev in Brunner *et al.,* 2015). In wood frogs, the greatest vulnerability appears to be in the late (prometamorphic) larval stages (Warne *et al.,* 2011).

Batrachochytrium dendrobatidis (Bd) is a chytrid fungus that infects amphibian skin causing the disease chytridiomycosis (Berger *et al.,* 1998; Longcore *et al.,* 1999; rev. in Rollins-Smith *et al.,* 2011). It is linked to ongoing global declines and extinctions of amphibians (Stuart *et al.,* 2004; Skerratt *et al.,* 2007; rev. in Fisher *et al.,* 2012). The infection is confined to the skin, and degeneration of the skin results in an imbalance in essential ions and eventual death due to cardiac arrest (Voyles *et al.,* 2009; 2012b). Both anuran amphibians (frogs and toads) (Berger *et al.,* 1998) and caudate (tailed) amphibians may succumb to chytridiomycosis caused by *Bd* (Thien *et al.,* 2001; Bovero *et al.,* 2008; Raffel *et al.,* 2015); however it seems to be of lower virulence in many salamander species (Chatfield, *et al.,* 2012; Pasmans *et al.,* 2013; Muletz, *et al.,* 2014; Bales, *et al.,* 2015). More recently, a second species in the genus *Batrachochytrium* was discovered. This new pathogen, *Batrachochytrium salamandrivorans (Bsal),* appears to have originated in Asia and is highly pathogenic to salamanders but appears to

229 spare their frog and toad cousins (Martel *et al.*, 2013; 2014). Both emerging fungal pathogens
230 threaten many amphibian species globally.
231
232 **6. Does stress impair immunity and increase disease susceptibility in larval amphibians?**
233 *6.1 Stress, corticosteroids, and ranavirus disease in tadpoles and recently metamorphosed*
234 *juveniles*
235 Given that ranavirus infections primarily cause mortality during the larval stage, studies
236 that were designed to investigate the effects of environmental stress on disease susceptibility
237 have focused on the this life history stage. In northern leopard frogs (*R.* pipiens), ranaviruses
238 have been associated with a number of mortality events in larval and metamorphosing frogs
239 (Greer *et al.*, 2005; Hoverman *et al.*, 2011). Crowding of premetamorphic leopard frog tadpoles
240 (Gosner stage 25) is a natural stressor that resulted in the elevation of whole body corticosteroids
241 to a level of approximately 0.15 to 0.2 ng/g (Glennemeier and Denver, 2002a). Under roughly
242 comparable conditions of crowding, exposure of premetamorphic tadpoles to ranaviruses
243 resulted in decreased growth of tadpoles, increased mortality, and a more rapid rate of death in
244 comparison with tadpoles infected and reared at somewhat lower densities (Echaubard *et al.*,
245 2010). These observations suggest that crowding stress may have induced modest increases in
246 corticosteroids and directed energy away from rapid growth and immune function and towards
247 maintenance physiology. Because there were no measures of immune function or corticosterone
248 levels in this virus-infection study (Echaubard *et al.*, 2010), it is unclear whether immune
249 defenses were impaired leading to increased disease.
250 In another study of the effects of natural stressors on premetamorphic wood frog
251 tadpoles, Reeve *et al.*, (2013) exposed Gosner stage 25 tadpoles to high-density, predator-cues,

or low-food conditions. After 14 days, the tadpoles were exposed to ranavirus (either by adding virus directly to water or by exposing the tadpoles to infected tadpoles introduced to the tank). Low food conditions significantly increased corticosteroids to a level of about 0.5 ng/g (and many tadpoles died), whereas high density and predator cues in this experiment did not lead to increased corticosterone (Reeve *et al.,* 2013). This study also showed that exposure to ranaviruses did not increase mortality or time to death beyond that of control tadpoles in any of the treatments. Although corticosterone was elevated to an average of less than 1 ng/g in the low-food group, this concentration would be in the range of concentrations sufficient to inhibit amphibian lymphocytes (1 ng/ml) (Rollins-Smith and Blair, 1993). Thus in this study, low-food stressed tadpoles or predator-stressed tadpoles may have experienced physiological or behavioral changes, but there was no higher rate of mortality than controls. The findings from this study question whether natural environmental stressors affect susceptibility to ranavirus infections in wood frogs, but the dose of ranavirus may have been too high to see differences.

6.2 *Stress, corticosteroids and chytridiomycosis in tadpoles and recently metamorphosed juveniles*

Batrachochytrium dendrobatidis (*Bd*) is generally not lethal to tadpoles because it infects only the keratin-bearing cells of the mouth parts (Berger *et al.,* 1998). Few studies have examined the possible role of the HPI axis in the setting of disease caused by *Batrachochytrium* fungi in tadpoles. Nutritional status of southern leopard frog tadpoles (*R. sphenocephala*) appeared to play a role in their capacity to resist *Bd* infection. Tadpoles raised on a low-protein diet had impaired immune defenses and were less resistant to infection in the mouth parts. The interpretation of these experiments was that tadpoles raised on a protein-rich diet had more

effective immune capacity which might have aided in resistance to infection or clearance of *Bd* from the mouth region after infection (Venesky *et al.,* 2012). Although corticosteroid hormones were not assessed in this study, it is possible that poor nutrition resulted in elevated corticosterone as shown in wood frog larvae (Reeve *et al.,* 2013) and may have inhibited development of immune capacity in these leopard frogs.

Although the mechanism of pathogenesis is not known, there are number of studies that suggest deleterious effects of *Bd* on development and survival of tadpoles and metamorphosing adults. Laboratory exposure of developing toad tadpoles [*Anaxyrus (Bufo) fowleri*, *Bufo bufo*) to *Bd* zoospores resulted in decreased mass at metamorphosis (Parris and Cornelius, 2004; Garner *et al.,* 2009) and increased mortality after metamorphosis (Garner *et al.,* 2009). One potential mechanism to explain reduced size is the effects of infection in the mouth on the ability to forage for food and consume food. In a study of Fowler's toads (*A. fowleri*), *Bd*-infected tadpoles spent less time foraging than uninfected or unexposed tadpoles and had less food in their digestive tracts (Venesky *et al.,* 2009). If these *Bd*-infected tadpoles were nutritionally compromised, they might have had increased corticosteroids (not measured in this study) and decreased capacity to resist or clear infections in the mouth parts.

7. Does disease alone alter the stress axis in tadpoles and metamorphosed juveniles?

7.1 Ranaviruses as agents of stress

An alternative interpretation of studies that show deleterious effects of ranaviruses or *Bd* on tadpole development is that the pathogen, itself, is a "stressor" that results in increased chronic release of corticosteroids. Exposure of wood frogs at prometamorphic stages (Gosner 37-39) to a local New York isolate of ranaviruses resulted in some early mortality, modestly

298 increased whole body levels of corticosterone (approximately 2 ng/g), and decreased mass in
299 comparison with unexposed control tadpoles (Warne *et al.,* 2011). This suggests that viral
300 exposure itself induced a stress response greater than the normally elevated levels of
301 corticosterone expected of tadpoles nearing metamorphosis (rev. in Denver, 2013). Disease-
302 induced stress in late tadpole stages has the potential to accelerate metamorphosis (Warne *et al.,*
303 2011; Reeve *et al.,* 2013). This could be considered to be adaptive (allowing individuals to
304 escape the pond to reduce exposure) or maladaptive (leading to death because of the excessive
305 use of reserve energy resources or further reduction of immune capacity as the frogs emerge
306 from metamorphosis).

307 A separate study of the effects of food-deprivation and reduced water volume during later
308 stages of larval development in wood frogs (*R. sylvatica*) showed that the low-food and low-
309 water treatment groups of tadpoles had elevated corticosterone and reduced mass and body
310 condition as they neared metamorphosis (Crespi and Warne 2013). After metamorphosis, these
311 "stressed" froglets were unable to catch up in terms of growth and fat storage. Although disease
312 was not a part of this study, it is reasonable to hypothesize that these froglets that were stressed
313 at the larval stage might be more susceptible to infections as postmetamorphic juveniles. The
314 Crespi and Warne (2013) study also showed that the "doubled stressed" tadpoles had a blunted
315 response to handling stress at 10 weeks after metamorphosis (reduced corticosterone). A further
316 study of size variability in developing wood frogs showed that the smallest tadpoles leaving the
317 pond last also had a reduced corticosteroid stress responses to mechanical stress at 10 weeks
318 after metamorphosis (Warne and Crespi, 2015). This may also suggest a blunted corticosteroid
319 response that could be adaptive to enable these small frogs to preserve immune capacity or the
320 capacity to mobilize energy reserves if they are hit with a disease pathogen.

One of the first innate immune responses to frog virus 3 (FV3, a well-characterized amphibian ranavirus) in adult frogs (*X. laevis*) is the production of tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) (Morales *et al.*, 2010) that would be expected to activate the HPI axis (rev. in Dunn, 2006). This response is muted in tadpoles (De Jesús Andino *et al.*, 2012), and tadpoles are much more susceptible to ranavirus-induced pathogenesis and death (Gantress *et al.*, 2003; rev. in Grayfer *et al.* 2012). Tadpoles, however, have a higher basal level of mRNA for TNF-α and IL-1β, and tissue damage from the viral infection may exacerbate elevated inflammatory responses resulting in mortality rather than clearance (rev. in Grayfer *et al.*, 2012). In adult frogs, the activation of the HPI axis would be expected to result in increased corticosteroids that could modulate the immune response (rev. in Webster and Sternberg, 2004). In prometamorphic tadpoles, the HPI axis may be "ramping up" to promote metamorphic changes, and an added infection and resulting elevated corticosteroids may reduce immune functions. In turn, this may limit the capacity of late-stage tadpoles to control a viral infection.

7.2 Batrachochytrium as an agent of stress

Several recent studies have evaluated the effects of natural infections by *Bd* on glucocorticoid release by individual tadpoles (Gabor *et al.*, 2013; 2015). Natural populations of larval midwife toads (*Aletes obstetricans and Aletes muletensis*) infected or not with *Bd* were examined for corticosterone release in the water. Infected populations of both species had higher rates of corticosterone release than uninfected populations (Gabor *et al.*, 2013). In nature, higher elevation populations of *A. obstetricans* were infected with *Bd* and shed more corticosterone than lower elevation populations. In a laboratory experiment, *A. muletensis* tadpoles were exposed repeatedly to a highly virulent global panzootic lineage isolate (*Bd* GPL) or a less virulent isolate

(*Bd* CAPE) or no pathogens for 46 days, and then corticosterone was measured. The *Bd* GPL exposed tadpoles shed more corticosterone. This suggests that more severe disease results in a greater stress response.

In nature, *Bd* infected metamorphosed froglets of A. *obstetricans* in the final stages of chytridiomycosis (unable to right themselves) shed more corticosterone than healthier froglets that were able to right themselves (Gabor et al., 2015). These studies strongly suggest that *Bd* infection of tadpoles and metamorphosed juveniles is physiologically stressful and the stress response may be maladaptive.

8. **Does stress impair immunity and increase disease susceptibility in adult amphibians?**

8.1 Stress, corticosteroids, and ranavirus disease in adult amphibians

Adult frogs naturally experience elevated corticosteroids during periods of explosive breeding when heightened activity is required to call and mate, and frogs may be fasting (Harvey *et al.,* 1997; Orchinik *et al.,* 1988). In a recently published study, the health and disease status of populations of adult wood frogs (*R. sylvatica*) returning to spring breeding ponds provided a good measure of possible environmental stressors and their relationship to circulating corticosteroids and sex hormones (Crespi *et al.,* 2015). Prevalence of *Bd* and ranavirus in these male frogs showed no correlation with potentially immunosuppressive corticosterone. Although only a small fraction of the frogs carried *Bd* (2%), about 39% of frogs tested positive for the presence of ranaviruses. There were no outbreaks of ranavirus disease in adults, but males returning to ponds with poor body condition were the most likely to be infected. Previous studies of *X. laevis* adults infected with FV3 showed that apparently healthy adult frogs carried quiescent or latent infections in macrophage populations (Robert *et al.,* 2007; Morales *et al.,*

2010; reviewed in Grayfer *et al.*, 2012). Thus, a combination of marginal health status and latent virus infections may contribute to a few sick individuals that spread ranaviruses to other previously uninfected adults who come to the breeding sites.

An important question to ask is whether temporary natural stressors such as increased activity for breeding or long-distance migration might elevate corticosteroids and alter immune responses. One recent study radio-tracked cane toads [*Rhinella (Bufo) marina*] migrating during rainy nights in Australia (Brown and Shine, 2014). After application of radio transmitters and tracking for seven days, blood corticosterone was measured immediately at capture and again after 24 hours of confinement stress. Several immune parameters were measured at the same time. Corticosterone levels were somewhat elevated (6 ng/ml), and they increased with confinement stress (18 ng/ml) (Brown and Shine, 2014). These concentrations are comparable to levels measured in amplexing toads (Orchinik *et al.*, 1988). Bacterial killing activity and phagocytic activity were reduced in toads that had traveled the greatest distance, but corticosterone levels were no greater than controls that had also been radio-tracked but traveled a shorter distance. A previous study by the same group showed that confinement stress increased corticosterone levels in cane toads and decreased bacterial killing activity (Graham *et al.*, 2012). Another study of a different toad species [cururu toads (*Rhinella icterica*)] by this group also showed that severe confinement stress reduced bacterial killing activity. At the same time they noted an increased neutrophil/lymphocyte ratio (de Assis *et al.*, 2015). This suggests that the temporary natural stress of movement or confinement may affect complement-mediated bacterial killing while increasing neutrophil activity. It is unclear from studies of this type whether, the apparent increase in neutrophils is due to greater release of neutrophils from hematopoietic tissues or results from a decrease in lymphocyte numbers in the blood. In contrast to bacterial

killing activity, PHA-induced swelling (due to the likely infiltration of macrophages and lymphocytes by 48 hours) was enhanced in the furthest migrating marine toads (Brown and Shine, 2014). Thus, these somewhat stressed toads were quite competent to mount a delayed-type hypersensitivity (DTH) response to PHA. Although natural stressors such as breeding activity or long-distance travel on rainy nights have not been conclusively linked to greater susceptibility to ranaviruses, it is possible that reduced phagocytic activity could allow for a less robust response to ranaviruses. In *X. laevis*, macrophages are key defenders of adults from experimental ranavirus infections (Morales, *et al.*, 2010; De Jesus Andino, *et al.*, 2012; Grayfer and Robert 2015).

8.2 Stress, corticosteroids, and chytridiomycosis in adult amphibians

Unlike ranavirus disease, which is a systemic infection affecting multiple organs, *Bd* infections are confined to the skin (Berger *et al.*,1998; Longcore *et al.*,1999; Pessier *et al.*,1999; Berger *et al.*, 2005). Thus, if stress is able to elevate corticosteroids and affect the macrophage and lymphocyte populations in the skin, then affected amphibians might be more susceptible to repeated rounds of infection and reinfection within the skin compartment. The link between stress, corticosteroids, and *Bd* is not well-established. The question remains whether environmental stressors can alter the HPI axis and allow for infection or whether infection with *Bd* itself is a severe stress that elevates corticosteroids. As mentioned above, metamorphs of *A. obstetricans* dying with *Bd* infections (unable to right themselves) had higher levels of corticosterone release in urine than more healthy individuals that could right themselves (Gabor *et al.*, 2015). Similarly, male Stony Creek frogs (*Litoria wilcoxii*) identified as positive for *Bd* infection had slightly higher baseline urinary corticosterone concentrations in comparison to *Bd*

negative male frogs (Kinderman *et al.,* 2012). A similar result was observed in Australian green tree frogs (*Litoria caerulea*) experiencing chytridiomycosis. Diseased frogs had elevated levels of corticosterone, decreased plasma sodium and potassium, fewer circulating lymphocytes, and elevated numbers of neutrophils in the blood (Peterson *et al.*, 2013). Thus, elevated corticosteroid hormones may be an attempt by infected frogs to return to physiological homeostasis, but the response may instead exacerbate the disease.

9. How might climate change alter the stress axis and disease outcomes in amphibians?

At present, many groups are in the process of trying to predict how future climate change may impact survival of ectothermic animals such as amphibians. According to the most recent report of the Intergovernmental Panel on Climate Change (IPCC) (Pachauri *et al.*, 2014), temperatures will likely continue to increase resulting in extreme heat waves and extreme rain events. Changes in rainfall will not be uniform, increasing at high latitudes and mid-latitude wet regions but decreasing in many mid-latitude and subtropical regions. However, climate change is also expected to affect the variability of temperature and precipitation, and host-pathogen interactions may be complex under future climate scenarios (Schär *et al.*, 2004, Pounds *et al.*, 2006, Yeh *et al.,* 2009). For example, longer and colder winters in the USA due to the "Greenland block" effect (Gramling 2015) could stress adults, delay breeding, and shorten the period for larval development such that newly metamorphosed amphibians would have less effective immune defenses including fewer lymphocytes in thymus and spleen (Rollins-Smith *et al.,* 1988) and delayed expression of AMPs in the skin (Holden *et al.,* 2015). An increase in temperature variation may also impair amphibian defenses and increase the risk of *Bd* epidemics (Rohr and Raffel 2010, Raffel *et al.,* 2013).

436 The diverse and extensive literature describing the effects of cold on immune functions in
437 amphibians spans more than four decades and was recently reviewed (Rollins-Smith and
438 Woodhams, 2012). Many of the previous studies describe the effects of natural hibernation in
439 cold-adapted species such as northern leopard frogs, *R. pipiens*, or the European common frog
440 (*R. temporaria)*. In general, immune system functions are dramatically reduced during
441 hibernation. Such changes may fall into the category of immune system accommodations or
442 trade-offs. It is energetically costly to produce lymphocytes in winter when metabolism is
443 reduced. The potential limitation of this biological strategy is that there is often a lag of weeks
444 before the immune system recovers full capacity in the spring.
445 In addition to changes in immune function due to hibernation, immune responses in
446 amphibians also change due to temporary fluctuations in temperature. The antibody response of
447 the marine toad (*B. marinus, now R. marina*) was delayed and reduced when the toads were kept
448 at 15°C rather than 25°C. Toads immunized at 25°C and transferred to 15°C also showed a
449 delayed switch to low molecular weight antibodies (Lin and Rowlands, 1973). Marine toads
450 kept at 20°C had significantly reduced antibody titers against horse red blood cells in comparison
451 with toads at 37°C (Cone and Marchalonis, 1972). Another classic measure of T cell function in
452 amphibians, skin allograft rejection is also strongly affected by temperature. Skin allograft
453 rejection in the eastern newt, *N. viridescens*, showed no differences in the kinetics of rejection at
454 23°C or 30°C. However, at 20°C rejection was delayed, and at 10-15°C, no rejection occurred.
455 When cold newts were transferred to warmer temperatures, rejection occurred within 36 days
456 (Cohen, 1966). In adults of other species [*Rana esculenta (now considered to be Pelophylax*
457 *lessonae and Pelophylax redibundus)*; *Bombina bombina*; and, *B. bufo*), there was a noticeable
458 delay in the timing of skin allograft rejection at 10°C in comparison with 22°C (Jozkowicz and

459 Plytycz, 1998). In *X. laevis*, even a small (3°C) difference in temperature significantly delayed
460 rejection by young postmetamorphic frogs. At 24°C, rejection occurred within 15 days, but was
461 delayed to about 18-19 days at 21°C (DiMarzo, 1980; rev. in Cohen et al., 1985). Thus, both B
462 cell and T cell responses are impaired when temperatures are reduced from the optimal
463 temperature for the host.

464 Since amphibian immune defenses are impaired in the cold, it might be expected that
465 some disease outbreaks would be associated with colder temperatures. Chytridiomycosis has
466 been associated with high elevation and cooler temperatures in multiple studies (Bradley et al.,
467 2002; Berger et al, 2004; Drew et al., 2006; McDonald et al., 2005; Kriger et al., 2007; Savage et
468 al., 2011; Olson *et al.*, 2013; rev. in Fisher et al., 2009; rev. in Rollins-Smith and Woodhams
469 2012). Furthermore, *B. dendrobatidis* thrives at cool temperatures (zoospores have longer period
470 of activity) (Woodhams et al., 2008; Voyles et al., 2012a). Thus, prevalence of infection can
471 increase in cooler seasons (Woodhams and Alford, 2005; McDonald et al., 2005).

472 The effects of temperature on development of ranavirus disease seem to be more
473 complex. Colder temperatures appeared to affect the probability of infection and mortality of
474 leopard frog (*R. pipiens)* and wood frog (*R. sylvatica*) tadpoles exposed to ranaviruses in a
475 controlled laboratory study. A temperature of 14°C significantly increased the infection rate and
476 mortality in comparison with 22°C (Echaubard et al. 2014). In contrast, exposure of European
477 common frog (*R. temporaria*) tadpoles to frog virus 3 (FV3), at 15 or 20°C showed that
478 mortality was greater at 20°C (Bayley et al., 2013). In field studies, greater prevalence of
479 ranavirus infected tadpoles or larval salamanders were sometimes linked with winter
480 temperatures (Gray et al., 2007; Rojas et al., 2005) and sometimes linked with warmer fall
481 temperatures (Hoverman et al., 2012). These conflicting results suggest that the interactions of

amphibian hosts with ranaviral pathogens are complex. Cool temperatures may impair immunity, but viruses can replicate more rapidly and may be shed at higher rates under warmer conditions.

A growing body of evidence suggests that warming temperatures strongly affect amphibian interactions with *Batrachochytrium* pathogens. In culture, *Bd* and *Bsal* are killed at temperatures above 28ºC for *Bd* and 25ºC for *Bsal* (Piotrowski *et al.*, 2004, Woodhams *et al.*, 2008, Martel *et al.*, 2013), and survival of exposed amphibians is often greater at warmer temperatures (Berger *et al.*, 2004, Andre *et al.*, 2008, Bustamante *et al.*, 2010). These studies suggest that an increase in mean temperature, as is predicted with global climate change, might be favorable to amphibians exposed to *Batrachochytrium* species, decreasing the likelihood of outbreaks and declines due to chytridiomycosis. However, warming temperatures in cooler regions may also allow for greater chytrid propagation and greater ranavirus shedding, increasing the possibility of infection and mortality.

10. Concluding Remarks

The immune system of amphibians is exquisitely tuned to respond to pathogen challenges, and the HPA/I axis acts as a brake to prevent collateral damage to host tissues. Natural stressors (nutritional deficits, crowding, predators, breeding activity etc.) can elevate corticosteroid levels, but they do not necessarily predispose healthy well-nourished hosts to greater disease vulnerability. Metamorphosing tadpoles and recently metamorphosed juveniles may be an exception (more vulnerable to disease) because the immune system is rapidly being reorganized and innate skin defenses may be diminished if the tadpoles were nutritionally stressed. Ranaviruses or *Batrachochytrium* pathogens alone are significant causes of

physiological stress and in severe disease when disease symptoms emerge, elevated corticosteroids may impair immune defenses and exacerbate the disease state. Prolonged stress, whether from disease or from environmental causes, may drain immune capacity. Climate change may benefit some amphibian species that can adapt to warm temperatures that kill *Batrachochytrium* pathogens or increase defenses against ranaviruses. On the other hand, warmer temperatures that are still below the lethal limits for *Batrachochytrium* species may also increase the presence of infectious zoospores or ranaviruses. Some species may be unable to find suitable habitats and may experience more events of acute and prolonged stress that will likely impair their immune defenses. Thus, the future for many amphibian species remains in doubt.

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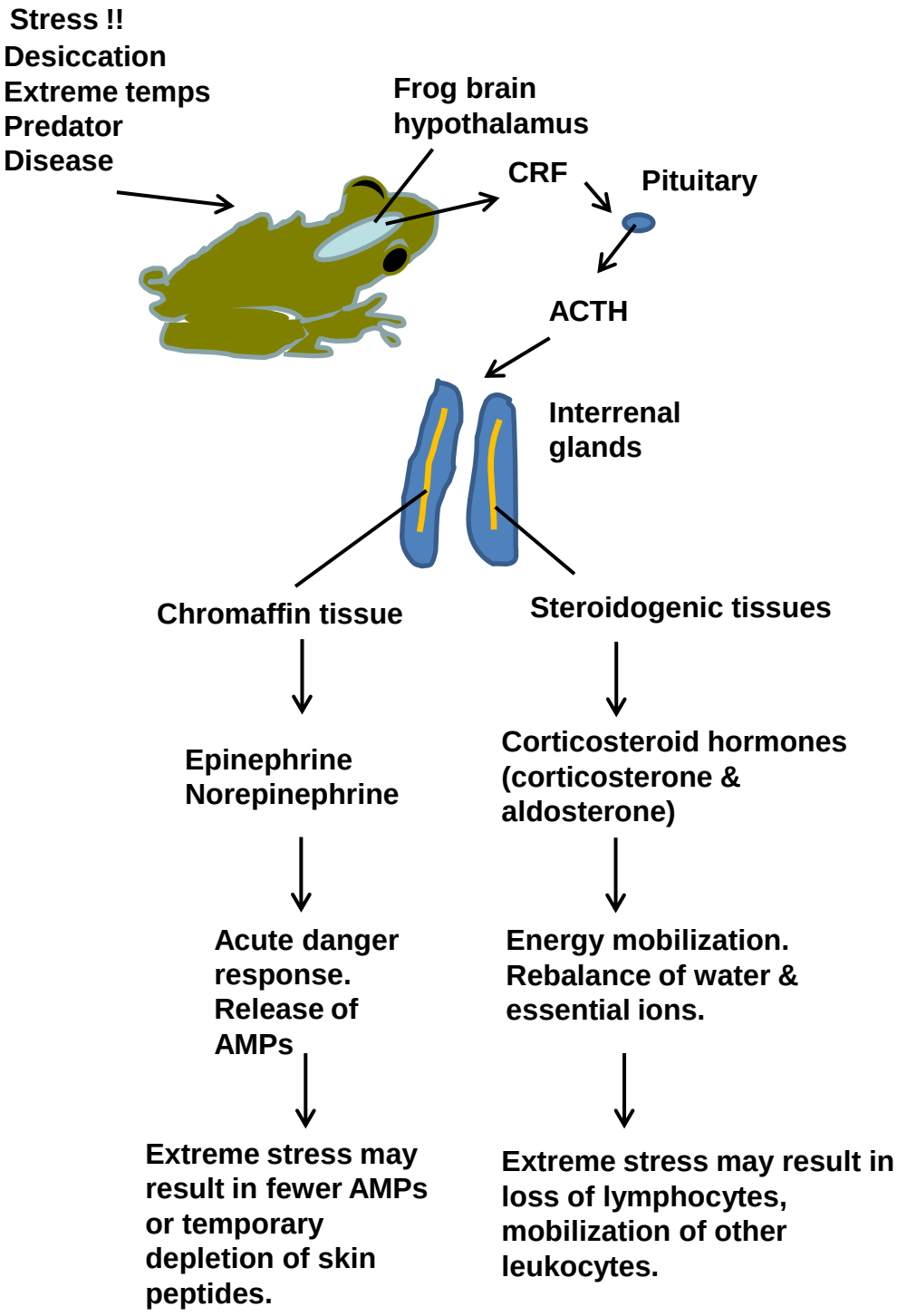
Figure Legend

Figure 1. Schematic diagram of stress and HPI axis in amphibians. Stress activates the hypothalamus to release corticotropin-releasing factor (CRF). CRF acts on the pituitary to induce release of corticotropin (homologous to adrenocorticotropic hormone, ACTH). Chromaffin tissue and steroidogenic tissues in the interrenal glands respond by release of epinephrine and norepinephrine or corticosterone and aldosterone, respectively. Extreme stress may result in diminished AMPs or lymphocyte loss and impaired immune defenses.



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