Contributed Paper

Effects of Two Amphibian Pathogens on the Developmental Stability of Green Frogs

VALÉRIE ST-AMOUR,* TRENTON W. J. GARNER,† ALBRECHT I. SCHULTE-HOSTEDDE,* AND DAVID LESBARRÈRES*††

*Biology Department, Laurentian University, Sudbury, Ontario, P3E 2C6, Canada
†Institute of Zoology, Zoological Society of London, Regents Park NW1 4RY, London, United Kingdom

Abstract: *Developmental instability, measured as fluctuating asymmetry (FA), is often used as a tool to measure stress and the overall quality of organisms. Under FA, it is assumed that control of symmetry during development is costly and that under stress the trajectory of development is disturbed, resulting in asymmetric morphologies. Amphibian emergent infectious diseases (EIDs), such as* Ranavirus *and chytrid fungus, have been involved in several mortality events, which makes them stressors and allows for the study of FA. We analyzed nine populations of green frogs* (Rana clamitans) *for the presence or absence of* Ranavirus *and chytrid fungus. Individuals were measured to determine levels of FA in seven traits under the hypothesis that FA is more likely to be observed in individuals infected by the pathogens. Significantly higher levels of FA were found in individuals with* Ranavirus *compared with uninfected individuals among all populations and all traits. We did not observe FA in individuals infected with chytrid fungus for any of the traits measured. Additionally, we observed a significant association between* Ranavirus *infection and levels of FA in both males and females, which may indicate this viral disease is likely to affect both sexes during development. Altogether, our results indicate that some EIDs may have far-reaching and nonlethal effects on individual development and populations harboring such diseases and that FA can be used as a conservation tool to identify populations subject to such a stress.*

Keywords: amphibian population declines, *Batrachochytrium dendrobatidis*, chytrid fungus, developmental instability, fluctuating asymmetry, green frogs, *Rana clamitans*, *Ranavirus*

Efectos de Dos Patógenos de Anfibios sobre la Estabilidad del Desarrollo de Ranas Verdes

Resumen. *La inestabilidad del desarrollo, medida como asimetría fluctuante (AF), a menudo se utiliza como una herramienta para medir estrés y la calidad integral de los organismos. Bajo AF, se asume que el control de la simetría durante el desarrollo es costoso y que bajo estrés se perturba la trayectoria del desarrollo, dando como resultado morfologías asimétricas. Las enfermedades infecciosas emergentes (EIE) en los anfibios, como* Ranavirus *y hongo chytridio, han sido involucradas en varios eventos de mortalidad, por lo que son factores estresantes y permiten el estudio de AF. Analizamos nueve poblaciones de ranas verdes* (Rana clamitans) *para la presencia o ausencia de* Ranavirus *y hongo chytridio. Los individuos fueron medidos para determinar los niveles de AF en siete atributos bajo la hipótesis de que es más probable observar la AF en individuos infectados por los patógenos. Encontramos niveles de AF significativamente mayores en individuos con* Ranavirus *en comparación con individuos no infectados entre todas las poblaciones y todos los atributos. No observamos AF en individuos infectados con el hongo chytridio para ninguna de los atributos medidos. Adicionalmente, observamos una asociación significativa entre la infección por* Ranavirus *y los niveles de AF tanto en machos como hembras, lo cual puede indicar que es probable que esta enfermedad viral afecte a ambos sexos durante el desarrollo. En conjunto, nuestros resultados indican que algunas EIE pueden tener efectos de largo alcance y no letales sobre el desarrollo de individuos y poblaciones que hospedan tales enfermedades y que la AF puede ser utilizada como una herramienta de conservación par identificar poblaciones sujetas a tal estrés.*

Introduction

Developmental instability (DI) occurs when individuals are unable to maintain stable development due to environmental stress (Van Valen 1962; Palmer & Strobeck 1986; Møller 1997). Conversely, developmental stability is an individual's ability to avoid or reduce this effect (Møller 2006). Developmental instability is often measured by the level of fluctuating asymmetry (FA) observed in one or several morphological traits. In fact, any difference in bilateral symmetry between paired body parts—where bilateral symmetry is expected—results in FA (Palmer & Strobeck 1986). Although it is predicted that individuals faced with environmental stressors are more likely to exhibit asymmetry (Lagesen & Folstad 1998; Hendrickx et al. 2003), both natural and sexual selection are expected to favor lower levels of FA because developmental noise is assumed to negatively affect fitness (Møller 1997; Björksten et al. 2000; Fuller & Houle 2002).

Although fluctuating asymmetry has been long proposed as a potential surrogate for fitness estimation with implications for conservation biology (Leary & Allendorf 1989; Clarke 1995), the utility of FA as an index of environmental stress remains debatable (Palmer 2000; Van Dongen 2006). Some studies have reported positive correlations between FA and stressors (Brown & Brown 2002; Hendrickx et al. 2003), whereas other studies failed to detect a relationship (Kimball et al. 1997; Bosch & Marquez 2000; Kruuk et al. 2003). For example, a significant relationship between FA of feathers and ectoparasite load was detected in populations of Cliff Swallows (*Petrochelidon pyrrhonota*) (Brown & Brown 2002). In contrast, FA was not associated with stress in secondary sexual traits (male antlers) in red deer (*Cervus elaphus*), although sexual traits are costly to maintain and should therefore be good indicators of FA (Kruuk et al. 2003). It has also been recognized that the level of FA is highly dependent on the level of stress within populations, which makes the detection of such association context dependant (Hendrickx et al. 2003). In fact, the discrepancy around FA results may increase with more stressful conditions because less-robust individuals may not survive under stressful conditions, which means only high-quality individuals can be assessed for FA.

Because of conflicting findings and possible oversights (see reviews by Palmer 2000; Møller 2006; Van Dongen 2006), several researchers have sought to determine appropriate methods and measurements for estimating FA. For example, it is best that many traits are measured in each individual (Hendrickx et al. 2003) because on an individual basis some traits may exhibit FA, whereas others may not (Bergstrom & Reimchem 2005). This also allows for traits to be analyzed individually or for traits to be grouped for a more-reliable measure of FA. Furthermore, this method makes it possible to determine a wider range of FA; higher levels of FA may be more informative than lower levels. It has been proposed that high levels of FA are more likely to be due to true DI rather than developmental noise (Fuller & Houle 2002).

Research on the relationship between FA and environmental stress in amphibians is lacking (but see Parris & Cornelius 2004; Woodhams & Alford 2005; Söderman et al. 2007; Delgado-Acevedo & Restrepo 2008) even though amphibians have long been regarded as model organisms for studying developmental stability (e.g., Helff 1934; Alford et al. 1999). The presence of FA may be an early warning of environmental stress in amphibian populations (Lamb et al. 1990; Gallant & Teather 2001; McCoy & Harris 2003), and given current concerns regarding amphibian population declines and the limited evidence that FA is correlated with environmental stress, the underuse of FA as a measure of population "health" is disturbing (McCoy & Harris 2003; Parris & Cornelius 2004; Söderman et al. 2007).

One major cause of amphibian declines is the emergence of infectious diseases (Daszak et al. 1999; Ouellet et al. 2005), the origins of which are still debated (Rachowicz et al. 2005). The two emerging infectious diseases (EIDs), *Ranavirus* and the chytridiomycosis-causing fungus *Batrachochytrium dendrobatidis* (*Bd*), are globally emerging pathogens, the latter of which has been linked to elevated FA (Daszak et al. 1999; Daszak et al. 2003; Parris & Cornelius 2004). *Ranavirus* was first identified in *Rana pipiens* and has since been found in several anuran hosts (Chinchar 2002). The external signs of infection include hemorrhagic lesions, skin sloughing, erosion, and ulceration, but death can sometimes occur without external signs of infection (Chinchar 2002). *Batrachochytrium dendrobatidis* is a waterborne pathogen transmitted by motile, spermatozoa-shaped, aquatic zoospores that prefer cooler climates, and it has been linked to several amphibian population declines (Lips 1999; Blaustein et al. 2005). Although it is one of the most serious EIDs (Daszak et al. 2003), prevalence and severity are still poorly understood (Bosch et al. 2007).

In the general context whereby developmental instability has been linked repeatedly with parasite burden (Reimchen & Nosil 2001; Brown & Brown 2002; Møller 2006) and amphibian EIDs are known to incur developmental instabilities (Johnson et al. 1999; Parris &

Cornelius 2004), we tested the hypothesis that the EIDs *Ranavirus* and *Bd* are associated with increased levels of FA in wild populations of the green frog (*Rana clamitans*). We predicted a positive relationship between the level of FA and the increased presence of either EID.

Methods

Sampling

The study sites ($n = 11$) were located in central and northeastern Ontario, Canada, and a previous survey indicated that green frogs (*Rana clamitans*) were present at all sites. Sites presented similar landscape features, such as pond size and vegetation, and were far from each other to represent independent samples. We sampled over a short period of time (June and July 2005) to avoid seasonal variation in the prevalence and intensity of EIDs that could affect our conclusions (Kriger & Hero 2006). On average, 25.18 (SD 6.16) adult frogs were collected from each site. We wore disposable gloves when we caught each frog individually. All individuals were kept in separate containers to avoid cross-contamination. All frogs were euthanized with an overdose of buffered MS22 (Syndel Laboratoires, Vancouver, Canada) and transported to the laboratory for measurement and assessment of infection.

Trait Measurements and Estimators of FA

We measured seven traits to increase the reliability of determining true FA as opposed to determining only measurement error (Leung et al. 2000). Five of these traits were morphological and thus were not influenced by sexual selection: length of tibiofibula (TF), length of foot (FO) from the calcaneum to the distal tip of the longest phalange, length of femur (FE), length of radioulna to the distal tip of the longest phalange (RP), and length of the thumb (TH). Although TH and RP were previously classified as sexually selected traits (Söderman et al. 2007) because of the occurrence of sexual dimorphism, we did not detect any significant difference in the level of FA among the sexes when analyzed separately (see Results). For this reason, we grouped these two traits with the other three traits that were not under sexual selection. We also measured two sexually selected traits: horizontal length (HT) and vertical length (VT) of the tympanum. These two traits are sexually dimorphic and are considered under selection because vocalizations of male frogs are used to communicate to potential mates and competitors during breeding (Purgue 1997; Bosch & Marquez 2000). Each trait was measured and recorded three times on each side. To avoid bias, all traits were measured before infectious status was determined.

DNA Extraction and Pathogen Detection

Liver tissue and a toe clip were removed from each frog, and forceps and scissors were sterilized with a 30% bleach solution between frogs to avoid cross contamination (Johnson et al. 2003). We used liver for *Ranavirus* detection because liver is the preferential tissue for infection (Cunningham et al. 2001). DNA was extracted with QIAmp DNeasy Kits following the standard protocol (Qiagen, Mississauga, Canada). Extraction negatives consisting of pure lysis buffer and extractions from frogs known to be unexposed to *Ranavirus* were used to assess the likelihood of contamination among samples (Harp & Petranka 2006). We also used samples known to be infected from a previous study as positive controls (St-Amour & Lesbarrères 2007). We used a standard polymerase chain reaction (PCR) protocol for *Ranavirus* known to amplify a broad cross-section of *Ranavirus*-like iridoviruses (Mao et al. 1997; Pearman et al. 2004). Samples were run on a 1% agarose gel at 100 V for 1 hour and gels were then stained with EtBr before visualization under an ultraviolet light. We considered a frog infected when we successfully amplified a product of approximately 500 base pairs.

We used a bead-beating protocol to extract toe clips (Boyle et al. 2004) and extractions were diluted 1/10. Dilutions were then tested for the presence of *Bd* following the real-time PCR method (Boyle et al. 2004). All the samples were amplified in replicate along with four known concentration standards (genomic equivalents [GE] of 100, 10, 1, and 0.1) and duplicated negative controls. We considered an amplification positive for *Bd* if both replicates amplified, if mean GE estimated from the two amplifications was at minimum 0.1, and the standard error of the estimate of GE was at least one order of magnitude lower than the mean GE. In the event that only one of two replicates amplified, real-time PCR was repeated. If this repeat PCR met the criteria outlined above, we then considered this animal positive for infection. If not, the individual was scored as negative for *Bd*.

Statistical Analysis

Several analyses were conducted to prevent measurement error (ME) and handedness from influencing estimates of FA. First, to determine ME we used the following equation (Yezerinac et al. 1992; Schulte-Hostedde & Millar 2000):

$$\%\text{ME} = \frac{s_w^2}{s_w^2 + s_a^2} \times 100,$$

where s_w is the variance within individual measurements and s_a is the variance among individual measurements. Second, handedness of the measurer was tested with a two-tailed t test to determine whether measurements were consistently skewed to one side or the other. Handedness of the measurer was calculated per trait for all individuals and separately for males, females, and

juveniles to determine whether differences were due to this type of error.

When analyzing pooled traits, we used the composite FA calculation (CF3) to rank the data points so as to avoid ME from skewed data (Leung et al. 2000). This method also corrects for the size of the animals being used and allows for large individuals to be compared with smaller individuals (Leung et al. 2000). To do this, we calculated the average of the three measurements taken for each trait and each side and subtracted the value for the right side from the left, which represents the relative amount of fluctuating asymmetry. We then pooled the absolute value of these averaged calculations across traits and among individuals to strengthen the test (Leung et al. 2000). We used a nonparametric ranked test (Mann–Whitney) to compare individuals infected with either *Ranavirus* or *Bd* with uninfected individuals. We also estimated the mean FA within populations. Finally, individual traits were analyzed separately to determine which of the traits displayed asymmetry.

Results

Forty individuals tested positive for *Ranavirus,* whereas 229 did not (17.5% prevalence), and prevalence among populations ranged from 0% to 63%. Three populations with prevalence of 0% were excluded from the population-based analysis. Thirty of the 269 individuals tested positive for *B. dendrobatidis* (11.2% prevalence). Prevalence among populations for this pathogen again ranged from 0% to 36%. There was no relationship between ranaviral presence and *Bd* presence within the same populations ($\chi^2 = 2.40$, $p = 0.13$).

We detected little evidence of bias due to handedness because all but one of the measurements were not skewed from left to right ($p > 0.05$ for all traits except HT). The one exception was horizontal measurement of the tympanum (HT), which was skewed to the right across all individuals ($t_{268} = -3.82$, $p < 0.001$) and in males, females, and juveniles ($t_{79} = -3.62$, $p < 0.001$; $t_{96} = -2.86$, $p < 0.01$ and $t_{91} = -3.49$, $p < 0.001$, respectively). Measurement error for each individual trait ranged from 10.9% to 66.6% (Table 1) (well within the common range for morphological studies [Schulte-Hostedde & Millar 2000]). We did not detect any significant difference in the level of FA between males and females (Mann–Whitney test, $z = 0.89$, $p = 0.37$), even for traits that are supposedly sexually dimorphic (HT: $z = 0.44$, $p = 0.75$; VT: $z = 0.23$, $p = 0.87$). We also did not observe a significant difference in levels of FA among sexually selected traits and traits presumed not to be under sexual selection ($t_{268} = 1.98$, $p = 0.24$).

Four populations with low prevalence of *Ranavirus* (5–9%) showed no significant differences in composite FA among uninfected and infected individuals

**Table 1. Measurement error for samples of green frogs ($n = 269$) for each of the traits analyzed for asymmetry.**

Trait	Right-side error (%)	Left-side error (%)
Tibiofibula	22.7	22.6
Foot	12.4	10.9
Femur	22.2	13.9
Radioulna	38.6	23.8
Thumb	43.6	58.6
Horizontal tympanum	50.7	52.3
Vertical tympanum	49.9	66.6

(Mann–Whitney test, $z = 1.46$, $p = 0.144$; $z = 1.37$, $p = 0.124$; $z = 0.00$, $p = 1.00$; $z = 0.00$, $p = 1.00$). Nevertheless, we detected a significant difference in composite FA in the four populations with higher prevalence of *Ranavirus* infection (13–63%; $z = 2.29$, $p < 0.05$; $z = 4.28$, $p < 0.001$; $z = 3.14$, $p < 0.01$; $z = 3.67$, $p < 0.001$); infected individuals exhibited significantly greater composite FA than uninfected individuals. By contrast, we could not detect any significant effect of population prevalence of *Bd* and composite FA in any of the ponds ($z = 0.00$; $p = 1.00$ to $z = 0.82$; $p = 0.411$).

When we pooled all frogs across all populations, composite FA was significantly greater in individuals infected with *Ranavirus* (mean = 119.96 [SD 26.18]) than in uninfected individuals (88.16 [30.71]; Mann–Whitney test, $z = 7.83$, $p < 0.001$). No such relationship was detected between composite FA and *Bd* ($z = 1.58$; $p = 0.113$). Even when we removed HT from the composite measure of FA, results remained significant for the comparison with *Ranavirus* prevalence ($z = 7.74$, $p < 0.001$) and not significant for the comparison with *Bd* prevalence ($z = 1.58$; $p = 0.113$). When all seven traits were examined individually, we detected significantly greater FA in frogs infected with *Ranavirus* (Fig. 1), but there was no effect of *Bd* on FA.

Discussion

The most salient result of our study was the consistent and significant association between FA and *Ranavirus* infection. This effect was influenced by sample size. In populations with low *Ranavirus* prevalence, composite FA was not significantly associated with infection. This may explain why we did not find a congruent relationship between FA and *Bd* because overall prevalence of the fungal pathogen was approximately two-thirds that of *Ranavirus*. Although no relationship between infection prevalence and FA was observed in eight species of stream-dwelling frogs (Woodhams & Alford 2005), exposure to *Bd* led to increased hind-limb asymmetry of recent metamorphs of two species of north-American anurans (Parris & Cornelius 2004). Our results suggest
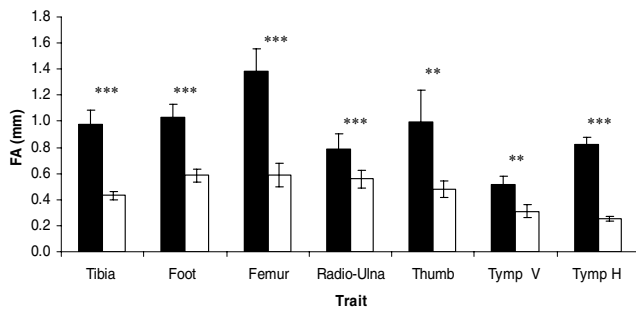
Figure 1. Levels of fluctuating asymmetry (FA) for each trait measured in Ranavirus *infected (solid bars) versus uninfected (white bars)* Rana clamitans. *Traits measured were length of tibiofibula (tibia), foot, femur, radioulna to tip of phalanges, thumb and horizontal length of the tympanum (Tymp H), and vertical length of the tympanum (tymp V) (**p < 0.01; ***p < 0.001).*

that lack of a relationship between FA and *Bd* could be the result of lethal effects of fungal infection (Møller 1997; Woodhams & Alford 2005). Much mortality attributed to *Bd* occurs at or soon after metamorphosis (Bosch et al. 2001; Bosch & Martínez-Solano 2006) which, if in effect in populations of green frogs, would result in the removal of individuals most affected by *Bd*. Alternatively, it is possible that animals were only recently infected by *Bd*, and thus there was little effect on their development. Quantitative results of real-time PCR analysis of *Bd* infection supported both these hypotheses because the majority of infection burdens were low (at or below 1 genomic equivalent, data not shown). Thus, the infected green frogs we sampled harbored relatively weak infections. Infection burden is related to virulence and thus to FA (Agnew & Koella 1997), but more work is needed to disentangle the relationship between the pathogens and FA.

Although the investigation of amphibian immune response to *Bd* has been limited to the expression of antimicrobial peptides (Woodhams et al. 2006, 2007), *Ranavirus* appears to incur typical costs associated with immune function. Virulence is dose dependent (Pearman et al. 2004), and certain host genotypes have the ability to clear infection, whereas others do not (Gantress et al. 2003). Frogs develop long-lasting and presumably costly humoral immunity to *Ranavirus* (Maniero et al. 2006), and such immune functions correlate with FA (Rantala et al. 2004; Møller 2006). For example, results of numerous studies show the link between activation of the immune system and increased asymmetry in developing individuals (Sibly & Calow 1989; Schmid-Hempel 2003; Møller 2006). The asymmetry might reflect host ability to mediate immune responses and prevent lethal infection (Robert et al. 2005; Bradley & Altizer 2007).

Although studies involving FA have decreased since the late 1990s, it is important that they be continued so

that the role of FA in measuring DI can be determined (Van Dongen 2006). Our results support the use of FA in determining stressors within natural populations and its implications for conservation biology (Clarke 1995). As the debate continues regarding the usefulness of FA, our results show this index is an efficient measure of stress (Leary & Allendorf 1989). Our results were highly significant in our examination of FA and viral presence, which suggests that the asymmetry seen was due to the stressors and not the measurement bias. Furthermore, our study species develops rather quickly, 1–2 years, compared with some larger organisms that may be used in FA studies, such as large vertebrates (Lagesen & Folstad 1998). In the context of frog development, the virus would need to be present for only one to two seasons to influence the growth of an entire generation of frogs. This assumption is further supported by the predictions of the residual asymmetry and compensatory growth hypotheses, which suggest that levels of asymmetry reflect recent growth history (Kellner & Alford 2003). Our results therefore highlight the potential benefit of using FA for measuring stress in amphibians relative to larger organisms that develop more slowly.

Although significantly different levels of FA in sexual traits compared with nonsexual traits have been noted previously (Söderman et al. 2007), we did not observe such differences. We found no differences in the levels of FA among all traits between males and females, although such differences have been reported in moor frogs (acidification increased levels of FA only in males; Söderman et al. 2007) and in stalk-eyed flies (food deprivation revealed significant levels of FA only in males; Björksten et al. 2000). By contrast, we observed a significant association between *Ranavirus* infection and levels of FA in both sexes, which indicates this viral disease is likely to affect both sexes during the development. This lack of difference between sexes with regards to levels of FA in secondary sexual traits may be attributed to the sampling period. We sampled frogs throughout the summer, a period that is outside the breeding season, when sexual traits such as the male thumb are exaggeratedly enlarged. Summer sampling allowed us to avoid any bias toward an increased level of FA that may have been attributed to the strong selection occurring on this trait during the breeding season (Hosken 2001). Finally, that all measurements but one (HT) showed no directional bias, suggested that the horizontal diameter of the tympanum was naturally skewed. Certain organisms, particularly vertebrates, tend to exhibit some bias toward trait sizes from left to right, possibly as a consequence of handedness (tree frog, Dill 1977; owl, Norberg 1977).

In light of the concern about amphibian declines, our methods are also valuable in assessment of the health of infected populations. Because it is only possible to sample those individuals that are still alive, it is difficult to know without experimental investigation whether less-robust

individuals succumbed to *Ranavirus* or FA. It appears, however, that the virus is not only causing mass mortalities within populations, but may influence the development of remaining individuals within these populations. It is therefore possible that individuals infected with the virus are compromised at an early stage, before the threat of mortality due to viral infection even occurs, because they also face the costs associated with DI. Infection with the *Ranavirus* may result in asymmetry, and those individuals must additionally cope with this evolutionarily challenging phenotype because FA is believed to indicate overall genetic quality (Polak & Taylor 2007), which influences mating success. For instance, FA inhibits the ability to locate mates in the midwife toad (*Alytes obstetricans*) (Bosch & Marquez 2000), and increased levels of FA are correlated with decreased clutch sizes in populations of wolf spiders (*Pirata piraticus*) (Hendrickx et al. 2003).

The decline of amphibian populations is of great concern, and further efforts should be made to gain insight into all aspects of these declines, particularly in the case of infectious diseases. EIDs represent a considerable stress for amphibian populations, and the ability to detect such stresses before they seriously affect a population can enhance the effectiveness of conservation programs. Consequently, we suggest that developmental stability may represent a surrogate for more-direct fitness estimation for use in conservation biology and biomonitoring programs (Alford et al. 1999). In the case of EIDs, where the stress is internal and the symptoms are not always apparent, measuring developmental stability may prove useful because stress possibly influences developmental stability before it affects life-history parameters. Moreover, this analysis is relatively inexpensive and simple to perform, as opposed to molecular investigations. Our results suggest that the threats associated with EIDs are beyond simply prevalence of the disease. Further studies should be conducted to determine the long-term consequences of EIDs, including nonlethal effects of both the virus and FA in natural populations.

Acknowledgments

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