Spot symmetry predicts body condition in spotted salamanders, *Ambystoma maculatum*

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Abstract. Reports of global amphibian declines necessitate a focus on measures of population health. Fluctuating asymmetry (FA) is a potential measure of the developmental stress experienced by individuals in different environments, but few studies have linked FA with measures of individual quality in amphibians, which is an important assumption of FA. The spotted salamander, *Ambystoma maculatum*, has two rows of yellow spots on its dorsal surface which might represent useful characters in FA analyses and population monitoring, provided they truly reflect the quality of individuals. In this paper we compared left-right asymmetry in spot features (spot number, size and shape) and leg lengths of this species with traditional measures of body size and body condition among museum specimens to address this question. Of all three spot symmetry variables, the simplest (the difference in left-right spot numbers) was the most important. Individuals with asymmetrical spot numbers were also more asymmetrical in hind leg length, evidence that spots are supposed to be symmetrical in this species. Moreover, salamanders with symmetrical numbers of left-right dorsal spots had higher body condition scores than those with asymmetrical spot numbers. Combined, our results indicate that spot number symmetry provides a good index of individual quality. Further, as many proximate factors can influence body condition (such as recent foraging history), FA in characters such as spots or limbs may provide a more stable metric for assessing the developmental health or quality of individuals, which would prove valuable in amphibian conservation programs.

Key words: *Ambystoma maculatum*; fluctuating asymmetry; spots; spotted salamanders.

Introduction

World-wide reports of declines in amphibian populations (Beebee and Griffiths, 2005; Pounds et al., 2006) have made amphibian conservation an important issue. Potential causes for these declines include habitat loss (Beebee and Griffiths, 2005), pathogens (Pounds et al., 2006), ultraviolet radiation (Trenham and Diamond, 2005), and global warming (Rohr and Madison, 2003), or more likely a combination of these issues. Declines have also necessitated an increased focus

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on population monitoring in amphibians. Most monitoring programs focus on estimates of population size (e.g. Welsh and Ollivier, 1998; Semlitsch, 2000; Steele et al., 2003), although recent work has indicated that measures of body condition can be useful as an additional measure of population viability (MacCracken, 2002; Karraker and Welsh, 2006; Lauck, 2006). Body condition is usually some index of the relationship between size (i.e. length) and mass (Rohr et al., 2004; Lauck, 2006), although some researchers simply use the mass of individuals (MacCracken, 2002) to assess the performance of populations.

A lesser-used but potentially effective way to assess population viability is by measuring aspects of fluctuating asymmetry (FA) among animals in the population. The degree of character asymmetry in bilateral animals is thought to reflect instabilities during the animals' development and is thought to be caused by either genetic or environmental stress, or both in combination (reviewed in Polak, 2003). Asymmetries have the potential to occur whenever there is growth in bilateral characters. Animals such as amphibians clearly have a larval period, where there is no doubt potential for asymmetrical growth, however they continue to grow larger throughout their adult stage as well, which provides additional opportunities for minor deviations in bilateral characters, particularly within environmentally or genetically stressed individuals. While conventional measures of condition in amphibian studies, such as the ratio between size and mass may be prone to fluctuations based on recent foraging (Lamoureux et al., 2002), measures of FA may give a more reliable and integrated measure of the "developmental health" of an animal over its life.

Identifying useful characters for any FA analysis requires demonstration that a character is supposed to be symmetrical and is associated with a measure of animal health or fitness. To be useful in monitoring, characters that can be easily quantified may be particularly useful. To date the vast majority of studies using FA have focused on differences in the linear lengths of bilateral characters, such as leg lengths in insects (e.g. Nosil and Reimchen, 2001), salamanders (McCoy and Harris, 2003), lizards (e.g. Martín and López, 2001), and frogs (Lauck, 2006) or feathers of birds (e.g. Møller, 1992; Bize et al., 2004). Leg length is a character that is obviously symmetrical by nature, which makes it an ideal candidate for FA studies. More recently, more novel characters have been included in FA studies, such as counts of head scales in snakes (Herczeg et al., 2005). Further, with the recent advances in digital image analysis techniques (e.g. Davis et al., 2004; Davis et al., 2005), researchers now have the opportunity to measure subtle variations in surface areas and shapes of skin patterns that occur on both sides of certain animals, many of which are potentially useful in FA studies, but have not been well-studied.

The spotted salamander, *Ambystoma maculatum*, is a common and widespread amphibian for which FA may be a useful character in assessing developmental stability. This species has brown to black skin with a row of bilateral yellow spots leading down its back, which are formed in the months following metamorphosis (Davis, pers obs). The total number of spots in this species varies amongst individual to individual, but does not differ between sexes (Pierce and Shayevitz, 1982). Wright and Zamudio (2002) showed that the total area of dorsal spots in this species had increased in asymmetry in a pond after the area around it had been converted to a golf course, but had remained symmetrical at a nearby pond that had remained undisturbed. This was one of the first studies to suggest salamander spot asymmetry might be linked to environmental quality. However two drawbacks to this study were that its results hinged upon the untested assumption that spotted salamanders are supposed to have symmetrical spots, and there was no indication of whether spot symmetry was related to individual "health" or quality. One way to address this first issue would be to compare measures of spot symmetry to measures of skeletal symmetry (leg lengths, for example), which are unquestionably symmetrical (or at least supposed to be) in bilateral vertebrates. If spots in this species are supposed to be symmetrical there should be a positive relationship between spot symmetry and leg length symmetry. To address the second idea, measures of symmetry could be compared to more conventional measures of individual quality, such as body size and body condition.

In this study we used digital image analysis of museum specimens of spotted salamanders to explore the two questions raised above. Specifically, we asked if there is a relationship between spot symmetry and leg length symmetry, and if there is a relationship between either body size or condition and aspects of spot symmetry.

Materials and Methods

Digitizing specimens

A total of 73 museum specimens of *A. maculatum* were obtained from the Georgia Museum of Natural History in Athens, GA. We had originally obtained 94 specimens but we excluded specimens that were preserved in a contorted position, since those tended to have obscured or partially-obscured spots when laid flat. The visibility of spots was also a factor in selecting specimens (below). Digitizing specimens involved removing them from their preservative and air-drying for 5 minutes. When completely dried each was individually placed on a stage with a white background and photographed from above with a Canon Digital Rebel XL SLR camera with the camera mounted at a fixed distance from the stage for all specimens. Care was taken to ensure that all specimens were positioned directly under the camera lens so that both left and right sides of the specimen were in full view in the pictures (fig. 1a). At the end of the digitizing session a standard metric ruler was photographed to provide a calibration picture for the image analysis software (explained further below). Finally, the sex of each specimen was recorded where possible, based on the presence of sexual characteristics (enlarged vent for males, distended abdomen for females). Of the 74 specimens, 30 were males, 19 were females and 25 were of unknown sex.

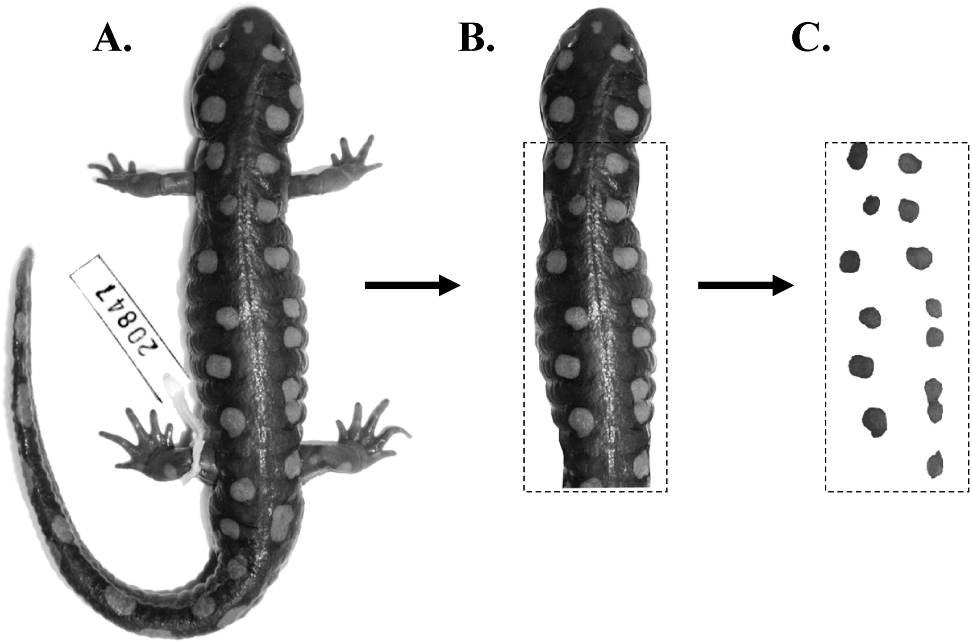

Figure 1. Progression of measurements of museum specimens used in this study. From the initial image (1a), we digitally removed the limbs and tail (1b) and measured the length and surface area of this feature. Next, the spots on this feature were digitally selected (1c) and their number, sizes (area) and shapes (roundness) measured.

Measuring specimens

All pictures were analyzed using Fovea Pro (Reindeer Graphics, Inc). The image analyses methods we used generally followed Davis (2004). Briefly, image analysis software calculates the dimensions of selected objects in digital images by calculating the number of on-screen pixels in user-selected parts of the object, then outputting the actual dimensions of the parts based on a user-defined pixel-to-millimeter ratio, which is obtained from the calibration picture of the ruler.

We first generated measures of body length and body area. Body length was calculated by measuring the length of a line drawn down the center of the specimen from the tip of the snout to the point where the hindlimbs arise. On most specimens this was a straight line, although for some moderately curved specimens the line was drawn so that it followed the curvature of the body. Whether or not the line was curved did not matter for the image analysis measurements (i.e. the software calculates the length of the line, curved or not). Body area was calculated by measuring the area of a selection drawn around the body that excluded the legs and tail (fig. 1b). We next digitally selected the spots on this body selection (fig. 1c). We only included spots that fell more than 50% within a rectangular selection that was drawn with top corners on the junction between the head and shoulder, and bottom corners at the posterior point of hindlimb attachment (dotted line selection area in fig. 1b). Although we did not include specimens that were contorted, some individuals that were included were crooked enough so that one or two individual spots were partially obscured in the photograph (which is a drawback when making a two-dimensional image from a three-dimensional object). To minimize this problem in our analyses, if any spot was more than 50% obscured we did not include that specimen.

Once all spots were isolated, we ran the Fovea Pro measure routine which measures all selected objects and outputs the variables of interest. In our case, these were the number of spots, the spot area (in mm^2) of each selected spot, and the roundness of each spot. The software calculated spot roundness from the formula $4 \times \text{Area}/\pi \times \text{Length}^2$, and it assigned each spot a number from 0-1, with 1 being a perfect circle.

With these data we created three variables relating to spot asymmetry. First was simply the absolute difference in left side-right side spot number. For spot area, we first summed the spot area for each side, then calculated the absolute difference in left-right total spot area following Wright and Zamudio (2002). Finally, we calculated the 'side average' spot roundness values for the left and right sides separately and from these averages, we calculated the absolute difference in the left-right sides to give us a single roundness asymmetry value for each individual.

On a subset of individuals ($n = 58$), we also measured the asymmetry in hind leg lengths (i.e. the absolute difference in left-right lengths). Specimens with missing toes were not measured, nor were individuals that were positioned so that their legs did not lay flat on the table. The leg measurement was accomplished in the same manner as the body length measure, in that a line was drawn from the point of leg attachment to the tip of the longest digit, following the curvature of the leg along the way.

Statistical analyses

Where necessary, variables were log-transformed + 1 to meet assumptions of normality before analyses. To address our first objective (to compare spot symmetry to leg length symmetry), we used general linear model analyses to examine the effects of sex (a categorical variable: male, female or unknown) and the three spot symmetry variables on leg symmetry. For our second objective, we first obtained a measure of body condition by comparing body length to body area using simple linear regression, and used the residuals of this regression as our measure of condition. This regression was significantly linear ($F = 359.9$, df $= 72$, $P < 0.001$). Positive residuals from this regression therefore represented individuals that were larger (in surface area) than expected given their length. Then, for analyses of body size (which we indexed by body area) and body condition (residuals), general linear model analyses was used to examine the effects of sex, spot number asymmetry, spot size asymmetry, and spot roundness asymmetry. All tests were performed using Statistica software (Statistica, 2003).

Results

Spot symmetry and leg length symmetry

The results of our general linear model of leg asymmetry indicated that salamanders with more asymmetrical hind leg lengths tended to have asymmetrical numbers of dorsal spots ($F_{1,57} = 3.61$, $P = 0.063$; fig. 2). Parameter estimates of this effect indicated this was a positive relationship. There was no significant relationship between spot area asymmetry and leg length asymmetry ($F_{1,57} = 0.683$, $P = 0.412$), nor was there a relationship between spot roundness asymmetry and leg length asymmetry ($F_{1,57} = 0.293$, $P = 0.590$). Finally, there was no difference in leg asymmetry between sexes ($F_{2,57} = 1.02$, $P = 0.365$).

Spot symmetry and body area

Not surprisingly, in our GLM analysis of body area there was significant variation between sexes ($F_{2,71} = 8.60$, $P < 0.001$). Females were larger than males (1280 mm^2 versus 919 mm^2), with individuals of unknown sex being intermediate in size (1131 mm^2). There was no measurable relationship between spot number asymmetry and body area of spotted salamander specimens ($F_{1,71} = 1.99$, $P = 0.163$). However there was a highly significant relationship between body size and spot area asymmetry ($F_{1,71} = 7.95$, $P = 0.006$). Parameter estimates of this effect indicated a positive relationship between these variables, so that larger individuals

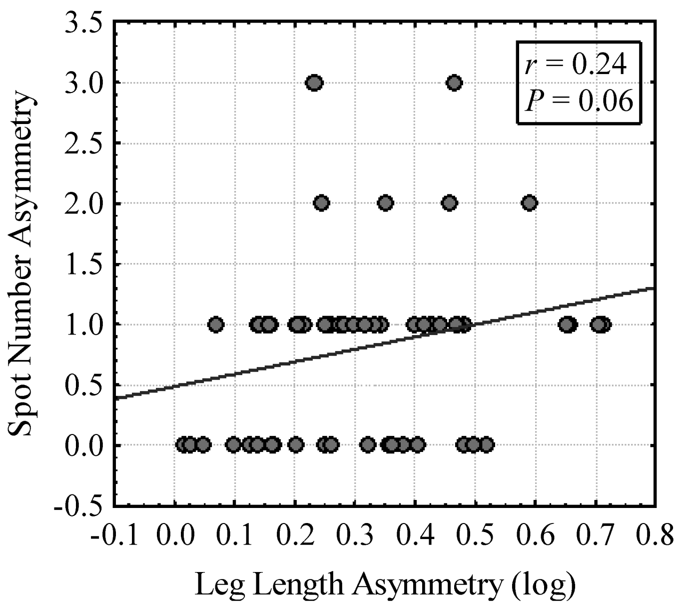

Figure 2. Relationship between spot number asymmetry (absolute difference between number of left spots minus number of right spots) and asymmetry in hind leg lengths (log-transformed + 1) of museum specimens.

had larger spot area asymmetries. Finally, there was no relationship between spot roundness asymmetry and body area ($F_{1,71} = 0.847$, $P = 0.361$).

Spot symmetry and body condition

In our GLM analysis of body condition there was a significant effect of spot number asymmetry ($F_{1,70} = 6.21$, $P = 0.015$). Parameter estimates indicated a negative relationship, so that individuals with more asymmetrical spot numbers had poorer body condition scores than those with symmetrical numbers (fig. 3, top). There was no relationship between spot area asymmetry ($F_{1,70} = 0.11$, $P = 0.747$) or spot roundness asymmetry ($F_{1,70} = 0.002$, $P = 0.960$) and body condition. There was no significant effect of sex on body condition ($F_{2,70} = 0.457$, $P = 0.635$).

Although it was not a specific objective of this study, given the observed negative relationship between spot number asymmetry and body condition and the positive relationship we found between spot number asymmetry and leg length asymmetry, we compared the body condition and leg length asymmetry variables. These variables were significantly negatively correlated (Pearson correlation, $r = -0.35$, $P = 0.007$; fig. 3, bottom).

Discussion

Surprisingly, of all three spot asymmetry variables we measured, the one most related to leg asymmetry and to body condition (although not size) was also the simplest – asymmetry in left-right spot number. Individuals with more symmetrical hind leg lengths tended to have symmetrical numbers of spots. This result suggests that spots (at least the left-right numbers) are supposed to be symmetrical in this species. Furthermore, although there was no relationship with body size, symmetry of spot numbers on the left and right sides of salamanders was associated with high body condition scores in this study. Combined, these results provide compelling evidence that spot numbers can serve as useful measures of asymmetry, at least in spotted amphibian species, and that spot symmetry can serve as an index of individual quality, which are both critical assumptions of fluctuating asymmetry studies.

Assessing spot symmetry of spotted salamanders can provide additional information that may not be obtainable from measures of skeletal symmetry (i.e. leg lengths), which would reflect environmental or genetic stress incurred during bone growth throughout the animal's lifetime. Dorsal spots in this species appear to be formed in the weeks just after metamorphosis (Davis, pers. obs). If these spot patterns remain fixed throughout the life of the individual, then the symmetry (or asymmetry) of dorsal spot patterns in an adult salamander could reflect the degree of "historical" stress imposed on the individual, during the period when spots are formed post-metamorphosis. It is not clear though, whether spot patterns indeed remain fixed in this species. Spot patterns do appear to remain fixed in red spotted newts

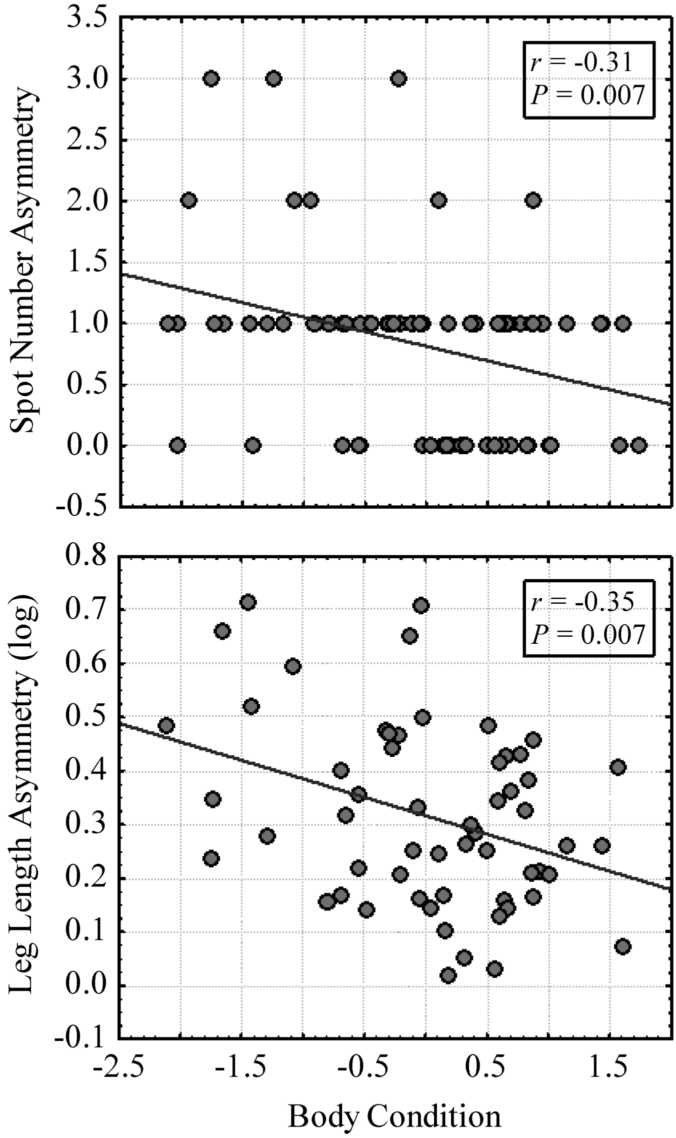

Figure 3. Relationship between body condition (residuals of body area-length regression) and spot number asymmetry (top) and log-transformed (+1) leg length asymmetry (bottom).

(*Notophthalmus viridescens*), which conveniently allows researchers to identify individuals by their 'spot fingerprint' (Gill, 1978). Our own data supports the idea of fixed spot patterns throughout life in this species, since we found no relationship between body size (as a proxy for age) and spot number symmetry. Furthermore, while it was not a goal of this study, comparison between the *average* left-right spot number and body size revealed no significant relationship (Pearson correlation, $r = 0.01$, $P = 0.91$), indicating that the number of spots on a salamander does not change with age. Meanwhile, comparison of *average* spot size with body size indicated a positive relationship ($r = 0.57$, $P < 0.001$), suggesting that spots increase in size with age. In contrast, Pierce and Shayevitz (1982) found a positive relationship between body size and total spot number (spot symmetry and spot size were not assessed) and suggested that either the number of spots increases with age in this species (i.e. that spots are not fixed), or that selection acts to remove individuals with fewer spots early in life.

If spot asymmetry indeed reflects “historical” developmental stress incurred by the animal, the relationship we found with body condition raises interesting questions. The body condition of an amphibian is known to fluctuate considerably depending on its recent feeding history (e.g. Lamoureux et al., 2002). Thus, the condition of the preserved specimens in this study could simply reflect the feeding history of those individuals in the week prior to their collection and preservation. Why then would asymmetry in spots be related to current body condition? It may be that individuals living in polluted or low-quality areas suffer continued stress throughout their lives, which acts both to create asymmetrical spots early in life, and to result in chronically reduced body conditions. On the other hand, stress incurred during early life could produce low-quality (and therefore asymmetrical) individuals that are then less able to procure food resources on a given feeding bout later in life (regardless of their environment), leading to a lowered body condition in the long term.

Other features of spot symmetry besides their left-right number did not appear to be related to size, body condition or leg symmetry. The exception was the positive relationship between spot size asymmetry and body size, a result consistent with that found by Wright and Zamudio (2002), who pointed out that larger individuals have larger spots, and therefore would also have larger differences between left-right areas. Spot *number* asymmetry was not related to size though, and conveniently, this is also the simplest measure to obtain, and needs no diagnostic tools. Spot symmetry is also easier to obtain than is leg length symmetry, which is difficult to measure on live salamanders (McCoy and Harris, 2003). These, plus the strong association with individual quality we observed here, means this variable could serve as a useful field index of FA in wild populations. Indeed, this approach is not that different from that of Wright and Zamudio (2002), who used the asymmetry in total area of left-right spots as a measure of environmental stress within salamander populations. Our results build on theirs by demonstrating the links between spot symmetry and individual quality, and provide a framework for future research into this important conservation tool.

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