Hindlimb abnormality reduces locomotor performance in *Pelobates cultripes* metamorphs but is not predicted by larval morphometrics

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Abstract

Locomotor performance is a fundamental feature commonly related to many animals' fitness. In most cases, locomotor performance is closely related to morphology of the structures responsible for it, which is therefore under strong selective pressure. Hence, limb abnormality could hinder locomotion and, for that reason, be eradicated by selection, which could explain its overall low prevalence that makes proper research difficult. Here, we took advantage of the moderately high prevalence of hindlimb abnormality in a sample of Iberian spadefoot (*Pelobates cultripes*) metamorphs developed from tadpoles captured and transferred to the laboratory before selection could act against metamorph abnormality. We tested the hypothesis that limb abnormality impairs locomotor performance. Moreover, we measured several larval and metamorph morphometrics, and checked for differences between normal and abnormal-limbed individuals. We also assessed correlations between hindlimb ratio (hindlimb length/SVL) and jumping performance in normal and abnormal-limbed metamorphs. Larval traits measured could not predict hindlimb abnormality. In metamorphs, only hindlimb ratio differed between normal and abnormal-limbed individuals, being shorter in the latter. Abnormal-limbed metamorphs jumped considerably shorter distances than normal-limbed conspecifics. Therefore, selection against reduced locomotor performance could eliminate limb abnormality from populations. Hindlimb ratio was included in the model as a covariable, and thus controlled for. Consequently, other factors besides shorter hindlimbs, probably hindlimb abnormality itself, could play a role in worse jumping capability of abnormal-limbed individuals. Hindlimb ratio was positively related to jumping distance in both groups, although the relationship was weaker in abnormal-limbed metamorphs.

Key Words

anomaly, anuran, jumping distance, morphology, spadefoot

Introduction

Locomotor performance is key to most animals' fitness (Miles 2004; Meyer-Vernet and Rospars 2016; Lailvaux and Husak 2017; Kraskura and Nelson 2018). Individuals with more efficient locomotor performance often dominate in social interactions (Perry et al. 2004), control more extensive territories (Peterson and Husak 2006), and better defend their mates (Husak et al. 2008), which in-creases reproductive success (Husak et al. 2006). Sexual selection favours maximal locomotor performance (Husak and Fox 2008). Moreover, locomotor performance enhances prey hunting (Budick and O'Malley 2000; Higham 2007), optimises refuge use (Martín and López 2000), and increases the chances of escaping predators' attacks (McGee et al. 2009; Oufiero et al. 2011). Predator pressure favours better locomotor performance (Ingley et al. 2016). Consequently, a better locomotor performance

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increases survival (Husak 2006a, 2006b), and is therefore directionally increased by both sexual and natural selection (reviewed in Irschick et al. 2008).

In most vertebrates, locomotor performance is closely related to morphology of the appendages responsible for locomotion, such as fins in fish (Li et al. 2016), limbs in amphibians (Zamora-Camacho 2018), reptiles (Zamora-Camacho et al. 2014) and mammals (Day and Jayne 2007), and wings in birds (Moreno-Rueda 2003). Morphology is commonly subject to strong natural selection (Calsbeek 2008) driven by locomotor performance (Botton-Divet et al. 2017; Higham et al. 2017). Even slight defects in appendage symmetry reduce locomotor performance (Martín and López 2001). Severe appendage abnormalities are likely to jeopardize movement ability, and could be rapidly eradicated by natural selection. That could be one reason why appendage abnormality prevalence in nature is often anecdotal, generally below 5% (Ouellet 2000; Mester et al. 2015). Nevertheless, this low prevalence does not allow proper investigation of its potential effects.

However, anurans are increasingly susceptible to limb abnormality (Lanoo 2008; Johnson et al. 2010; Laurentino et al. 2016), which makes its study particularly relevant. The most common abnormalities in anurans include partially or totally missing limbs, supernumerary limbs, and deformed limbs (Johnson et al. 2010; Reeves et al. 2013), and are more frequent in the hindlimbs of metamorphs. Therefore, such abnormalities might arise during the larval period due to environmental factors (besides a genetic component): ultraviolet radiation, contamination, agrochemicals, parasite infections (often synergistically), and predator attacks may provoke limb abnormality in metamorphs (Pahkala et al. 2001; Johnson and Bowerman 2010; Roberts and Dickinson 2012; Bernabo et al. 2016; Haas et al. 2018). The increase in amphibian abnormality rates could be related to global change (reviewed in Blaustein and Johnson 2003).

In this paper, we took advantage of a cohort of metamorphs of Iberian spadefoot toads (*Pelobates cultripes* Cuvier, 1829) resulting from tadpoles captured from a wild population and transferred to the laboratory before selection could act against metamorph abnormality. This procedure enabled us to obtain a prevalence higher than in nature and hence to perform analyses with an appropriate sample size. Among those metamorphs, an abnormality in hindlimb morphology, consisting of misshaped limbs (see below), was present in a sufficiently high number of metamorphs to study its effect on locomotion. We predict that locomotion will be impaired in abnormal-limbed metamorphs as compared to normal-limbed conspecifics. Abnormal morphology of amphibian metamorphs could reflect a deficient larval development (Fox 1983). In such a case, larvae becoming abnormal-limbed metamorphs could show a smaller body size, a poorer body condition, or a reduced tail. Based on this rationale, we checked whether larval biometrics could predict metamorph abnormality. Finally, we compared biometrics between normal and abnormal-limbed metamorphs, and assessed potential differences in the relationships between biometrics and locomotion between both groups.

Methods

Pelobates cultripes is a medium-to-large sized toad widely distributed throughout most of the Iberian Peninsula and in Southern France (Recuero 2014). These toads inhabit sandy habitats where they can burrow using their metatarsal tubercles, transformed into sharp, hard spades (Recuero 2014). Reproduction takes place mainly during rainy, relatively warm periods (from autumn to spring depending on the local climate). Toads mate in large temporary ponds where tadpoles can complete their larval periods, which take at least eleven weeks (Recuero 2014). Tadpoles feed on both plant and animal matter, while metamorphs and adults feed on diverse invertebrates (Recuero 2014). In turn, water arthropods such as diving beetles or dragonfly nymphs commonly prey on tadpoles, while vertebrates such as birds, mammals, and snakes (among others), often consume tadpoles, metamorphs, and adults (Recuero 2014).

Animal collection took place in Pinares de Cartaya (SW Spain, 37°20'N, 7°09'W), a sandy stone pine (*Pinus pinea*) grove. In March 2017, we captured 71 recently hatched larvae in a small pond (a suboptimal habitat for these tadpoles; Recuero 2014) with a dip net, and transported them to the laboratory, as a part of a planned research project. Tadpoles were pooled into two 14-L plastic aquaria. Both aquaria were placed next to each other, at the same room temperature (around 18–20 °C) and light conditions. A nearby window let natural daylight in, permitting adjustment of circadian rhythms. Tadpoles were fed dry cat food (which contains animal and plant matter) *ad libitum*. We daily replaced 80% water volume with untreated, natural spring water. For water exchange, the aquaria were gently tilted to pour out water, with a soft mesh lid so that tadpoles could not jump out of the water or suffer any other damage. When tadpoles reached Gosner stage 42 (Gosner 1960), we weighed them with a balance model CDS-100 to the nearest 0.01g (after carefully drying them with a disposable napkin), and measured their snout-vent length (hereafter, SVL) and tail length with a digital calliper to the nearest 0.01mm. The sum of SVL and tail length was considered as total length. Then, we calculated tadpole scaled mass index (SMI; Peig and Green 2009) according to the formula:

$$SMI = W_i \left[\frac{L_0}{L_i} \right]^{b_{SMA}}$$

where W_i and L_i are, respectively, body mass and total length of each tadpole, L_0 is the arithmetic mean of total length in the sample, as a valid value to which the individual values can be standardised, and b_{SMA} is the slope of a standardised major axis regression, calculated here as the result of dividing the slope from the ordinary

least squares regression of body mass on total length by the Pearson's correlation coefficient, r (Peig and Green 2009). In the case of tadpoles, b_{SMA} was 2.9505. We also calculated the percentage of total length occupied by the tail. At this stage, limb malformities were already evident when present. From that moment until the end of metamorphosis, tadpoles were individually kept in 0.5-L plastic aquaria. Water was completely replaced with untreated spring water every day, with extreme caution not to hurt tadpoles, and the animals were no longer fed, because in these last stages of their metamorphoses mouthpart and gut morphology are undergoing changes that do not allow feeding (Gosner 1960; Jenssen 1967). At the last stages of metamorphosis, tadpoles were transferred to individual 0.5-L tilted aquaria that allowed them to leave the water at any moment. When metamorphosis finished (tail completely resorbed, stage 46; Gosner 1960), we weighed metamorphs with a balance model CDS-100 to the nearest 0.01g (after carefully drying them with a disposable napkin), and measured SVL and hindlimb length using a mm-marked ruler, from the insertion point in the pelvis to the extreme of the longest toe. In order to take this measure, we firmly but gently stretched metamorph hindlimb. Then, we calculated metamorph SMI as described for tadpoles (in the case of metamorphs, b_{SMA} was 2.9800), and hindlimb ratio as hindlimb length divided by total length.

In total, 23 out of 71 metamorphs showed abnormal hindlimbs. Abnormality consisted of a malformation in the angle of insertion of the hindlimbs in the pelvis, both inserted with an angle of approximately 270° with respect to each other (Fig. 1). Also, in the resting position, dysfunctional knee-joints made metamorph unable to fold hindlimbs close to hindquarters normally (Fig. 1). The rest of the metamorphs had normal hindlimbs (Fig. 1).

Within 12 hours of metamorphosis having completed, metamorphs were tested for jumping distance. Metamorphs were not fed prior to the trials, which reduces variability due to nutritional status. In the laboratory, metamorphs were transferred to a box of expanded polystyrene (50×50×20 cm) for the trials. To that end, they were individually released next to a corner of the box, with a drawing pin stuck in the polystyrene immediately behind the urostyle. Then, we gently prodded their hindquarters to stimulate jumping, and stuck a new drawing pin immediately behind the urostyle in the spot where metamorphs touched down. We stimulated five successive jumps, and stuck a drawing pin as described each time. Finally, we measured the distance between drawing pins consecutively stuck, corresponding with the distance of each jump. This way, we could calculate maximum jumping distance as that of the longest jump of each individual. Trials were performed at 22 °C room temperature, because body temperature can affect locomotor performance in anurans (Navas et al. 2008). Laboratory measures of anuran maximum jumping distance accurately describes actual maximum jumping capacity in the wild (Astley et al. 2013). Hence, we analysed here maximum jumping distance, because it is a most ecologically relevant trait, and is related
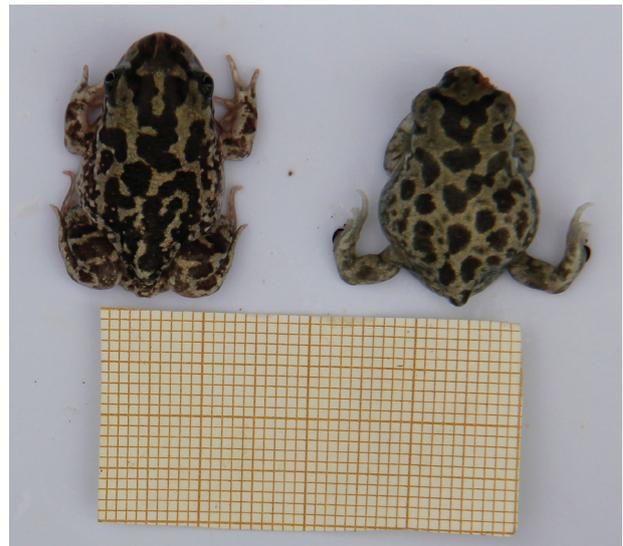

Figure 1. Normal (left) and abnormal-limbed (right) metamorphs, with a piece of graph paper below. Photo by Francisco Javier Zamora-Camacho.

to fitness (Irschick and Garland 2001). Metamorphs were released at the point where larvae had been captured as soon as the experiment finished.

We conducted parametric statistics after the appropriate transformations (maximum jumping distance needed to be log-transformed, in order to meet those criteria) to accomplish homoscedasticity and residual normality (Quinn and Keough 2002). Firstly, morphological differences between normal and abnormal-limbed metamorphs (and larvae going to turn into normal and abnormal-limbed metamorphs) were assessed with a one-way MANOVA including larval SVL, tail percentage, body mass, and SMI, as well as metamorph SVL, hindlimb ratio, body mass, and SMI. Then, we conducted an ANCOVA to test for differences between normal and abnormal-limbed metamorphs in maximum jumping distance, controlled for hindlimb ratio included as a covariable. Finally, we used simple regressions to test correlations between hindlimb ratio of normal and abnormal-limbed metamorphs with maximum jumping distance. Statistical analyses were conducted using software Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA).

Results

Sample sizes were 48 normal- and 23 abnormal-limbed individuals. The MANOVA analysis for morphological variables was significant ($F_{8, 62}$ = 5.200; $P < 0.001$). When the separate effects on each variable within the model were checked, only that on hindlimb ratio was significant ($F_{1, 69}$ = 34.111; $P < 0.001$; Fig. 2). Hindlimb ratio was greater in normal- than in abnormal-limbed metamorphs (mean ± SE; normal-limbed metamorphs: 1.033 ± 0.019; abnormal-limbed metamorphs: 0.836 ± 0.028; Fig. 2). All the other morphological variables of larvae and metamorphs
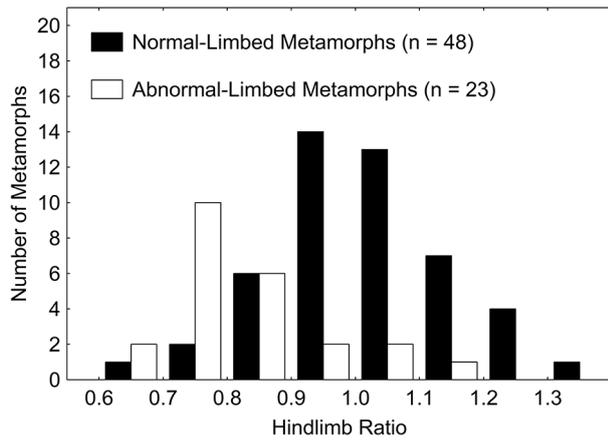
Figure 2. Hindlimb-ratio distribution of normal (black bars) and abnormal-limbed metamorphs (white bars). Sample sizes are indicated.
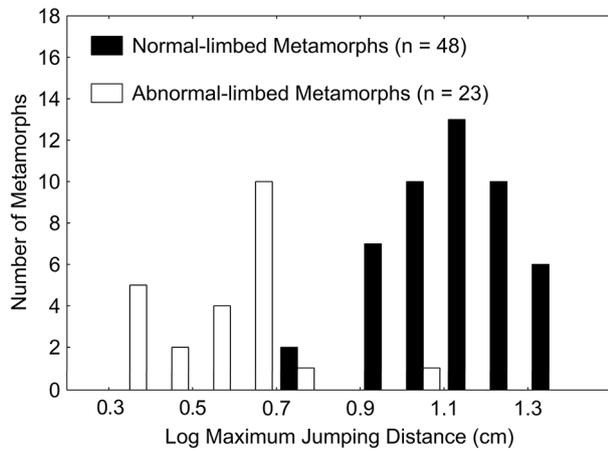

Figure 3. Maximum jumping distance (log-transformed) distribution of normal (black bars) and abnormal-limbed metamorphs (white bars) when hindlimb ratio was included as a covariable. Sample sizes are indicated
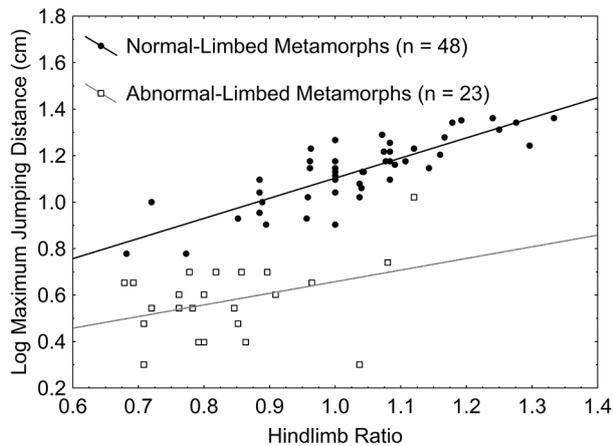

Figure 4. Correlations between maximum jumping distance (log-transformed) and hindlimb ratio of normal (black dots; $r = 0.830$; $P < 0.001$) and abnormal-limbed (empty squares; $r = 0.374$; $P = 0.079$) metamorphs. Sample sizes are indicated.

did not differ between normal- and abnormal-limbed individuals (in all cases, $F_{1, 69} < 0.409$; $P > 0.524$). Maximum jumping distance differed between normal- and abnormal-limbed metamorphs ($F_{1, 68} = 139.275$; $P < 0.001$; Fig. 3), with hindlimb ratio included as a covariable (effect of hindlimb ratio: $F_{1, 68} = 59.083$; $\beta = 0.412$; $P < 0.001$). Normal-limbed metamorphs performed longer maximum jumping distance (mean ± SE; normal-limbed metamorphs: $1.083 ± 0.018$ cm; abnormal-limbed metamorphs: $0.678 ± 0.027$ cm; Fig. 3). Maximum jumping distance was positively correlated with hindlimb ratio in normal-limbed metamorphs ($r = 0.830$; $P < 0.001$; Fig. 4), but this correlation was marginally non-significant in abnormal-limbed metamorphs ($r = 0.374$; $P = 0.079$; Fig. 4).

Discussion

According to our prediction, abnormal-limbed metamorphs jumped considerably shorter distances. Reduced jumping capacity in abnormal-limbed metamorphs could severely compromise their survival, because of their reduced capacity to flee from predators (Heinen and Hammond 1997), among other factors (see Introduction). Plus, limb abnormality could misadjust antipredator behaviour: *Pseudacris regilla* frogs with parasite-induced abnormal limbs allowed predators to approach closer and fled shorter distances (Goodman and Johnson 2011). In addition, in the particular case of *P. cultripes*, abnormal-limbed metamorphs were invariably unable to dig the vertical burrows where these toads normally take shelter from predators and adverse weather (personal observations). Thus, besides exposure to predators, heat and/or desiccation during the summer, or freezing during the winter, could also play a role in selection against limb abnormality in this species (Recuero 2014). In the light of these results, limb abnormality is likely eradicated by selection on morphology, mediated by locomotor performance (Jayne and Bennett 1990; Calsbeek and Irschick 2007). Such strong selection against limb abnormality linked to compromised locomotor performance is probably the cause of its low prevalence in natural environments. If abnormality has a genetic basis, the chances of abnormal-limbed individuals surviving until maturity, mating, and producing offspring that would spread their condition are scarce. On the other hand, if abnormality has an environmental origin, abnormal-limbed individuals are also likely to be preyed on and/or to fail in competition for food (or even just in food acquisition) early in their lives. The cause of limb abnormality in these toads remains unknown, though. Sampling was carried out in a natural, well-preserved habitat, so an anthropic origin of limb abnormality is improbable. Nevertheless, larvae were caught in a suboptimal habitat. In this species, larger males arrive first to the reproduction sites, where agonistic interactions among males are frequent (Lizana et al. 1994). Low-quality individuals may be forced to reproduce in

suboptimal sites by competitive exclusion from optimal habitats by high-quality conspecifics, as described in other taxa (Morrison et al. 1992; Morris 2003), which might suggest a low quality of parents and thus a genetic origin of abnormality.

Morphological variables measured in tadpoles were not able to predict hindlimb abnormality in metamorphs. This coincides with findings by Ponssa and Abdala (2016) that anuran morphological abnormalities are not visible before stage 42 Gosner. Among morphological variables measured in metamorphs, only hindlimb ratio differed between normal and abnormal-limbed metamorphs: abnormal limbs were moreover proportionally shorter than normal limbs. Since hindlimb ratio is directly related to jumping distance in *P. cultripes* metamorphs (Zamora-Camacho and Aragón 2019), shorter hindlimbs of abnormal-limbed metamorphs could play a role on their reduced locomotor performance. However, separate correlations between jumping distance and hindlimb ratio for normal and abnormal-limbed metamorphs showed that, for a given limb ratio, abnormal-limbed metamorphs performed shorter jumping distances than normal-limbed conspecifics. These findings indicate that other factors besides hindlimb ratio, probably hindlimb abnormality itself, could reduce the jumping distance of abnormal-limbed metamorphs.

Limb length is typically related to locomotor performance in anurans (Tejedo et al. 2000; Gomes et al. 2009; Zamora-Camacho 2018; Zamora-Camacho and Aragón 2019). We detected that hindlimb ratio was positively correlated with jumping distance in both normal and abnormal-limbed metamorphs. Nevertheless, this relationship was stronger in normal than in abnormal-limbed metamorphs. The relationship between maximum jumping distance and hindlimb ratio was only marginally non-significant in abnormal-limbed metamorphs. This result could be an effect of lower sample size of abnormal-limbed metamorphs, but it could also reflect irregularities in locomotor performance caused by abnormal limbs.

Acknowledgements

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