The developmental biology of *Calotriton arnoldi*: an analysis of the speed of growth of accidentally amputated extremities

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Resumen: A pesar de ser objeto de varios proyectos de conservación, gran parte de la biología y fisiología del amenazado tritón del Montseny (*Calotriton arnoldi*) es aún desconocida. Este estudio compara la velocidad de regeneración de extremidades accidentalmente amputadas en juveniles y adultos. Medimos las longitudes de las extremidades y el cuerpo de tritones en cautividad, así como la temperatura del agua. En juveniles, las extremidades amputadas tardaron seis años en crecer hasta longitudes normales; en adultos, las regeneradas alcanzaron 4 mm menos que las normales. Además, la tasa de regeneración fue más elevada en juveniles que en adultos. Es probable que las mayores tasas de regeneración en juveniles se deba al hecho de que éstos aún están desarrollándose.

The Montseny Newt (*Calotriton arnoldi*) is endemic to Catalonia, where it is only found in the Montseny mountains (NE Iberian Peninsula) (Amat & Carranza 2005). It is one of Europe's most threatened vertebrates and is classified by the International Union for Conservation of Nature (IUCN) as Critically Endangered due to its extremely small and fragmented range (less than 8 km^2) and a total population estimated at fewer than 1500 adults (Carranza & Martínez-Solano, 2009). In view of this situation, the local administrative bodies (Catalan Government and Barcelona Provincial Council) in charge of the conservation set up in 2006 a programme to study and conserve this species, which includes, amongst other undertakings, a number of *ex situ* projects. The Generalitat de Catalunya (Catalan Government) set in motion a captive breeding programme of this species in 2007 whose aims were to establish a solid genetic reserve, improve knowledge of its biology, and produce and select newts for reintroduction, thereby increasing the rare species' range. Nevertheless, given that it is an only recently discovered species (Amat & Carranza, 2005), many unknown but basic aspects of its biology and physiology still need to be identified if handling in captivity is to be improved.

To date, the regeneration of amputated extremities has been documented in an adult newt (Obón *et al*, 2004) but not in juvenile specimens. This study analyses the speed with which accidentally amputated extremities in juvenile newts regenerate in comparison with adult newts.

In summer-autumn 2013, a number of Montseny newts in the captive breeding programme were discovered to have lost limbs accidentally, which enabled us to compare the speed of regrowth of these extremities in four juvenile newts born in 2011 with that of an adult newt born in 2007. To detect whether or not there was a difference in the speed of growth of normal and amputated limbs in juvenile newts that were still growing, we measured the length of all extremities, as well as the body weight and total length of each newt.

All measurements were taken by the same person using digital callipers (*Powerfix*®, Hunan E&K Tools Inc) every two weeks for a year. Given the importance of temperature in the growth and development of amphibians (Wells, 2007), we also recorded the temperature in each aquarium in which the newts were housed to test whether or not this variable influenced the development of the newts' extremities. Temperature differences between juvenile and adult aquariums were less than 1º C, and so were regarded as being equivalent. Throughout the year, temperatures fluctuated between 10º and 15º C. A comparison of the temperatures in the aquariums showed no significant differences (Two Sample Test; $t_{(13)}$= -0.19; *P*= 0.85) and so any effect of temperature differences on subsequent results was ruled out. To compare adults and juveniles, a single-case test was carried out between the juveniles and the adult using the software PAST 3.25 (Hammer *et al*, 2001).

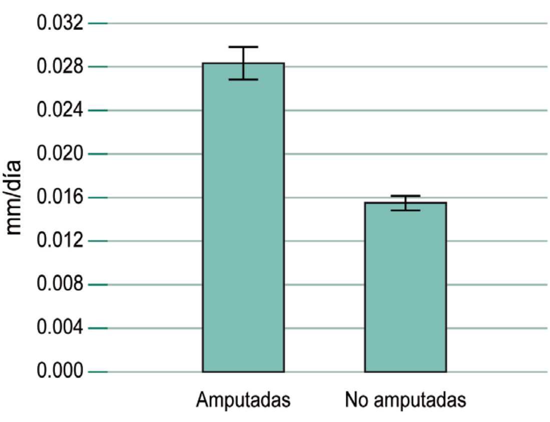

Figure 1: Growth rate (mm/day) in young newts of amputated limbs *vs*. non-amputed limbs.
Figura 1: Velocidad de crecimiento (mm/día) de extremidades amputadas en jóvenes *vs.* no amputadas.

Three months after amputation the blastema and phalanges began to form in the new limb in all newts (Obón *et al*, 2014). The amputated extremities eventually developed into apparently normal limbs: within six years in all the juvenile newts the amputated extremity was as long as the other extremities, while in adults it was 4 mm shorter.

The difference in the speed of growth in juveniles and in the adult was significantly greater in the juveniles (t-test: $t_{(4)}$= 4.90; *P* = 0.012). In the juveniles, the speed of growth was significantly quicker in the amputated than in the non-amputated extremities (Paired Test; $t_{(4)}$= 14.48; $P \leqslant$ 0.0007) (Figure 1).

The results of this study suggest that young Montseny newts can completely regenerate their extremities and do so faster than adults. Some studies indicate that there is a correlation between the degree of development and the regenerative capacity (Galis *et al* 2003; Godwin & Rosenthal 2014). In *Xenopus laevis*, the potential for regenerating extremities is gradually lost as the individual develops and, after a number of amputations, it became clear in all cases that the regenerative capacity diminished as the development of the individual progressed (Muneoka *et al*, 1986). Thus, it is possible that the greater speed of regrowth in the juvenile Montseny newts was due to the fact that they were still developing. We suspect that it may be easier for tissues in embryonic or juvenile newts that are still growing to regenerate than in adults whose cellular regeneration mechanisms have been inactive for months or even years (Poss, 2010).

Recent studies indicate that a localized injury may slow down the metamorphosis of a larva in order to ensure that development continues to be synchronized (Poss, 2010). This could explain why the amputated extremities grew more quickly, thereby allowing the animal to continue full simultaneous development. In addition, the fact that many juvenile amphibian phases suffer much higher predation rates (Turner, 1962) implies that rapid regeneration in juveniles could be a response this predation (Tsasi *et al*, 2009). The loss of an extremity is a severe handicap when facing predation and will dramatically affect an individual's biological efficiency and so those individuals with greater regeneration potential will have an adaptive advantage. This hypothesis has been proposed for reptiles (e.g. Tsasi *et al.*, 2009) where high predation rates may encourage more rapid tail regeneration as a mechanism for overcoming the costs of amputation. This could in turn explain why the functionality of extremities in juveniles needs to be effective as quickly as possible and that the amputated extremity should reach full size as soon as possible. Nevertheless, more experimental work is still required to demonstrate this hypothesis in amphibians. An amputated limb is a serious handicap for the survival of a larva that is still developing. Our results provide new data on the developmental physiology of the juveniles of this little-known species, which seems to develop in a similar way to other species of amphibians.

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