
Article

On the identification of paedomorphic and overwintering larval newts based on cloacal shape: review and guidelines

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Abstract

Paedomorphosis is an alternative process to metamorphosis in which adults retain larval traits at the adult stage. It is frequent in newts and salamanders, where larvae reach sexual maturity without losing their gills. However, in some populations, larvae overwinter in water, while remaining immature. These alternative ontogenetic processes are of particular interest in various research fields, but have different causes and consequences, as only paedomorphosis allows metamorphosis to be bypassed before maturity. It is thus relevant to efficiently identify paedomorphs versus overwintering larvae. In this context, the aim of this article was 3-fold: firstly, to perform a meta-analysis of the identification procedures carried out in the literature; secondly, to determine the effectiveness of body size to make inferences about adulthood by surveying natural newt populations of *Lissotriton helveticus* and *Ichthyosaura alpestris*, and thirdly, to propose easy guidelines for an accurate distinction between large larvae and paedomorphs based on an external sexual trait, which is essential for reproduction—the cloaca. More than half of the studies in the literature do not mention the diagnostic criteria used for determining adulthood. The criteria mentioned were the presence of mature gonads (10%), eggs laid (4%), courtship behavior (10%), and external morphological sexual traits (39%) including the cloaca (24%). Body-size thresholds should not be used as a proxy for paedomorphosis, because overwintering larvae can reach a larger size than paedomorphs within the same populations. In contrast, diagnosis based on cloacal external morphology is recommended, as it can be processed by the rapid visual assessment of all caught specimens, thus providing straightforward data at the individual level for both sexes.

Key words: amphibians, cloacal morphology, facultative paedomorphosis, neoteny, review, secondary sexual traits, sexual dimorphism, sexual maturity.

Many species of newts and salamanders are biphasic and show the successive use of aquatic and terrestrial habitats across life stages (Griffiths 1996; Petranka 1998). This involves the metamorphosis of an aquatic gilled larva into a terrestrial juvenile phenotype. One of the features of this shift is the resorption of the external gills and the closure of gill slits (Ivanović et al. 2011). However, in some populations, a part of the aquatic larvae do not metamorphose and acquire sexual maturity while retaining larval morphology,

including gills (Džukić et al. 1990; Breuil 1992; Whiteman 1994; Denoël et al. 2005). The polymorphism, named facultative paedomorphosis, therefore produces two alternative adult phenotypes: paedomorphs and metamorphs. The term “paedomorphosis” is here used to describe the acquisition of sexual reproduction in larvae, that is, in gilled individuals (Garstang 1922; McKinney and McNamara 1991; Denoël et al. 2005). Although paedomorphosis is rarer than metamorphosis, it is found in a large number of species

and is often locally abundant in the geographic areas where it is expressed. In newts, facultative paedomorphosis is particularly observed in *Ichthyosaura alpestris*, *Lissotriton helveticus*, and *Lissotriton vulgaris* in Europe (Džukić et al. 1990; Andreone and Dore 1991; Denoël 2007; Denoël et al. 2009a) and in *Notophthalmus viridescens* in North America (Wilbur and Collins 1973; Takahashi et al. 2011). It is also present in other families, such as ambystomatid salamanders (Sexton and Bizer 1978; Shaffer and Voss 1996; Whiteman et al. 2012).

However, the persistence of aquatic larvae for longer than usual does not necessarily involve a strictly speaking paedomorphic process (i.e., involving maturity). Indeed, in some populations, larvae do not metamorphose before winter and are then found the year after they hatched from eggs. These larvae are larger than the typical larval cohorts born after winter and coexist with them (Ernst 1952; Grossenbacher 1979; Denoël et al. 2016). They are called overwintering (immature) larvae (Harris 1987) and they can metamorphose during their first year, that is, before the second winter. However, they can also spend more than one winter in water before metamorphosing, or can be present at intermediate developmental stages before the acquisition of sexual maturity in a paedomorphic stage (Sexton and Bizer 1978; Breuil 1992; Wissinger et al. 2010). As they are larger than the usual size at metamorphosis, they can be seen as branchiate juveniles in comparison with the terrestrial juvenile stage that becomes a metamorph at adulthood (Fasola and Canova 1992; Denoël and Joly 2001) and can also reach similar sizes to some paedomorphs, but cannot reproduce.

Both phenomena, that is, the overwintering of larvae and paedomorphosis, are interesting processes in ecology and evolution as well as in conservation, because they represent examples of intra-specific diversity (Emel and Bonett 2011; Denoël and Winandy 2015; Denoël et al. 2016). However, it is essential to distinguish between both states, because they can be produced by various processes and have different implications. Overwintering larvae can be the result of late breeding, second breeding as a maximization on autumnal rains or slow development that prevents metamorphosis before winter (Ernst 1952; Andreone and Dore 1992; Wissinger and Whiteman 1992). In contrast, paedomorphosis involves maturity and can be regarded as a longer-term strategy. Facultative paedomorphosis has indeed been shown to be adaptive in newts and salamanders in allowing either early reproduction or an optimal use of resources and adaptation to local environmental conditions (Semlitsch 1987; Denoël et al. 2005; Denoël and Ficetola 2014). Because maturity is a key life-history trait (Ryan and Semlitsch 1998), the presence of a sexual trait would be expected to be a major diagnostic trait for separating these processes. However, this is often not the case, as body size is also used to describe paedomorphs (see e.g., van Gelder 1973). Therefore, it might be difficult to establish whether studied specimens are larvae (i.e., immature gilled individuals) or paedomorphs (i.e., adult gilled individuals). Unfortunately, no review is currently available to determine what are the diagnostic sexual traits used in the literature, and their respective advantages and drawbacks.

In this context, the aim of this study was 3-fold: firstly, to perform a meta-analysis of the identification procedures carried out in the literature; secondly, to survey natural newt populations to determine the effectiveness of body size to make inferences about adulthood, and thirdly, to propose easy guidelines for an accurate distinction between larvae and paedomorphs based on an external sexual trait, which is essential for reproduction—the cloaca (Sever and Staub 2011).

Material and Methods

Literature analysis

An analysis of the literature on paedomorphosis in the last five decades (1966–2015) was performed to draw conclusions on the presence and absence of criteria used to identify paedomorphs in pond-breeding newts (genera *Ichthyosaura*, *Lissotriton*, *Notophthalmus*, *Ommatotriton*, and *Triturus*; family Salamandridae). A focus was done on this group because facultative paedomorphosis is often reported in species of these genera and because they share similar life-history, morphological and behavioral traits. Reference data (articles in journals and proceedings) were extracted from Scopus, the ISI Web of Science, and the OVID and Google Scholar databases, using combinations of keywords on newts and paedomorphosis (and associated terms), which were then supplemented by searches of the citations in the obtained papers. The meta-analysis was based on 160 references (Supplementary Table S1).

Field data

Newts at either of the two life stages (i.e., overwintering larvae and paedomorphs) were sampled by dip-netting in two natural populations. Because male and female newts are sexually dimorphic, both sexes of paedomorphs were collected. The studied larvae had spent the previous winter in water, as indicated by their large size. Palmate newts (*L. helveticus*) were sampled, in a pond from a traditional agricultural area (La Clastre, Larzac, Hérault, France; 43°50'40"N–3°30'44"E) while alpine newts (*I. alpestris*) were sampled from an alpine lake (Lac de la Cabane, Alpes de Haute Provence, France; 44°24'7"N–6°24'40"E). Sampling occurred during the reproductive period (May 2014) at the first site and after the reproductive period (August 1997 and July 2014) at the second site, to show the persistence of traits in these two periods and their presence in species from different genera. These two sites were taken as representative examples over more than one hundred populations personally studied in Europe (see e.g., Denoël et al. 2009a; Denoël and Ficetola 2014). Newts were photographed (Nikon D610, 60-mm lens) during the 2014 surveys. Special attention was paid to the cloaca, to provide data on a qualitative trait that can be used for pond-breeding newts regardless of the species or population considered. This criterion was used to distinguish paedomorphs from larvae. Gilled individuals (including paedomorphs) were distinguished from metamorphs by the presence of gill slits and external gills. Newts were measured (snout-vent length, i.e., from the tip of the snout to the posterior edge of the cloaca), to determine the largest size of larvae and the smallest size of adults, therefore allowing to verify the validity of a size threshold in the identification of paedomorphs ($n=44$ for *L. helveticus* in May 2014 and $n=168$ for *I. alpestris* in August 1997).

Results

A meta-analysis of the literature

In 46% of the references ($n=74$ out of 160), the criteria for the identification of paedomorphs were indicated, which included the presence of mature gonads (10% out of the 160 references), eggs laid (4%), courtship behavior (10%) and external morphological traits (39%), including the cloaca (24%) (Figure 1). About 26% of papers contained at least one photograph of the branchiate newts in support of the descriptions.

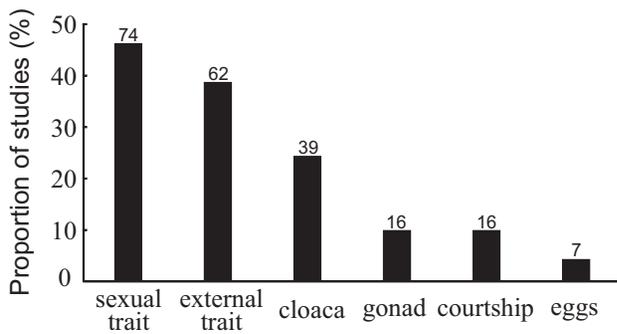


Figure 1. Proportion of studies on newt paedomorphosis (1966–2015) that mentioned sexual traits, morphological external sexual trait, cloacal shape, mature gonads, courtship behavior, and laid eggs to identify paedomorphs (i.e., gilled adults).

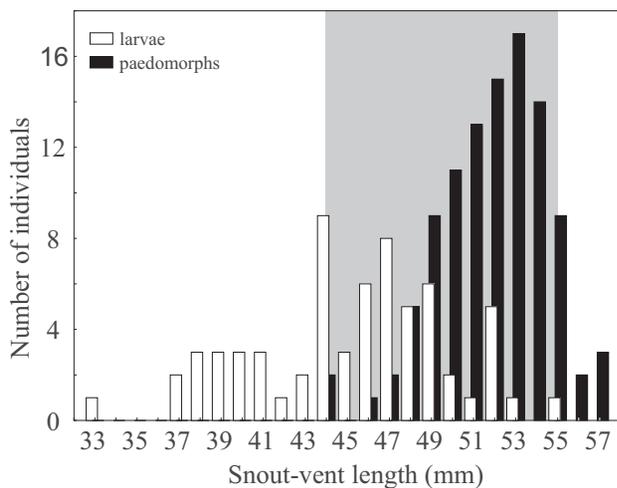


Figure 2. Size structure of overwintering larvae (i.e., gilled immature individuals) and paedomorphs (i.e., gilled adults) in alpine newts *Ichthyosaura alpestris* in La Cabane Lake (France). The gray area represents the size overlap between both life stages.

Differences between paedomorphs and overwintering larvae

In the two studied populations, the largest overwintering larvae were larger than the smallest paedomorphs. In *L. helveticus* from La Clastre, there were few overwintering larvae (i.e., 7%) in the sampling of gilled overwintering individuals. The snout-vent length of the smallest paedomorph was 33 mm versus 37 mm in the largest larva (mean \pm SE = 38.1 \pm 0.4 mm and 34.3 \pm 1.8 mm, respectively). There were 39% size overlap between the two life stages. In *I. alpestris* from Lac de la Cabane, there were numerous overwintering larvae (branchiate juveniles) (i.e., 39%). The smallest paedomorph was 44 mm versus 55 mm for the largest branchiate juvenile (mean \pm SE = 51.8 \pm 0.3 mm and 45.3 \pm 0.6 mm, respectively). There were 86% size overlap between the two life stages (Figure 2).

In both species, paedomorphs and larvae were characterized by a different cloacal shape, as illustrated in both the lateral (Figures 3 and 4) and ventral views (Figure 5). In larvae, including the 1st-year overwintering cohort and the older branchiate juveniles, the cloaca was only a slit, which was slightly swollen in the largest individuals, but remained thin and smooth, that is, much less developed than in adults. In paedomorphic males, the signs of maturity were a

well-swollen cloaca that did not contain grooves on its external surface. The male cloaca had a rather spherical shape, which occasionally possessed some surface relief, and a vent that was more or less open and bordered by papillae. In paedomorphic females, the cloaca always contained some types of grooves on its external surface and was generally relatively small and had varied shapes (elongated, spherical, or heart-shaped). Outside the reproductive period, the cloaca was less developed; however, it usually maintained a larger and less smooth shape, and a more pronounced coloration that differentiated it from that of larvae.

During the reproductive period in May, paedomorphic male palmate newts not only possessed an enlarged cloaca, but also secondary sexual traits, such as a more conspicuous coloration, a tail filament, and partially developed foot-webbing. These traits were absent in larvae. After the reproductive season, paedomorphic male alpine newts had a more conspicuous coloration than that of larvae. In both species, paedomorphic females more resembled larvae than the males, but in addition to the cloacal difference, they often had a more conspicuous coloration. However, it was not always possible to distinguish large overwintering larvae from adults by coloration only. In particular, not all paedomorphs showed the conspicuous adult nuptial dress. As paedomorphs have the typical dorso-caudal crest of the larval stage, this sexually dimorphic trait was not always as obvious as in metamorphs.

Discussion

Although finding large branchiate newts can suggest they are adults, caution is needed, due to individual, population, and species variations in the size at maturity (Miaud et al. 2000; Ficetola et al. 2010; Colleoni et al. 2014). The same large body size can be then associated with reproduction in some populations but not in others (Denoël and Joly 2000; Denoël et al. 2016). As shown here, even within the same population, some branchiate larvae can reach larger sizes than paedomorphs. Although larvae are smaller than paedomorphs in some other populations (see e.g., Denoël and Winandy 2014), the large size overlap between life stages that was found here indicates that using only body size to make inferences concerning paedomorphosis (i.e., sexual maturity while retaining gills) is not a valid approach (see also Semlitsch 1985 for ambystomatid salamanders). The only way to make a confident assessment is to find evidence of primary or secondary sexual traits.

A search of the literature of the last five decades (1966–2015) showed that more than half the published papers did not mention any sexual criteria for the identification of paedomorphs. This does not mean that these studies did not use other traits than size to make inferences about adulthood and it is probable that in many studies, the authors accurately identified paedomorphs and overwintering larvae. However, the absence of diagnostic criteria in the literature leads to doubts, especially for field-based studies in which the paedomorphic process is described for the first time at a new locality and for very few individuals. Furthermore, even in-depth research might contain some biases in the identification of paedomorphs, particularly at the level of individuals. For instance, Gabrion and Sentein (1976) considered paedomorphosis on the basis of the development of sexual traits, and also incorporating a minimum size threshold. Although this means that paedomorphs were clearly present in the studied palmate newt populations, this does not exclude the possibility that some large branchiate individuals might have been counted as paedomorphs and *vice-versa* around the transitional size threshold. Indeed, smaller paedomorphs and larger larvae than



Figure 3. Lateral view of the branchiate life stages of palmate newts *Lissotriton helveticus* during the breeding season (left; Larzac, May) and alpine newts *Ichthyosaura alpestris* after the breeding season (right; Lac de la Cabane, July): overwintering larvae (top), paedomorphic females (center) and paedomorphic males (bottom). Sexual traits (e.g., developed cloaca) are only present in paedomorphs (i.e., gilled adults).

the threshold sizes can be found in the populations studied by Gabrion and Sentein (Denoël M, personal observation). However, as the authors considered sexual traits thoroughly, the examined paedomorphic specimens were probably mature. In contrast, in a long-term field study of paedomorphic palmate newts, van Gelder (1973) could not always distinguish paedomorphs from larvae and used an arbitrary threshold size for classification.

Various sexual traits were listed in the literature to support the adult state of branchiate newts. The presence of functional gonads and mature gametes is clearly proof of reproduction (Kalezić et al. 1996). Such primary traits were used in 10% of the studies. Another effective way was to carry out field or laboratory observations of courtship behavior and sperm transfer (Bovero et al. 1997; Denoël et al. 2001) and to report eggs laid by paedomorphs (Kaya et al. 2008; Baškale et al. 2011; Gvoždík et al. 2013). These methods were performed relatively rarely in the literature, with only 10% of references reporting courtship, and 4%, eggs.

Although determining the presence of traits such as functional gonads, gametes, courtship, or eggs provides insight into the study of paedomorphosis, this cannot be performed for all the individuals of a population, for both ethical and conservation reasons. Knowing the abundance of each morph and their sex, and therefore, their proportions in the populations, is essential in many scientific disciplines (Whiteman 1997; Arntzen 2002; Denoël and Ficetola 2014). Using rapidly assessed traits for large sample sizes and numerous populations is then particularly relevant. Therefore, it is not surprising that 39% of the studies on newt paedomorphosis used external (morphological) sexual traits for diagnosis. This approach was particularly used for species that are characterized by a conspicuous sexual dimorphism (Griffiths 1996; Petranka 1998). For instance, male palmate newts have a long tail filament and webbing between their toes appears at the time of breeding (Halliday 1975; Cornuau et al. 2012). However, these secondary sexual traits are less developed in paedomorphs than in metamorphs (Denoël et al. 2001; Winandy

and Denoël 2015) and the tail filament can occasionally be damaged (Denoël M, personal observation). Color patterns can also be effectively used to distinguish larvae and paedomorphs (Kalezić and Džukić 1985; Ceacero et al. 2010; Denoël M, personal observation). However, large larvae can sometimes have similar color patterns to paedomorphs, particularly females (see also Breuil and Thuot 1983). Males exhibit more conspicuous traits than females, but some paedomorphic individuals lack the nuptial coloration that is typical of metamorphic males (Denoël M, personal observation). The external criterion that is most useful in both sexes is the cloaca; despite this, only 24% of studies reported its use for identifying adulthood in branchiate newts.

The cloaca of newts is an essential organ for reproduction (Sever and Staub 2011). In both sexes, but particularly in males, cloacal glands produce sex pheromones that allow the attraction of mates during courtship (Malacarne and Vellano 1987; Kikuyama et al. 1997; Treer et al. 2013; Janssenswillen et al. 2015; Van Bocxlaer et al. 2015). In males, the cloaca is also involved in the synthesis of spermatophore components (Osikowski and Cierniak-Zuzia 2013) and is displayed to females during sperm transfer (Denoël 2003). In females, the cloaca is important for sperm transfer and storage, as it is more open in receptive females and protracted when females follow the males after spermatophore deposition and then pick up the spermatophore, which allows the sperm to be stored in the spermathecae (Halliday 1974, 1975; Sever 2002; Janssenswillen and Bossuyt 2016). As all these functions are only required in adults, the cloacal glands are not present in larvae (Sever and Staub 2011). The external shape of the cloaca can therefore allow the rapid identification of overwintering larvae versus paedomorphs. The smallest paedomorphs identified as such were mature, because the males displayed courtship behavior, including the deposition of spermatophores and the females laid fertilized eggs that gave viable larvae in laboratory experiments in both palmate and alpine newts (Denoël et al. 2001; Denoël M, unpublished data). Moreover,



Figure 4. Lateral view of the cloaca of the branchiate life stages of palmate newts *Lissotriton helveticus* during the breeding season (left; Larzac, May) and alpine newts *Ichthyosaura alpestris* after the breeding season (right; Lac de la Cabane, July): overwintering larvae (up), paedomorphic females (middle), and paedomorphic males (bottom). Paedomorphs are gilled adults.

a correspondence between the development of gonads and that of the cloaca has been established in newts (Vilter and Vilter 1963). The size of the cloaca is also correlated with seasonal secondary traits such as the foot-web and tail filament in palmate newts (Winandy and Denoël 2015). On another hand, large or overwintering larvae, determined as such by the shape of the cloaca, were never observed to display courtship behavior or lay eggs (Denoël M,

personal observation). The shape of the cloaca is thus a qualitative criterion that should be used to distinguish between paedomorphs and overwintering larvae.

Although body size and proportions can be permanently sexually dimorphic in adult newts (Denoël et al. 2009b; Reinhard and Kupfer 2015), the traits associated with mating (e.g., the tail filament, the dorsal crest, or the tail height) show seasonal patterns of



Figure 5. Ventral view of the cloaca of the branchiate life stages of palmate newts *Lissotriton helveticus* during the breeding season (left; Larzac, May) and alpine newts *Ichthyosaura alpestris* after the breeding season (right; Lac de la Cabane, July): overwintering larvae (top), paedomorphic females (center), and paedomorphic males (bottom). Paedomorphs are gilled adults.

expression and consequently cannot be always assessed all the year round (Griffiths and Mylotte 1988; Andreone and Dore 1992; Secondi et al. 2009; Bloch and Grayson 2010). Courtship behavior and egg-laying are also inherently linked to the reproductive period only (Verrell and McCabe 1988). The same applies to the formation of gametes in gonads (Verrell et al. 1986). However, the breeding period is usually long in newts (often several months) (Gabrion et al.

1977) and external morphological traits can remain visible throughout its duration in both metamorphs and paedomorphs (Griffiths and Mylotte 1988; Winandy and Denoël 2015). Specifically, repeated observations on the same paedomorphs (in both the alpine and palmate newts) across time showed that they maintained external sexual traits during and even beyond the mating period (Winandy and Denoël 2015; Denoël M, unpublished data). Most

research occurs mainly during the breeding period, thus efficiently making use of seasonal external sexual traits. Moreover, even if the cloaca of adults becomes smaller over time (Vilter and Vilter 1963), it retains its shape characteristics in paedomorphs. One exception is particularly for skinny paedomorphs that can show a less differentiated cloaca than healthy newts (Denoël M, unpublished data). In case of doubts, it is recommended to avoid classification of the individuals as paedomorphs.

This study focused on newt species because they share ecological and morphological traits. In addition to the two examples presented here in detail, the qualitative external diagnosis of paedomorphs on the basis of their cloaca can be generalized to other populations and has already been applied in over 100 localities in several European countries (Denoël M, unpublished data; see locations of the main populations in Denoël 2007; Denoël et al. 2009a). This is also valid for other salamandrid genera, such as *Triturus* (Djorović and Kalezić 1996) and *Notophthalmus* (Takahashi et al. 2011). Although the cloaca is more diverse in other families, such as ambystomatids, distinguishing sex based on the cloacal shape has also been successfully performed during the breeding season (Trauth et al. 1994; Whiteman and Semlitsch 2005). Moreover, studies in ambystomatids showed that paedomorphs have a similar cloacal structure to that of metamorphs (Licht and Sever 1991; Trauth et al. 1994).

Only one-quarter of studies provided photographs of the paedomorphs or overwintering larvae, which often clearly showed the adulthood of the specimens examined (Dörr et al. 1985; Covaciu-Marcov et al. 2013; Gvoždík et al. 2013). However, when images of females did not depict the cloaca in detail, it became more difficult to verify the maturity status. Although it was previously difficult to include pictures in many journals due to space limitations, the current development of online archives now makes this possible.

In conclusion, future studies should avoid using only body size as a proxy for paedomorphosis, and should detail the criteria that are used for identifying sexual maturity, and should possibly use a combination of approaches, depending on the objectives and the characteristics of the studied populations. The advantage of using the shape of the cloaca is that adulthood can be rapidly assessed in both sexes at the individual level and throughout the whole year, thus providing accurate data for research.

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SUPPLEMENTARY MATERIAL

Supplementary material can be found at <http://www.oxfordjournals.org/>.

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