

1 **Heterochrony, cannibalism, and the evolution of viviparity in *Salamandra***  
2 ***salamandra***

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4 Running head: Evolution of viviparity in *S. salamandra*

5

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11 **SUMMARY**

12

13         The way in which novelties that lead to macroevolutionary events originate is a  
14 major question in evolutionary biology, and one that can be addressed using the fire  
15 salamander (*Salamandra salamandra*) as a model system. It is exceptional among  
16 amphibians in displaying intraspecific diversity of reproductive strategies. In *S.*  
17 *salamandra*, two distinct modes of reproduction co-occur: the common mode,  
18 ovoviviparity (females giving birth to many small larvae), and a phylogenetically derived  
19 reproductive strategy, viviparity (females producing only a few large, fully metamorphosed  
20 juveniles, which are nourished maternally). We examine the relationship between  
21 heterochronic modifications of the ontogeny and the evolution of the new reproductive  
22 mode in the fire salamander. The *in vitro* development of embryos of ovoviviparous and  
23 viviparous salamanders from fertilization to metamorphosis is compared, highlighting the  
24 key events that distinguish the two modes of reproduction. We identify the heterochronic  
25 events that, together with the intrauterine cannibalistic behavior, characterize the derived  
26 viviparous reproductive strategy. The ways in which evolutionary novelties can arise by  
27 modification of developmental programs can be studied in *Salamandra salamandra*.  
28 Moreover, the variation in reproductive modes and the associated variation of sequences of  
29 development occur in neighboring, conspecific populations. Thus, *S. salamandra* is a  
30 unique biological system in which evolutionary developmental research questions can be  
31 addressed at the level of populations.

## 32 INTRODUCTION

33

34 Amphibians possess complex life cycles; in many, the cycle consists of free-living  
35 aquatic larvae that metamorphose into terrestrial adults (Duellman and Trueb 1986). This  
36 biphasic life cycle involves two, morphologically distinct developmental stages, each of  
37 which occurs in a different environment—i.e., larvae in water and terrestrial adults on land.  
38 The evolutionary potential of amphibians is tied to the biphasic life cycle and the distinct  
39 selective pressures that are brought to bear on the individual in the different ecological  
40 contexts (Wake and Roth 1989, Hanken 1999).

41 The presumably ancestral biphasic life cycle has been modified repeatedly in the  
42 three orders of extant amphibians. Modifications range from species with perennial larvae  
43 (e.g., permanent paedomorphic salamanders, summarized by Duellman and Trueb 1986,  
44 Wiens et al. 2005, Bonett and Chippindale 2006, Safi et al. 2006) to direct developers, in  
45 which the free-living larval stage is absent and adult structures form directly from the  
46 embryos (e.g., *Eleutherodactylus* frogs, many salamanders of the family Plethodontidae,  
47 and some caecilians of the family Caeciliidae [Wake 1982, 1989]). Many variants are found  
48 within this broad reproductive continuum, including the repeated and independent  
49 transitions from oviparity to viviparity in the three amphibian orders (e.g., Wake 1982,  
50 1993, 2004) and the evolution of environmentally driven reproductive strategies (e.g.,  
51 facultative paedomorphic urodeles, Whiteman 1994, Ryan and Semlitsch 2003, Denoël et  
52 al. 2005), as well as the evolution of elaborate mating systems and parental care strategies  
53 (e.g., Duellman and Trueb 1986, Beck 1998, Duellman 2003, Lehtinen and Nussbaum  
54 2003, Nussbaum 2003, Haddad and Prado 2005, Summers et al. 2006).

55 Variations in life-history traits of amphibians often relate to modifications of the  
56 developmental sequences (Hanken 1992, Bruce 2003). Ontogenetic modifications may be  
57 key events in the evolution of larval and adult structures (e.g., Alberch 1989). Many such  
58 modifications are heterochronic—i.e., they involve shifts in the relative timing of  
59 developmental events in a descendant organisms compared to the timing of the same events  
60 in an ancestor (Gould 1977, Alberch et al. 1979, McKinney and McNamara 1991, Zelditch  
61 2001, McNamara and McKinney 2005). As several authors have pointed out (e.g., Fink  
62 1982, Alberch 1995, Smith 2002), in practice almost all studies of heterochrony involve a

63 comparative analysis among phylogenetically related taxa, because information on the  
64 timing of developmental events in ancestors is not available. Heterochrony is considered to  
65 be a linking concept between development and evolution and, as such, it is a paradigm in  
66 the study of morphological evolution (Alberch and Blanco 1996, Poe and Wake 2004). The  
67 importance of heterochronic processes in amphibian evolution has been discussed  
68 extensively (e.g., Wake and Roth 1989, Hanken 1992, 1999 and references therein). These  
69 kinds of ontogenetic modifications are thought to underlie the evolution of structures or  
70 body parts (e.g., limb morphology or pigmentation: Blanco and Alberch 1992, Parichy  
71 2001), the evolution of some modes of reproduction (e.g., facultative paedomorphic  
72 urodeles: Gould 1977, Ryan and Semlitsch 1998, Denoël and Joly 2000, Denoël et al 2005),  
73 and the evolution of complex life cycles (Hanken 1992, 1999, Bruce 2003).

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## 76 ***SALAMANDRA SALAMANDRA*: THE BIOLOGICAL MODEL**

77

78 Intraspecific diversity in reproductive strategies is rare among vertebrates. Examples  
79 of polymorphisms that involve co-occurrence of oviparity and ovoviviparity, or even  
80 viviparity with various degrees of structural modifications, have been documented for  
81 several lizard species (e.g., Shine 1985, Mink and Sites 1996, Andrews 1997, Heulin et al.  
82 1997, Smith and Shine 1997, Qualls and Shine 1998). Facultative paedomorphosis in some  
83 species of urodeles is another example, although in these cases, reproductive bimodality in  
84 populations is environmentally induced (Whiteman 1994, Ryan and Semlitsch 1998,  
85 Denoël and Joly 2000, Denoël et al 2005).

86 Fire salamanders (*Salamandra salamandra*) represent a striking example of biological  
87 polymorphism within a species. Morphological diversification in *Salamandra salamandra*  
88 includes variation in size (13–22 cm in snout–vent length [SVL] for adult females), body  
89 proportions, head shape, and coloration. In addition, the duration of the larval period varies,  
90 extending from a few months to more than one year (Joly 1986, Salvador and García-París  
91 2001). There is also diversity in reproductive strategies. Female *S. salamandra* may be  
92 ovoviviparous, in which case 30–60 larvae are produced at some stage before  
93 metamorphosis is complete, with yolk being their only source of nutrition (–larviparity–

94 [*sensu* Greven 2003]). Alternately, female fire salamanders can be viviparous. In this  
95 phylogenetically derived reproductive mode (Veith et al. 1998, García-París et al. 2003,  
96 Weisrock et al. 2006), 1–15 fully metamorphosed juveniles are born; in addition to yolk,  
97 maternal nutrition is provided (–pueriparity– [*sensu* Greven 2003]; Blackburn 1994, Wake  
98 1989, 1992, 2002, 2004). Viviparity in *S. salamandra* is characterized by the early hatching  
99 of the embryos within the maternal oviducts and by the phenomena of oophagy and  
100 adelphophagy–i.e., intra-oviductal ingestion of eggs or larvae, respectively (Joly 1986,  
101 Wourms 1981, Dopazo and Alberch, 1994, Greven 1998.) Furthermore, the developmental  
102 rate of viviparous embryos is accelerated (Dopazo and Alberch 1994, Dopazo and  
103 Koremblum 2000).

104 Viviparity occurs in populations of the northern Iberian subspecies *Salamandra*  
105 *salamandra bernardezi* and *S. s. fastuosa*. Viviparous populations are not geographically  
106 isolated, but they occur within the continuous range of distribution of the species in the  
107 Iberian Peninsula. Thus, viviparous populations are surrounded by ovoviviparous  
108 populations of salamanders (Joly 1986, Thiesmeier and Haker 1990, Dopazo and Alberch  
109 1994, Alcobendas et al. 1996). A paleogeographic scenario and a population model based  
110 on the phylogenetic and phylogeographic analysis of mitochondrial and nuclear DNA  
111 markers was proposed to explain the evolutionary history of *S. salamandra* in the Iberian  
112 Peninsula (García-París et al. 2003). Intraspecific lineage divergence within *S. salamandra*  
113 is a consequence of vicariant processes in the late Pliocene. Viviparity likely arose in the  
114 isolated populations on the northern slopes of the Cantabrian Mountains (northern Iberian  
115 Peninsula). Posterior range shifts as a result of climate oscillations facilitated secondary  
116 contact among isolated lineages. Large demographic expansions, possibly favored by a  
117 selective advantage of the newly evolved reproductive mode, might have led to the  
118 admixture and homogenization of previously differentiated genomes. These genomic  
119 changes might have resulted in the spread of viviparity, together with other nuclear-  
120 encoded traits (e.g., striped coloration) to the current distribution of the derived  
121 reproductive mode in the northern Iberian Peninsula (García-París et al. 2003).

122 The preliminary data on viviparity in *Salamandra salamandra* and the importance of  
123 developmental heterochronic processes in the evolution of reproductive strategies and life-  
124 history traits in urodeles suggest that the evolution of viviparity in *S. salamandra* might be

125 related to ontogenetic modifications (Dopazo and Alberch 1994). Herein, the ontogenies of  
126 viviparous and ovoviviparous *Salamandra salamandra* are compared. We reared *in vitro*  
127 embryos of *S. salamandra* from fertilization to metamorphosis and identified the key events  
128 that distinguish reproductive modes. Heterochronic changes are associated with the  
129 occurrence of viviparity and ovoviviparity within a single species. Such events can lead to  
130 new adaptive pathways, and are examples of small developmental changes that produce  
131 evolutionary novelties in a lineage—i.e., the process through which microevolutionary  
132 events produce macroevolutionary patterns.

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### 134 **Comparing developmental sequences**

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136 We studied the development of embryos from fertilization to metamorphosis in  
137 eight viviparous *Salamandra* from Oviedo (Asturias, northern Spain, 43° 21' 5'' N 06° 09'  
138 02'' W); this is the type locality of *S. s. bernardezi* and the first population described as  
139 obligatorily viviparous in the species (Thiesmeier and Haker 1990). In addition, we studied  
140 nine ovoviviparous *S. s. bejarae* from Mijares, (Ávila, central Spain 40° 20' 01'' N 05° 11'  
141 21'' W) (Table 1). Females were captured after the mating season (March–May); they were  
142 kept in laboratory at 14–17°C in 12h light:12h dark cycles, and fed earthworms twice a  
143 week. Mating and fertilization of ova are decoupled in the fire salamander. Females store  
144 the sperm in a spermatheca until they complete the vitellogenic cycle and the eggs are  
145 ready to be fertilized (e.g., Joly 1986, Greven and Guex 1994). Fertilization occurs after  
146 ovulation, which can be assessed by a patent change in female body shape. Once ovulation  
147 was confirmed, we sacrificed one viviparous and one ovoviviparous female with an  
148 overdose of anesthesia (Benzocaine). The ova of females in early pregnancy were removed  
149 and cultured in a 10% Holtfreter solution (Armstrong et al. 1989) at 17°C in 9cm diameter,  
150 3.5cm high plastic culture dishes. All the eggs contained in one uterus were cultured  
151 together in one culture dish (2 dishes per female, 1 per uterus). The other females were  
152 sacrificed sequentially, every 3–4 to 10–15 days. The experimental design permits us to  
153 obtain a consistent and reliable sample of overlapping developmental series of embryos  
154 from fertilization to metamorphosis from different females. We followed and photographed  
155 the embryos with a Nikon FX-35WA camera coupled to a Nikon SMZ-10

156 stereomicroscope. We used the development stages of fore- and hind limbs, as well as a  
157 temporal axis, as the reference points to compare the developmental events of both  
158 ontogenetic sequences. Legal collecting permits, issued by the pertinent Spanish Regional  
159 Environmental Agencies, supported the collections. The University of California, Berkeley,  
160 Animal Care and Use Committee (ACUC) approved the experimental procedure described.

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### 163 **ONTOGENETIC SEQUENCES IN OVOVIVIPAROUS AND VIVIPAROUS** 164 **FIRE-SALAMANDERS**

165

166 We obtained fertilized eggs, embryos, or larvae from 14 of the 17 females studied;  
167 one viviparous and two ovoviviparous females did not reproduce. Samples analyzed are  
168 summarized in Table 1. A staging table for the regular developmental sequence in  
169 *Salamandra salamandra* (ovoviviparous) will be given elsewhere (Buckley et al. in prep.).  
170 Here, we highlight the primary differences between the development of viviparous and  
171 ovoviviparous embryos.

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173 Fertilization of eggs in *Salamandra salamandra* is associated with ovulation, which  
174 occurs during the first week of July in both viviparous and ovoviviparous females from the  
175 two populations studied. This pattern is consistent with the reproductive cycles described  
176 for populations occurring in similar habitats, although reproductive patterns may greatly  
177 vary in different environmental conditions (Joly 1986, Joly et al. 1994, Greven and Guex  
178 1994, Greven 2003, D. B. pers. observ.). Ovoviviparous females ovulate between 20 and 60  
179 eggs; upon fertilization, nearly all undergo intrauterine development to hatching. Hatching  
180 in ovoviviparous *S. salamandra* typically occurs during, or just prior to, the release of  
181 larvae into water. In our laboratory conditions, ovoviviparous females gave birth to larvae  
182 after 80–90 days of intrauterine development. The newly released larvae are large (20–35  
183 mm total length), with fully developed gills, limbs, and eyes, and conspicuous ventral and  
184 dorsal tail fins; they feed immediately. Development to this stage depends solely on yolk  
185 provision (lecithotrophy). The length of the larval period varies depending on  
186 environmental conditions (Alcobendas et al. 2004). Viviparous females also ovulate  
numerous eggs, but they develop through metamorphosis entirely within the maternal

187 genital tract, and only a few (1–15) fully metamorphosed terrestrial juveniles are born after  
188 80–90 days of gestation.

189 The following differences were noted in *in vitro* development of viviparous and  
190 ovoviviparous embryos (Fig. 1 and 2).

191 (1) Development is arrested in many eggs (up to 50% in some cases) in all the  
192 viviparous females analyzed (Fig. 1a), whereas in ovoviviparous salamanders, no  
193 developmental arrestment occurs (Fig. 1b). In each arrested egg, a yolk plug obliterated the  
194 blastopore, a phenomenon that may reflect a dysfunctionality of the neurulation process (cf.  
195 arrested developmental syndrome in newts [Sessions et al. 1988]).

196 (2) The degree of differentiation of the cephalic structures and gills is the same in  
197 ovoviviparous and viviparous embryos. However, the embryos are disposed differently on  
198 the yolk masses (Fig. 1c, d); whereas the cephalic and caudal regions are clearly elevated in  
199 the ovoviviparous embryos, the viviparous ones are tightly curled around the yolk. The  
200 latter attain such a degree of differentiation in 3 or 4 days, whereas the ovoviviparous  
201 embryos require 9 days.

202 (3) After 8 or 9 days of development, the cephalic and pharyngeal regions of the  
203 viviparous embryos are enlarged and advanced in development relative to those of  
204 ovoviviparous embryos (Fig. 1e). The cephalic and pharyngeal regions are separated from  
205 the yolk masses, which are now located in the posterior part of the body. The embryos have  
206 three pairs of unramified gills (G), a shallow oral groove (O), well-developed optic  
207 vesicles, two rudimentary balancers (B), and incipient forelimb buds (H). The embryos  
208 have some muscular activity. Melanophores are scattered along the dorsal line. The  
209 pharyngeal region and the anterior part of the digestive tract (D) are differentiated.  
210 Ovoviviparous embryos at the same stage (incipient forelimbs) differ morphologically (Fig.  
211 1f). The cephalic and caudal regions are well elevated from the yolk mass, which is  
212 centered in the ventral area, where the digestive tract will develop. Furthermore, viviparous  
213 embryos hatch precociously at this developmental stage. In contrast, ovoviviparous  
214 embryos hatch only after 80–90 days of development, just prior or during their release into  
215 water.



216 (4) Developmental rates of the viviparous embryos are heterogeneous (Fig. 1g),  
217 whereas ovoviviparous embryos develop in synchrony (Fig. 1h). This pattern is consistently  
218 found in all the ovoviviparous and viviparous females studied.

219 (5) Fore- and hind limbs of viviparous and ovoviviparous have the same degree of  
220 differentiation (Fig. 2. a, b, a', b'), but they differ dramatically in other respects. The  
221 mouths of the viviparous embryos (Fig. 2. a, a') open and the balancers are resorbed earlier  
222 in viviparous than in ovoviviparous embryos (Fig. 2. b, b'). Furthermore, the yolk mass of  
223 the viviparous embryos is drastically reduced and the anterior part of the digestive tract (D)  
224 is developed. Viviparous embryos attain this stage after 12 or 13 days, whereas the  
225 ovoviviparous embryos require approximately 19 days.

226 Viviparous embryos start to feed actively within the maternal oviducts. Although  
227 neither their mouths nor their limbs are fully developed, the embryos are capable of  
228 predation on arrested eggs (oophagy) and other embryos (adelphophagy) (Fig. 2.c, Fig. 3).  
229 The extra yolk fills the precociously differentiated portion of the digestive tract. As a result  
230 of the intrauterine cannibalism, viviparous embryos quickly attain their larval morphology,  
231 while retaining a large amount of yolk in their digestive tracts. The supplementary nutrients  
232 enable the viviparous individuals to reach metamorphosis, then birth, after 90 days of  
233 intrauterine development. In contrast, ovoviviparous embryos consume their yolk masses as  
234 they develop with no extra nutrients obtained through the ingestion of eggs or siblings (Fig.  
235 1d). At about the time yolk is exhausted and larval form is achieved, hatching, birth, or both  
236 take place in the time span in which viviparous forms have reached terrestriality. Larvae are  
237 aquatic for one to several months before they metamorphose and become terrestrial.  
238 Viviparous juveniles are, on average, smaller than the ovoviviparous ones, although the  
239 size of the former falls within the range of variability found in ovoviviparous populations  
240 (Rivera et al. 1999, D. B. pers observ.). Supplementary nutrients are available to viviparous  
241 embryos because the embryos develop more rapidly, but these nutrients do not cause an  
242 increase in size of the metamorphosed juveniles (Kopp and Baur 2000, Alcobendas et al.  
243 2004).

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## **HETEROCHRONIC PATTERNS IN VIVIPAROUS *S. SALAMANDRA***

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248 Heterochronic modifications of ontogenetic trajectories have been associated with  
249 the evolution of life-history traits in amphibians (Emerson 1986, Hanken 1992, 1999, Ryan  
250 and Semlitsch 1998, Denoël et al. 2005). We report an example in which heterochronic  
251 shifts seem to result in the evolution of a new reproductive strategy, viviparity, in the  
252 otherwise ovoviviparous fire salamander, *Salamandra salamandra*. The three main  
253 heterochronic patterns encountered are described below (Fig. 4), following definitions in  
254 Gould (1977), Alberch et al. (1979), and McKinney and McNamara (1991).

255 First, the developmental program of viviparous salamanders is accelerated relative  
256 to that of ovoviviparous conspecifics. Development of viviparous salamanders through  
257 metamorphosis takes 90 days; in the same amount of time, ovoviviparous larvae hatch and  
258 are born into water, with metamorphosis occurring often months later. The acceleration is  
259 possible in part because more food is available owing to intrauterine cannibalistic feeding  
260 (oophagy and adelphophagy). However, the acceleration also occurs during the pre-  
261 hatching, non-feeding lecithotrophic stages of development. Thus, the increased  
262 developmental rate in part must be determined intrinsically.

263 Second, pre-displacement and accelerated development characterizes cephalic and  
264 pharyngeal structures. These structures, especially those involved in feeding, such as the  
265 mouth and the digestive tract (e.g., stomodeum opening, development of the jaw system  
266 and of the stomach), develop earlier in viviparous than in ovoviviparous embryos and the  
267 sequence of developmental events in the two strategies is not parallel. Two heterochronic  
268 patterns (pre-displacement and acceleration) permit precocious feeding by viviparous  
269 embryos. It is noteworthy that the process of tooth development does not follow the same  
270 heterochronic scheme (data not included).

271 Third, hatching is pre-displaced in viviparous embryos (Figs. 1, 3). It is not known  
272 how hatching is mediated in *Salamandra salamandra*; therefore, we cannot determine  
273 exactly what process is pre-displaced in the viviparous ontogenetic sequence.

274 Dopazo and Alberch (1994) hypothesized that the presence of unfertilized or  
275 abortive eggs, a “physiological malfunction”, underlies the evolution of viviparity in *S.*  
276 *salamandra*. The extra nutrients available would have triggered the selection of embryos  
277 that grow faster and are capable of feeding on the available eggs or other siblings. The

278 acceleration of the rate of morphological change or the pre-displacement of the onset of  
279 metamorphosis would explain the heterochronic patterns observed. Dopazo and Korenblum  
280 (2000) formalized this evolutionary scenario; the number of eggs produced and fertilized  
281 per female is a trait under strong selection. However, the co-option of eggs for nutrition  
282 creates a new intrauterine environment in which selection would favor the acceleration of  
283 developmental rates and cannibalism. The exaptive scenario eventually would lead to the  
284 evolution of viviparity in the species.

285         The results obtained in our study support some of the Dopazo and Alberch (1994)  
286 hypotheses. We have shown that developmental rates are accelerated and that hatching is  
287 pre-displaced in viviparous embryos. The onset of metamorphosis, though, is not pre-  
288 displaced. Furthermore, we have demonstrated that the structures related to feeding develop  
289 earlier in viviparous embryos. However, our data do not help us to ascertain whether the  
290 co-option of unfertilized and abortive eggs triggered the evolution of viviparity or whether,  
291 instead, other developmental processes were responsible. One of the main problems in  
292 postulating evolutionary scenarios is that they are *ad hoc* hypotheses that cannot be  
293 falsified (Wake 1992); therefore, they are not useful frameworks for further analyses.  
294 Instead, we think that the study of the evolution of viviparity in *S. salamandra* requires  
295 integration of new approaches and techniques at different levels of biological organization.

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## 298           **AN INTEGRATIVE APPROACH TO THE STUDY OF THE EVOLUTION** 299 **OF VIVIPARITY IN *S. SALAMANDRA***

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301         We have identified the developmental clues that differentiate the ontogenies of  
302 ovoviviparous and viviparous salamander embryos at the morphological level. This work,  
303 together with the previous phylogenetic and phylogeographic studies (García-París et al.  
304 2003), constitutes an essential step towards a comprehensive mechanistic approach to the  
305 analysis of the evolution of viviparity in *Salamandra salamandra* (Autumn et al. 2002).  
306 The next step involves isolating the specific elements, and the cellular, genetic, and  
307 epigenetic developmental mechanisms responsible for the observed pattern (Smith 2003).  
308 In this context, we recently have undertaken the description of the heterochronic patterns at

309 different levels of hierarchical complexity (external morphology, tissue, cellular, or  
310 genetic). This approach will provide us with a robust framework within which to analyze  
311 the developmental mechanisms that underlie the ontogenetic modifications observed (Raff  
312 and Wray 1989).

313         The integrated framework also will permit us to explore the importance of the  
314 ontogenetic modifications in the evolution of viviparous adult salamander morphology and  
315 life-history traits. Little is known about derivatives of the embryonic structures in urodeles  
316 (Hanken 1999, Gross and Hanken 2005). A detailed analysis of the embryological origins  
317 of larval and adult structures will help us understand the developmental mechanisms that  
318 drive the evolution of larval morphology and the relation between larval and adult  
319 structures. We also will be able to determine the extent to which larval structures may limit  
320 or constrain the evolution of adult morphology (Wake 1991, Wake and Roth 1989). The  
321 highly specialized larval structure may limit the range of possible adult structures. Larval  
322 constraints on adult structures have been relaxed in several different ways—e.g., (1) the  
323 cellular lines that give rise to larval and adult structures are compartmentalized early in the  
324 embryo (e.g., Alberch 1987, 1989); (2) larval structures are co-opted for new adult  
325 functions (e.g., Alberch 1987, Hanken 1999); and (3) in the extreme, the free-living larval  
326 stage is eliminated in direct developers. The possible new developmental combinations that  
327 arise in direct developers under this “ontogenetic repatterning” (Roth and Wake 1985,  
328 Wake and Roth 1989) are fundamental to the morphological diversification of plethodontid  
329 salamanders, for example (Wake and Roth 1989, Wake 1991, Hanken 1999). Interestingly,  
330 *S. salamandra*, as do most viviparous amphibians, retains the larval developmental  
331 program, even though the larvae develop within the maternal oviducts. The presence of  
332 larval structures specialized for a free-living aquatic stage is thought to have facilitated the  
333 evolution of the derived reproductive mode by co-opting larval structures for new functions  
334 (Hanken 1999, Wake 2004). We are conducting a detailed survey of the cellular origins of  
335 the larval and adult structures in viviparous and ovoviviparous fire salamanders. We  
336 anticipate that this study will elucidate the relative importance of ontogenetic modifications  
337 related to viviparity to the evolution of adult traits such as coloration patterns (striped  
338 viviparous *vs.* spotted ovoviviparous adults), size (smaller viviparous *vs.* larger

339 ovoviviparous adults), and other morphological characteristics (e.g., rounded snouts in  
340 viviparous vs. pointed snouts in ovoviviparous adults).

341 Viviparity is an uncommon phenomenon in Caudata. It has evolved independently  
342 in only a few species of salamanders, all within Salamandridae (Wake 1993, Greven 1998,  
343 Veith et al. 1998). Sixteen genera and sixty-six species are recognized in the family (e.g.,  
344 Weisrock et al. 2006). Viviparity occurs in the seven species within *Lyciasalamandra*, in  
345 *Salamandra atra*, in *S. lanzai*, and in *S. salamandra* (Özeti 1979, Greven 1977, 1998,  
346 Nascetti et al. 1988, Veith et al. 1998). Also, it has been suggested that viviparity occurs in  
347 *S. algira*, although little is known about the physiological characteristics of live-bearing in  
348 this species (Martínez et al. 1997, Donaire Barroso 2001). Therefore, viviparity has evolved  
349 independently at least four times in 11 species, if we consider the seven *Lyciasalamandra*  
350 taxa formerly referred to subspecies of the genus *Mertensiella* to be valid species (Veith  
351 and Steinfartz 2004). The physiology of viviparity differs in salamandrids. For instance,  
352 gestation in *S. atra* is extended up to 3 or 4 years. Females produce two juveniles. Only one  
353 egg is fertilized in each oviduct and the unfertilized eggs serve as nutrients for the  
354 developing embryos. Furthermore, the mothers also supply the embryos with a nutritious  
355 material secreted in the uterus (e.g., Wake 1993, Greven and Guex 1994, Greven 1998).  
356 The integrated framework used in this work must be extended to the other species within  
357 the family. The developmental and phylogenetic approaches will enable us to differentiate  
358 between homologous and homoplastic patterns and processes among viviparous  
359 salamandrids, and to speculate about the association of these patterns and processes with  
360 the diversity of reproductive modes in Salamandridae. Homoplasies, especially  
361 parallelisms, may be informative about design limitations and developmental constraints  
362 (Wake 1991, Hodin 2000). Furthermore, the homoplastic coevolution of a set of characters  
363 may indicate that this set actually is behaving as a module or unit of evolution (e.g., Galis et  
364 al. 2003, Schlosser 2004). Therefore, the study of homoplastic patterns can inform us about  
365 the developmental processes underlying morphological evolution (Wake 1991). Eventually,  
366 the developmental and phylogenetic approaches should be extended to the rest of the  
367 urodele families, which have adopted different developmental “solutions” such as direct  
368 development or paedomorphosis. This will permit us to sort out how the different  
369 reproductive modes and life history traits have evolved in urodeles.

370

371 **CONCLUSION: *S. SALAMANDRA* AS A PARADIGMATIC ‘NON-MODEL’**  
372 **ORGANISM**

373

374 A fundamental problem in evolutionary theory concerns the relationship between  
375 microevolutionary processes, amenable to empirical testing, and the origin of key  
376 innovations at the macroevolutionary level. In the case presented here, evolutionary  
377 novelties that originate from heterochronic changes during embryonic development lead to  
378 viviparity, thereby facilitating access to resources both in the oviduct and in an otherwise  
379 non-accessible terrestrial ecological niche. Therefore, viviparity is a trait that fulfills the  
380 criteria to be considered a relevant feature of macroevolution. Moreover, the origin of  
381 viviparity as a local, intraspecific variant allows us to study the phenomenon from a strictly  
382 microevolutionary perspective; thus, viviparity is an unlikely and unique empirical system  
383 for the examination of the origin of key innovations. In previous phylogenetic and  
384 phylogeographic studies (García-París et al. 2003), we identified distinct contact zones  
385 where viviparous and ovoviviparous *Salamandra salamandra* meet and mate. These  
386 “reproductive hybrid zones” constitute unusual natural laboratories in which to study the  
387 dynamics of the evolution of the novelty in action. Thus, there is a two-fold interest in *S.*  
388 *salamandra* as a model organism in evolutionary biology. First, *S. salamandra* facilitates  
389 empirical analysis of the way in which evolutionary novelties arise through the  
390 modification of developmental programs (Hanken 1999, Wake 2003). Second, because  
391 variation in reproductive modes and the associated variation in sequences of development  
392 occur in neighboring conspecific populations, *S. salamandra* represents a unique biological  
393 model suitable to endow the evo-devo research program with a populational perspective  
394 (Baguñá and García-Fernández 2003). We present this research program in genetics,  
395 development, and evolutionary biology as case study for an integrative approach to the  
396 study of macroevolutionary processes.

397

398

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632 **Table 1:** Samples analyzed; **Vivip-** and **Ovovivip-**: viviparous and ovoviviparous  
633 females from Oviedo and Mijares, respectively; **Day/Month:** date when females were  
634 dissected; **E:** embryos; **O:** ova (either fertilized or arrested); **L:** larvae. In viviparous  
635 females we designate as “larvae” individuals that have already hatched and are actively  
636 feeding within the maternal oviducts. In ovoviviparous females, “larvae” are the individuals  
637 that have attained the larval morphology and are ready to initiate the free-living aquatic  
638 stage after delivery.

<b>Female</b>	<b>Day/Month</b>	<b>Right Oviduct</b>	<b>Left Oviduct</b>
<b>Vivip-1</b>	15/06	53 <b>O</b>	50 <b>O</b>
<b>Vivip-2</b>	14/07	4 <b>L</b>	4 <b>L</b>
<b>Vivip-3</b>	18/07	8 <b>E</b> + 33 <b>O</b>	3 <b>E</b> + 41 <b>O</b>
<b>Vivip-4</b>	18/07	4 <b>E</b> + 16 <b>O</b>	3 <b>E</b> + 27 <b>O</b>
<b>Vivip-5</b>	18/07	12 <b>L</b>	10 <b>L</b> + 1 <b>O</b>
<b>Vivip-6</b>	31/07	6 <b>L</b> + 30 <b>O</b>	9 <b>L</b> + 31 <b>O</b>
<b>Vivip-7</b>	31/07	No ovulation	No ovulation
<b>Vivip-8</b>	19/08	35 <b>O</b>	3 <b>L</b>
<b>Ovovovip-1</b>	06/05	No ovulation	No ovulation
<b>Ovovovip-2</b>	24/06	No ovulation	No ovulation
<b>Ovovovip-3</b>	12/07	16 <b>O</b>	26 <b>O</b>
<b>Ovovovip-4</b>	13/07	25 <b>O</b>	32 <b>O</b>
<b>Ovovovip-5</b>	18/07	31 <b>O</b>	29 <b>O</b>
<b>Ovovovip-6</b>	18/07	22 <b>E</b>	19 <b>E</b>
<b>Ovovovip-7</b>	31/07	24 <b>E</b> + 1 <b>O</b>	23 <b>E</b> + 1 <b>O</b>
<b>Ovovovip-8</b>	12/11	13 <b>L</b>	21 <b>L</b> + 1 <b>H</b>
<b>Ovovovip-9</b>	12/12	29 larvae delivered	

639 **Figure legends:**

640

641 **Figure 1:** Comparable developmental stages of viviparous and ovoviviparous  
642 salamander embryos. Comparable stages corresponding to the development of the fore- and  
643 hindlimbs are contrasted, highlighting the main differences found during the development  
644 of viviparous (left column) and ovoviviparous (right column) salamanders (see the text for  
645 a more detailed description of the developmental stages). **(a–b):** viviparous **(a)** and  
646 ovoviviparous **(b)** eggs at gastrulation. The presence of numerous abortive eggs is the rule  
647 in viviparous females. Some of them present a yolk plug that obliterates the blastopore **(a)**.  
648 Abortive eggs are barely found in ovoviviparous females **(b)**, the majority of the eggs  
649 following a normal gastrulation process **(B: blastopore)**. **(c–d):** viviparous and  
650 ovoviviparous embryos present the same degree of differentiation of the cephalic structures  
651 (although the head and the tail are already separated from the yolk mass in ovoviviparous  
652 embryos but not in viviparous ones). Viviparous embryos attain such a state in 3–4 days,  
653 while ovoviviparous embryos take around 9 days. The development of viviparous embryos  
654 is thus accelerated during the lecithotrophic phase of the ontogeny. **(e–f):** when the  
655 forelimbs are at the bud stage **(H)**, viviparous and ovoviviparous embryos are  
656 morphologically very different. Yolk has been quickly consumed by the viviparous  
657 embryos that, furthermore, have already hatched. Hatching in ovoviviparous individuals  
658 occurs much later, when they are laid in water to initiate the larval aquatic phase. The  
659 development of oral groove **(O)**, balancers **(B)**, and gills **(G)** is similar in ovoviviparous  
660 and viviparous embryos. The latter, however, present an early-differentiated pharyngeal  
661 region and digestive tract **(D)**. **(g–h):** Viviparous embryos are normally found at very  
662 different developmental stages within the oviducts **(g)**. This heterogeneity in developmental  
663 stages is never found in ovoviviparous females, in which the eggs are all fertilized and  
664 develop synchronously **(h)**. This difference has been consistently found in all the  
665 viviparous and ovoviviparous salamanders studied.

666

667 **Figure 2:** As in Fig 1, the main differences found during the development of  
668 viviparous (left column) and ovoviviparous (right column) salamanders are highlighted. **(a-**  
669 **b):** At this developmental stage [forelimbs enlarged as conical structures **(L)**], the mouth of



670 the viviparous embryos opens. Balancers have been resorbed and the foregut (**D**) is already  
671 differentiated (**a** and **a'**). None of these features occur in ovoviviparous embryos (**b** and **b'**).  
672 The mouth is still closed and the balancers (**B**) are present (**b'**). The yolk mass is still very  
673 prominent and is located where the digestive tract will differentiate later in development.  
674 (**c-d**): Once their mouths are opened, viviparous embryos feed actively on abortive eggs  
675 and sibs. Transition from the lecithotrophic phase to active feeding occurs very early during  
676 the development of viviparous embryos [forelimbs as conical or palm structures (**a'** and  
677 **c'**)]. The ingested nutrients fill the part of the digestive tract that differentiated  
678 precociously. Some examples of this intrauterine cannibalistic behavior are shown in **Fig.**  
679 **3**. Intrauterine cannibalism never occurs in ovoviviparous salamanders. The mouths of  
680 ovoviviparous embryos open when digits are differentiating in the forelimbs (**d** and **d'**); at  
681 this developmental stage, however, ovoviviparous embryos are still within the egg  
682 membranes. Hatching and the switch from the lecithotrophic phase to active feeding in  
683 ovoviviparous embryos occur when they are delivered in water as larvae and they start the  
684 aquatic free-living phase.

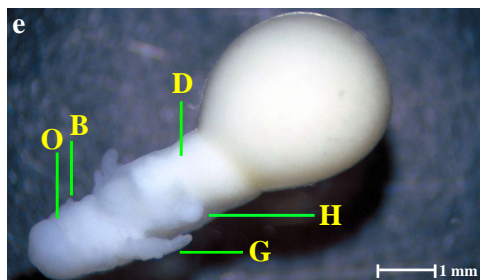
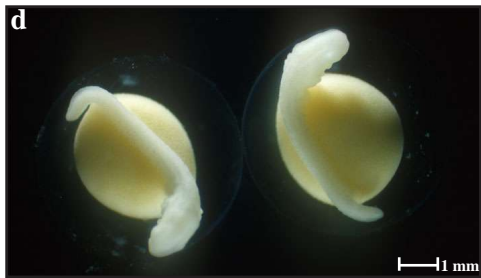
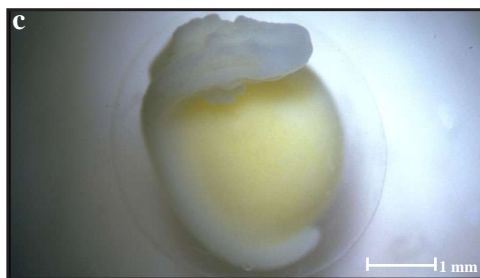
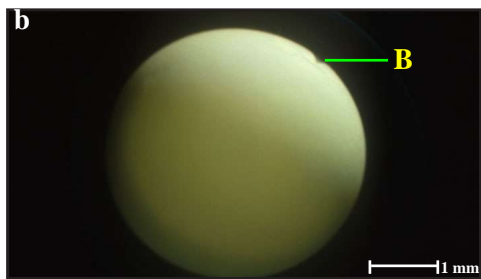
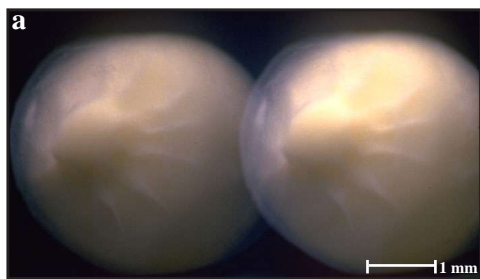
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686 **Figure 3:** Intrauterine cannibalism in viviparous *S. salamandra* embryos. The  
687 precocious hatching and opening of the mouth, together with the early differentiation of the  
688 digestive tract, permit the feeding of the embryos within the maternal genital tract.  
689 Viviparous embryos feed on abortive eggs [oophagy (**a**, **b**)], or on other siblings  
690 [adelphophagy (**c**, **d**)]. The intrauterine cannibalistic behavior never occurs in  
691 ovoviviparous embryos. **a:** Viviparous embryo feeding on an abortive egg. Although  
692 neither the mouth nor the dentition are completely developed, embryos are able to ingest  
693 the big and yolky eggs (3–3.5 mm in diameter). The ingested yolk fills the anterior part of  
694 the digestive tract, which differentiates precociously in viviparous embryos. **b:** Sibs from  
695 the same maternal oviduct. Viviparous embryos are found at different developmental stages  
696 within the maternal genital tract. We observe one non-hatched and one hatched embryo in  
697 similar developmental stages, together with an abortive egg and one embryo in an earlier  
698 development stage. The hatched embryo has two yolk masses within its digestive tract. The  
699 first one (**I**) contains the yolk remaining from the original provision of the egg. The second  
700 one (**II**) corresponds to the ingested yolk that comes from cannibalizing abortive eggs.

701 Within the digestive tract of the non-hatched embryo, we find only the remains of the  
702 original egg provision (**I**). The non-hatching of viviparous embryos within the oviducts is  
703 an uncommon situation. Normally, embryos hatch precociously during development. **c**:  
704 Adelphophagy in viviparous embryos. The arrow points to the tail tip of the cannibalized  
705 embryo, which is filling the anterior part of the digestive tract. **d**: This embryo presents a  
706 large and irregular yolk mass within its digestive tract due, again, to the intrauterine  
707 ingestion of abortive eggs and one sib. Arrows point to the head (**H**) and the tail (**T**) of the  
708 cannibalized sib.

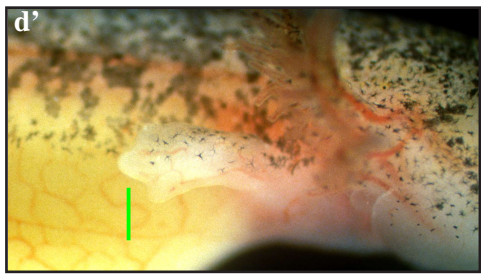
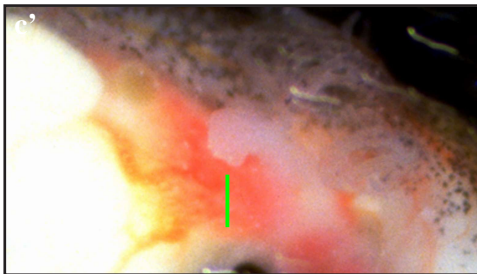
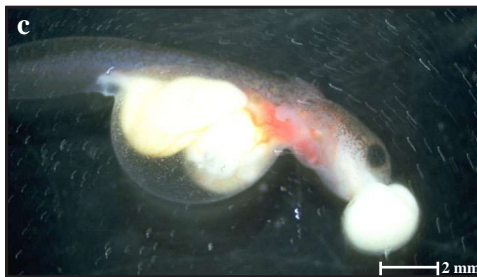
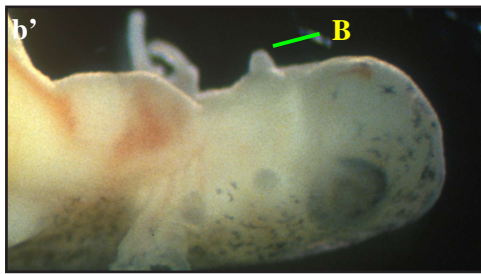
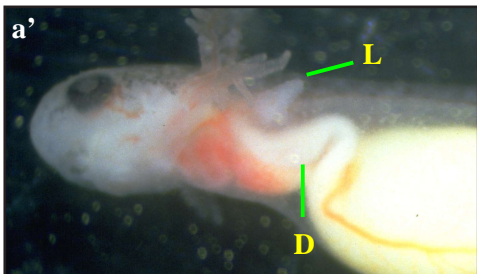
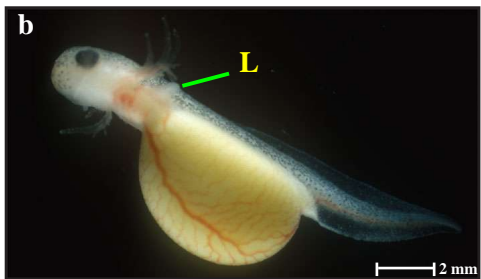
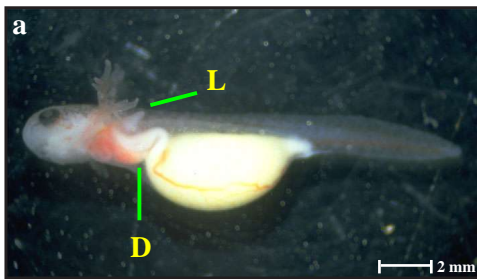
709 **Figure 4:** The “Heterochronic *S. salamandra* Pathway.” As a schematic summary,  
710 the heterochronic patterns revealed during the ontogeny of ovoviviparous and viviparous  
711 embryos are shown on a temporal axis. The axis starts at Stage I/ Day 0 (Gastrulation); key  
712 developmental events are represented on two parallel pathways for comparison.  
713 Developmental stages have been created for this study, based on the development of the  
714 limbs. **Roman Numerals:** comparable developmental stages; **H:** hatching; **F:** end of the  
715 lecithotrophic phase, start of active feeding; **STOP:** metamorphosis. The key features that  
716 characterize the development of the viviparous embryos involve the presence of abortive  
717 and unfertilized eggs (**I**), the acceleration of the developmental sequences, perceptible from  
718 the early stages of development (e.g., stage **II**), the pre-displacement of hatching (**H**, stage  
719 **III**), and the pre-displacement of structures related to feeding (**F**, stage **IV**). As a result, the  
720 developmental phase from fertilization to metamorphosis in viviparous embryos is  
721 drastically shortened compared to that in ovoviviparous individuals. The former  
722 metamorphose and are delivered as terrestrial juveniles after 90 days of intrauterine  
723 development (**VI**). Ovoviviparous individuals start their aquatic larval stage after 90 days  
724 (**V**), hatching and active feeding occurring at this point. They will stay in water from one to  
725 several months before completing metamorphosis (**VI**).

726



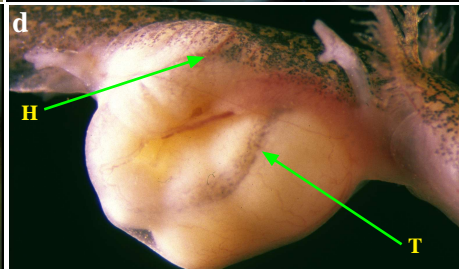
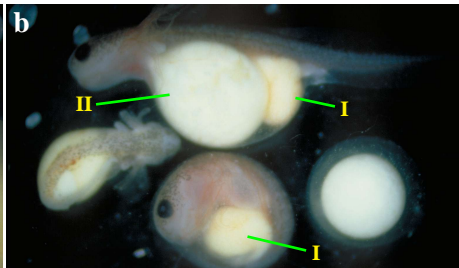
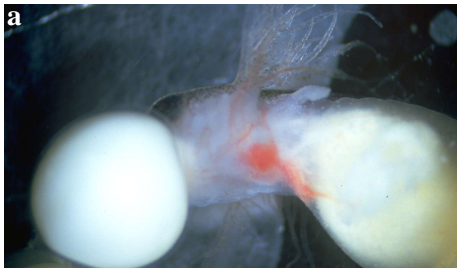
**VIVIPAROUS**

**OVOVIVIPAROUS**



**VIVIPAROUS**

**OVOVIVIPAROUS**



# The Heterochronic *S. salamandra* Pathway

