

1
2
3 **Life history of the Anji salamander (*Chordata*,**
4 **Amphibia, Caudata, Hynobiidae) from fertilized**
5 **eggs to subadults**
6

7 HAIYAN DONG, JIABAO XU, JIAYI HE, XIAOXIAO LUO, JIAXIN YAO, WENJING
8 WANG, MINGLAN DONG, LIPENG YU, YANGYANG HUAN, ZHEDONG LANG,
9 HONGCHANG ZHOU, XIANTING WANG
10

11 **THIS ARTICLE HAS BEEN ACCEPTED FOR PUBLICATION AND UNDERGONE FULL**
12 **PEER REVIEW BUT HAS NOT BEEN THROUGH THE COPYEDITING, TYPESETTING,**
13 **PAGINATION AND PROOFREADING PROCESS, WHICH MAY LEAD TO DIFFERENCES**
14 **BETWEEN THIS VERSION AND THE VERSION OF RECORD.**

15 **PLEASE CITE THIS ARTICLE AS:**
16

17 Dong, H., Xu, J., He, J., Luo, X., Yao, J., Wang, W., Dong, M., Yu, L., Huan, Y., Lang,
18 Z., Zhou, H., Wang, X. (2026): Life history of the Anji salamander (*Chordata*,
19 Amphibia, Caudata, Hynobiidae) from fertilized eggs to subadults. Acta
20 Herpetol. **21**. DOI: 10.36253/a_h-18605
21
22

23 **Life history of the Anji salamander (*Chordata*, *Amphibia*, *Caudata*,**
24 ***Hynobiidae*) from fertilized eggs to subadults**

25 Haiyan Dong^{1,2,3,*}, Jiabao Xu¹, Jiayi He¹, Xiaoxiao Luo¹, Jiaxin Yao¹, Wenjing Wang¹,
26 Minglan Dong³, Lipeng Yu², Yangyang Huan¹, Zhedong Lang², Hongchang Zhou¹,
27 Xianting Wang²

28
29 ¹*Department of Basic Medical Science, Huzhou University, 759 Erhuan East Road,*
30 *Huzhou, Zhejiang 313000, P.R. China*

31 ²*Anji Salamander (*Hynobius amjiensis*) National Nature Reserve Management Center,*
32 *No. 77-83, Building 1, Yongfu Commercial Street, Baofu Town, Anji County, Huzhou,*
33 *Zhejiang 313000, P.R. China*

34 ³*National-local Joint Engineering Laboratory of Aquatic Animal Genetic Breeding and*
35 *Nutrition (Zhejiang), Zhejiang Provincial Key Laboratory of Aquatic Resources*
36 *Conservation and Development, Key Laboratory of Aquatic Animal Genetic Breeding*
37 *and Nutrition of Chinese Academy of Fishery Sciences, Huzhou University, 759 Erhuan*
38 *East Road, Huzhou, Zhejiang 313000, P. R. China*

39 **Corresponding authors. E-mail: 02196@zjhu.edu.cn*

40

41 *Submitted on: 2025, 9th September; revised on: 2026, 1th April; accepted on: 2026,*
42 *10th April.*

43 *Editor: Mattia Falaschi*

44

45

46 **Abstract.** Anji salamander (*Hynobius amjiensis*) is a rare species endemic to China,
47 living in peat moss swamp at an altitude of 1,300 meters on Longwang Mountain in
48 Anji County, Zhejiang Province. Anji salamander larvae live in natural puddles in the
49 swamp, and the adults spend most of their time in the humus under the peat moss, only
50 briefly visiting the puddles during the breeding period to spawn and then disappear back
51 into the swamp. Therefore, the life history of Anji salamander is poorly understood. In
52 this study, we analyzed the developmental characteristics, behaviors and life habits of
53 Anji salamander embryos from fertilized eggs to subadults. Following previous studies
54 on the development of small salamanders and amphibians, we divided Anji salamander
55 development into six stages, including embryonic development, balance bar, limb
56 development, pre-metamorphosis, metamorphosis, and subadult development, with 34
57 developmental nodes. The period before the shedding of the balance bar was a relatively
58 sensitive period. The metamorphosis period, especially the late metamorphosis period,
59 was the most sensitive period from larvae to subadults, during which the small
60 salamanders undergo complex physiological and biochemical changes such as
61 relatively long fasting time, respiratory changes, and transition from aquatic to
62 terrestrial life. These data fill in some of the gaps in the life history of Anji salamander
63 from fertilized eggs to subadults, and are valuable for its conservation and future
64 endeavor on artificial breeding.

65

66 **Keywords.** Anji salamander, *Hynobius amjiensis*, life history, metamorphosis, molt,
67 subadult

INTRODUCTION

68

69 Anji salamander, first discovered in 1991 in Longwang Mountain, Anji County,
70 Zhejiang Province (Gu et al., 1999), and subsequently named *Hynobius amjiensis*,
71 belongs to the class Amphibia, order Caudata, family Hynobiidae, genus *Hynobius*. It
72 is a critically endangered (CR) species in China, one of only two families of Hynobiidae
73 in Zhejiang Province (Yang et al., 2016). Previous studies showed that Anji salamander
74 lives in peat moss swamps at an altitude of about 1300 m (Zhang et al., 2010a), and
75 only enters the swamp puddles to spawn during the breeding season from late
76 November to March each year (Liu et al., 2016). Anji salamander lives in peat moss
77 swamps during the non-breeding season. This cryptic habitat preference contributed to
78 its recent discovery (Zhang et al., 2010a). Anji salamander has strict requirements for
79 the quality of its living environment, and the scarcity of its spawning sites and
80 intraspecific competition have led to a very low survival rate of Anji salamander larvae.
81 Therefore, fewer than 1% of hatchlings typically survive to the subadult stage and
82 successfully emerge onto land (Gu et al., 1999; Dong et al., 2016; Liu et al., 2016). In
83 recent years, with global warming, the habitat suitable for its survival has been
84 decreasing (Pan et al., 2020). We work in the reserve to explore ways to improve the
85 survival rate through artificial breeding of hatchlings into subadults and then release
86 them back to the land (Chen et al., 2018a). During this process, we also carried out a
87 study on the life history of Anji salamander in order to provide valuable reference data
88 for subsequent conservation works.

89 Life history refers to the adaptive evolution of morphological, physiological, and
90 behavioral patterns under long-term natural selection in which animals develop their
91 own unique life responses based on their genetic characteristics as well as
92 environmental pressures (Williams, 1966; Begon et al., 1990; Nettle et al., 2019). Life
93 history traits generally involve biological characteristics such as growth, development,
94 and reproduction, and are specifically described as growth patterns, body size, age at
95 sexual maturity, longevity, and clutch size (Stearns, 1977). Amphibians are more
96 vulnerable to habitat loss and fragmentation than other terrestrial animal groups, due to
97 their specialized amphibious life history, relatively weak terrestrial migration, and high
98 dependence on habitat (Tan et al., 2023). With environmental degradation such as
99 reduced precipitation, diseases, and habitat loss, amphibians are facing tremendous
100 survival challenges. The study of internal and external factors such as amphibian life
101 history, habitat, etc., is of great significance to amphibian conservation efforts (Pottier
102 et al., 2025; Meletiadis et al., 2023).

103 Some studies have shown that the life history of the Hynobiidae is unique
104 compared to other amphibians, Mi et al. (2007) categorized the embryonic development
105 of *Hynobius guabangshanensis* into 21 developmental stages. The developmental
106 period of *Hynobius leechii* from fertilized egg to subadult is divided into 27
107 developmental stages. They suggested that the long period of time after metamorphosis
108 without emerging onto land would cause the death of *Hynobius leechii*, and that
109 *Hynobius leechii* completing metamorphosis may die due to foraging difficulties.
110 Therefore, special protection methods can be used to improve the survival rate of

111 subadults during this developmental period (Ma et al., 1994). The development of
112 *Hynobius maoershanensis* is divided into four stages: hatching stage, forelimb bud
113 stage, hindlimb bud stage, and metamorphosis stage. After the metamorphosis stage,
114 *Hynobius maoershanensis* has a strong crawling ability, but it still cannot survive in a
115 dry environment. This has significant relevance for artificial breeding. Therefore,
116 during metamorphosis, the developmental status should be closely monitored and it
117 should be placed in a safer environment to prevent unwanted mortality due to climbing
118 out of the culture tank (Ning et al., 2021). Data on the Anji salamander development is
119 limited to observational studies on the staging of developmental stages and
120 developmental processes from fertilized egg to embryo (Cao et al., 2024; Qiu et al.,
121 2024).

122 In this study, we illustrated the complete life history of Anji salamander from
123 fertilized eggs to subadults. We also analyzed the developmental characteristics of each
124 developmental stage and the ontogenetic habitat shifts of Anji salamander, advancing
125 our understanding of the physiological behavior of Anji salamander, contributing
126 additional data to conserve *Hynobius* species.

127

128 MATERIALS AND METHODS

129 *Objects of study*

130 In order to reduce the number of Anji salamander larvae that are unable to come
131 ashore due to cannibalism and to increase the larvae survival rate, we selected the last
132 batch of spawned eggs to be raised until the subadults and released in our sanctuary.

133 The reason for this is that most of the larvae hatched from the last batch of spawned
134 eggs in nature become the target of predation by larvae hatched earlier, and very few of
135 them can survive.

136

137 *Embryo incubation and animal breeding*

138 Fertilized eggs were brought into a glass tank (500×300×160 mm), waiting for
139 them to come out of the membrane, and then separated from the live bait and fed after
140 3 days, usually opening on the 3rd-5th day. The water environment is set according to
141 the data detected in the natural puddle in the reference habitat, maintained at 4-10 °C
142 before hatching, and 8-15 °C after hatching out of the membrane (the dissolved oxygen
143 was maintained at 7.700-7.900mg/L, the pH value maintained at 6.9-7.0, the phosphate
144 maintained at 0.045±0.005mg/L, and the zinc maintained at 0.05±0.01mg/L), and the
145 full-spectrum light was maintained for 12 hours each day. A quiet environment was
146 maintained.

147

148 *Staging criteria*

149 In this study, the staging criteria for Anji salamander were based on other species
150 of Hynobiidae, such as the growth and developmental stages of *Hynobius leechii* (Ma
151 et al. 1994), the postembryonic developmental characteristics of *Hynobius*
152 *guabangshanensis* (Mi et al., 2007), and the developmental stages of *Hynobius*
153 *maoershanensis* (Ning et al., 2021). Together with the developmental characteristics of
154 the Anji salamander itself, we divided Anji salamander development from fertilized egg

155 to sub-adult into six stages, namely embryonic development stage, balance bar stage,
156 limb development stage, pre-metamorphosis stage, metamorphosis stage, and subadults,
157 with a total of 34 developmental nodes. The first stage, the embryonic development
158 stage, spans from egg fertilization to hatching. The second stage, the balance bar stage,
159 is marked by the appearance and subsequent disappearance of the balance bar. The third
160 stage, the limb development stage, involves the growth of limbs, including the
161 appearance of the limbs, and the development of fingers and toes. The fourth to six
162 stages are pre-metamorphosis stage, metamorphosis stage, and subadult stage,
163 respectively.

164

165 *Data acquisition*

166 We used a soft ruler to measure the body length from the tip of the head to the tail,
167 and a Hored Portable Water Quality Detector (HD-S15) to measure the water quality
168 indicators. Images were taken using a ZEISS microscope or HUAWEI nova11. Pattern
169 diagrams were drawn based on the pictures of the samples and incorporating the main
170 characteristics of the development in each period using the Honor magicpad2 on the
171 GoPaint 3.1.2 software, Photoshop 2024 was used for photo layout, and Origin 2024
172 for statistical charts.

173

174

174 **RESULTS**

175 *Fertilized egg and embryonic stages of Anji salamander*

176 Adults Anji salamander pair up for spawning in natural puddles in peat moss
177 swamps from November through March each year. Fertilized egg particles (defined as
178 0h, Fig. 1 A1) were found in egg capsules. The fertilized egg then began the cell division
179 phase, a period of increasing cell ploidy, and at 16 ± 3 h(average \pm standard deviation)
180 of embryonic development, as the number of cell divisions increased, more and more
181 cells formed clusters of cells known as multicellular phases (Fig. 1 A2). At 132 ± 4 h
182 of embryonic development, as the embryo continued to develop, cells continued to
183 divide and increase, the blastoderm cells were rolled into the embryo to form the dorsal
184 lip, and the embryo was irregularly round with an uneven surface, known as the
185 protoganglionic embryonic stage (Fig. 1 A3). At 164 ± 4 h of embryonic development,
186 a shallow neural groove appeared in the embryo. The sides of the shallow neural groove
187 were relatively flat, and the edges had not yet fully formed the neural folds, which is
188 called the neural plate stage (Fig. 1 A4). At 176 ± 3 h of embryonic development, the
189 folds on either side of the neural groove continued to develop into irregular folds known
190 as the neural fold stage (Fig. 1 A5). At 200 ± 3 h of embryonic development, the embryo
191 elongated in the direction of the neural plate, the neural folds converged inward, and
192 the sulcus became deeper, referred to as the neural sulcus stage (Fig. 1 A6). At 260 ± 5
193 h of embryonic development, the gradual fusion of the embryonic neural folds and the
194 subsequent formation of the embryonic neural tube occurred. At this time, the embryo
195 formed a primitive head and distinct somites, which is called the neural tube stage (Fig.
196 1 A7).

197 At 315 ± 3 h of embryonic development, the embryo elongated along the neural
198 tube toward both ends, tail buds appeared, and both the primitive head and tail buds
199 curved ventrally, with a more pronounced expansion of the abdomen, referred to as the
200 early tail bud stage (Fig. 1 A8). The embryo's body size increased, and there was a slight
201 depression on both sides of the head. At 355 ± 5 h of embryonic development, the
202 abdominal expansion became markedly smaller, and the tail bud contour was clear,
203 referred to as the late tail bud stage (Fig. 1 A9). At 420 ± 5 h of embryonic development,
204 the embryo was larger and more extended, the larval body was largely formed, and three
205 primitive external gills and a balance bar appeared on both sides of the head, referred
206 to as the early external gill stage (Fig. 1 A10).

207 At 610 ± 3 h of embryonic development, the embryonic head organ development
208 was essentially complete. The eye was basically formed, and smaller gill filaments were
209 present on the three pairs of gills, referred to as the late external gill stage (Fig. 1 A11).
210 At 672 ± 3 h of embryonic development, as the embryo continued to develop, the
211 hatchling's morphology matured, the gill filaments on the three external gills became
212 abundant, the balance rods were clearly visible, forelimb buds were forming, and the
213 hatchling was occasionally seen to move within the egg, referred to as the incubation
214 period (Fig. 1 A12). At 694 ± 48 h of embryonic development, larvae broke through the
215 egg membrane and hatched out, usually with a small amount of yolk remaining. Most
216 lay on their sides at the bottom of the water and are referred to as out-of-membrane
217 larvae (Fig. 2 B1).

218

219 *Anji salamander balance bar stage*

220 We define the day of emergence of hatch as post-hatching day 1. Balance bars
221 began to appear and develop in the early stages of the external gills. Small bar-like
222 tissues appeared on both sides, and the stage from the appearance of the balance bars
223 to their complete shedding is referred to as the balance bar stage. The development of
224 the balance bars on both sides of the head was completed in the embryo 2-3 days before
225 exsanguination (Fig. 1 A10). After the membrane was removed, the balance bars
226 gradually changed from a short, thick state to a long, slender form. This transformation
227 essentially stopped around the 17.5 ± 2.5 day post-hatching, reaching a length of
228 approximately 2.00 ± 0.50 mm, and disappeared by the 19 ± 2 day post-hatching (Fig.
229 3). The balance bars of Anji salamander disappeared in an asymmetric shedding pattern,
230 and the balance bars were shed with either the left or right side of the bars shed first,
231 and the other side shedding within the next 24 h. Moreover, the entire stage in which
232 the balance bar exists is accompanied by the development of the forelimbs, and the
233 hindlimbs began to develop later in the development of the balance bar.

234 On day 5 ± 2 post-hatching, the larvae began feeding, and if there was no external
235 food as an opener, Anji salamander might resort to intraspecific predation. The
236 swallowing process typically involved biting the head or other body parts, with the prey
237 being gradually ingested. The digestive time varies depending on the size of the food,
238 and some of the digestive time in the mouth could reach up to 16 hours or even longer.
239 After hunting for a companion, Anji salamander hide in a quiet corner and slowly
240 enjoyed a meal on its own. However, if it was attacked or otherwise disturbed, it would

241 assess the risk of abandoning the food or taking it and running away. During this stage,
242 Anji salamander was relatively active, preferring dark and quiet water or even hiding
243 in the gaps between rocks or in the mud underwater, occasionally swimming out of the
244 water. Individuals at this stage were small, flexible, and preferred to be alone. They
245 exhibited agitation during balance bar shedding and gradually settled down afterwards.

246

247 *Anji salamander limb developmental stage*

248 The process of limb development, including the 4 fingers on the forelimbs and the
249 5 toes on the hindlimbs, from their emergence to complete development, is referred to
250 as the limb developmental stage of Anji salamander. As early as the embryonic stage,
251 the forelimb bud began to develop (Fig. 1 A12). On day 8.5 ± 1.5 post-hatching, the
252 forelimb bud developed into the forearm (Fig. 2 B2). On day 15.5 ± 1.5 post-hatching,
253 the first forelimb finger began to differentiate, but the longest clear skin was followed
254 by the development of the fourth finger (Fig. 2 B3). On day 18.5 ± 1.5 post-hatching,
255 the second finger of the forelimb began to differentiate (Fig. 2 B4). On day 18 ± 2 post-
256 hatching, the hindlimb bud also began to form (Fig. 2 B4). On day 19.5 ± 1.5 post-
257 hatching, the forelimb's third finger and the hindlimb's forearm began to develop (Fig.
258 2 B5). On day 20.5 ± 1.5 post-hatching, the fourth finger of the forelimb began to
259 differentiate, and capillaries started to appear within the longest transparent skin layer.
260 On day 25.5 ± 1.5 post-hatching, the rudimentary form of the four fingers was formed,
261 but there were still web-like connections between each finger (Fig. 2 B6).

262 On day 30 ± 1 post-hatching, the first toe of the hind limb began to differentiate
263 (Fig. 2 B6). On day 31 ± 1 post-hatching, the second toe of the hind limb began to
264 differentiate (Fig. 2 B7). On day 32 ± 1 post-hatching, the third toe of the hind limb
265 began to differentiate, and at the same time, the skin of the fourth toe had also formed,
266 but it had not yet undergone differentiation from capillaries (Fig. 2 B8). On day 33 ± 1
267 post-hatching, the fourth toe of the hind limb began to differentiate (Fig. 2 B9). On day
268 36.5 ± 1.5 post-hatching, the forelimbs had fully developed, with the fourth toe having
269 formed and there were no web-like connection between the fourth toe and the other toes
270 (Fig. 2 B9). On day 40.5 ± 1.5 post-hatching, the hind limb began to differentiate and
271 the fifth toe emerged (Fig. 2 B10). On day 43.5 ± 1.5 post-hatching, the rudimentary
272 form of the hind limb's five toes was formed, although there were still web connections
273 between the five toes of the hind limb (Fig. 2 B11). On day 51.5 ± 1.5 post-hatching,
274 limb development was completed. At this point, the hind limbs had developed the fifth
275 toe and there was no web connection between the five toes (Fig. 2 B12).

276 On day 34 ± 1 post-hatching, the ventral costal fovea begins to appear (Fig. 4 B9).
277 On day 49 ± 1 post-hatching, the ventral costal fovea gradually became deeper (Fig. 4
278 B10). The four fingers of the forelimbs of Anji salamander developed in the following
279 sequence from top to bottom: 3, 1, 4, 2 (Fig. 2 B12). That is, the finger at the top of the
280 picture developed as the third one, followed by the first, fourth and second fingers in
281 sequence. The sequential development order of the five toes of the hind limbs from top
282 to bottom was 3, 4, 1, 2, 5 (Fig. 2 B12).

283 The behavioral characteristics during this stage were mainly characterized by
284 using live prey as the main food source, with an increase in movement ability. They
285 could swim, or move on the bottom of the water, or reach the surface. The growth rate
286 was relatively fast. During this stage, if the larval Anji salamander lived in groups and
287 touched each other's bodies, they would engage in mutual attacks and biting, especially
288 on the limbs and external gills. By this stage, the limbs, the four pairs of fingers on the
289 front limbs and the five pairs of toes on the hind limbs had fully developed, with the
290 body length reaching 50.0 ± 5.0 mm.

291

292 *Anji salamander pre-metamorphosis stage*

293 The stage from the completion of the development of the limbs and fingers of the
294 Anji salamander to 12 ± 3 days before the metamorphosis stage is referred to as the pre-
295 metamorphosis stage. During this stage, the gill filaments of Anji salamander turned
296 red, the blood vessels became abundant, the body size increased larger and longer, the
297 limbs became strong and powerful, the body became plump, with a body length of 55.0
298 ± 5.0 mm. The dorsal fin gradually disappeared, the ventral costal fovea deepened, and
299 the eyeballs slightly protruded.

300 The behavioral characteristics during this stage are mainly characterized by a
301 significant increase in food intake, frequent movement at the bottom of the water and
302 in the upper water layer, and sometimes extending their heads close to the water surface
303 to exhale bubbles. They prefer to stay in dark corners alone, had less activity during the
304 day, and were relatively more active at night.

305 *Anji salamander metamorphosis stage*

306 The external gills began to retract inward until they completely disappeared. The
307 breathing method changed from gill respiration to using skin and lungs for breathing,
308 and the lifestyle shifted from aquatic to semi-aquatic. This process is what we call the
309 metamorphosis period. During the metamorphic stage, the gill filaments of Anji
310 salamander gradually became thicker and shorter, and the external gills also became
311 shorter (Fig. 5), and before the external gills had completely disappeared, Anji
312 salamander began to undergo its first molting process of life (Fig. 6). Subsequently, the
313 skin on the upper part of the gill cover and the skin on the neck gradually merged and
314 covered the area where the gills had been exposed, forming a neck fold. Eventually, the
315 outer gills completely disappeared. During this stage, the head of Anji salamander
316 became more pointed, its body color darkened, white spots appeared on its body surface.
317 There was a significant protrusion of the eyeball when compared to the larval eyeball,
318 and the ventral costal fovea continued to deepen.

319 The behavioral characteristics of Anji salamander during its metamorphosis stage
320 were that it spent a long time hiding in darker places, was sensitive to light and sound,
321 had a darker body color and more body spots, with enhanced color-changing and
322 concealment abilities. During metamorphosis stage, there was almost no phenomenon
323 of mutual attacks or cannibalism. After the external gills disappeared, Anji salamander
324 began to engage in long-distance crawling, leaving the water puddles and searching for
325 new habitats and food. Its dietary characteristics also underwent significant changes.
326 From the early stage of metamorphosis, its appetite significantly weakened and even

327 led to prolonged fasting. It took approximately 42 ± 3 days of fasting before it could
328 resume normal feeding.

329

330 *Anji salamander subadult stage*

331 We refer the period from the end of metamorphosis to sexual maturity as subadult
332 stage. During this stage, the color of Anji salamander was significantly different from
333 that of the larvae. The color of the subadult is darker and has white dot-like spots
334 compared to the larvae. The head of the larva was sharper, the eyes were more
335 prominent and had fully formed ventral costal fovea (Fig. 5 B12, B18). During the
336 subadult stage, the body length and weight of the salamander gradually increased, and
337 it underwent frequent molting. The aging skin usually fell off from the head to tail,
338 forming a complete "shell" (Fig. 6C). Under the microscope, the shed skin maintained
339 the body's shape (Fig. 6 C, C-1), and the pigment spots on the skin might also be clearly
340 observed (Fig. 6 C-2). The entire exfoliation process took approximately 12 hours.

341 In terms of behavior, Anji salamander at this stage seemed to prefer living in
342 groups, especially shortly after their abnormal development. There was almost no
343 phenomenon of cannibalism. Groups of three or four can be seen gathering together.
344 However, they had territorial awareness. If their territory was invaded by outsiders,
345 there might be biting behavior, mostly targeting the limbs and tail. During this stage,
346 although they could live away from water, they still required a high level of humidity.
347 If they left the water environment and the humidity was insufficient, they might suffer

348 from dehydration, leading to skin wrinkling, tail curling, and in severe cases, it could
349 even endanger their lives.

350

351 *General characteristics of the development from fertilized egg to subadult stage of Anji*
352 *salamander*

353 In aquatic environments at 4 - 15°C, the embryonic development period of Anji
354 salamander lasted for 30 ± 5 days. The higher the temperature, the faster the
355 development. From the fertilized egg to the subadult stage, there were a total of six
356 major developmental stages: embryonic development stage, balance bar stage, limb
357 development stage, pre-metamorphosis stage, metamorphic stage, and subadult stage.
358 The balance bar stage required 18 ± 3 days, the limb development stage 50 ± 5 days,
359 the pre-metamorphosis stage 10 ± 5 days, the metamorphosis stage 10 ± 5 days, and after
360 the metamorphosis is completed, it took 30 ± 5 days to develop into subadult stage (Fig.
361 7).

362 There were 34 developmental nodes, which are Fertilized egg, Multicellular stage,
363 Gastrula stage, Neural plate stage, Neural fold stage, Neural groove stage, Neural tube
364 stage, Early tail bud stage, Late tail bud stage, Early external gill stage, Late external
365 gill stage, Incubation stage, Hatching stage, Forelimb forearm stage, Forelimb first
366 finger stage, Forelimb second finger stage, Hindlimb buds stage, Balance bar falling
367 off stage, Forelimb third finger stage, Forelimb fourth finger stage, Forelimb four -
368 finger initial stage, Hind limb first finger stage, Hind limb second finger stage, Hind
369 limb third finger stage, Hind limb fourth finger stage, Forelimb four-finger mature stage,

370 Hind limb fifth finger stage, Ventral costal fovea appearance stage, Hindlimb five - toe
371 initial stage, Ventral costal fovea deepening stage, Hindlimb five - toe mature stage, Pre
372 metamorphosis stage, Metamorphosis stage, and Subadult stage (Fig. 8). The changes
373 and characteristics of the 34 developmental nodes throughout the entire development
374 process were presented in Table 1 and Table 2.

375

376 DISCUSSION

377 *Embryonic development of Anji salamander*

378 The egg capsule of Anji salamander could develop normally under normal
379 conditions without any infection, except for a few unfertilized eggs. The unfertilized
380 eggs would gradually disintegrate, and the cells eventually died as the eggs became
381 concave. The embryonic development process of Anji salamander is extremely similar
382 to that of other Hynobiidae species, including other vertebrates. For instance, *Hynobius*
383 *leechii*, *Hynobius guabangshanensis*, Hynobiid Salamander, *Onychodactylus japonicus*,
384 and fish all undergo the developmental process of fertilized eggs, cleavage, blastula,
385 gastrula, organogenesis, and finally membrane formation (Iwasawa and Kera, 1980;
386 Iwasawa and Yamashita, 1991; Ma et al., 1994; Mi et al., 2008; Mousavi et al., 2022).
387 The difference is that the forelimbs of Anji salamander were merely a raised bud-like
388 structure before hatching, while those of the Chinese salamander have already
389 developed into a bifurcated shape and have formed the rudimentary form of two fingers
390 before hatching.

391 The development time varies even among different species and within the same
392 species. During the embryonic development stage, the development time we measured
393 is slightly different from two recent reports (Cao et al., 2024; Qiu et al., 2024). This
394 might be due to environmental factors, such as light exposure, water temperature,
395 oxygen saturation, etc., resulting in different developmental speeds. Under natural
396 conditions, the development speed of the eggs laid by Anji salamander varied at
397 different times. Generally, eggs incubated in warmer water developed more rapidly.

398

399 *The balance bar of Anji salamander*

400 In other species of *Hynobius*, the presence of balance bars has been observed
401 during a similar stage (Zhang et al., 1987b; Ma et al., 1994; Mi et al., 2008).
402 Furthermore, Ning et al. also discovered on *Hynobius maoershanensis* that the balance
403 bar will fall off (Ning et al., 2021). In this study, we not only reported the times of the
404 appearance and disappearance of the balance bar but also the fact that the balance bar
405 fell out in an asymmetric manner. This asymmetric detachment might imply that the
406 development of the balance bars on the left and right sides was not synchronous.

407 So far, there have been no relevant studies on the function of the balance bar in
408 salamanders. We hypothesize that the balance bars of Anji salamander are similar to the
409 lateral lines and antennae of other vertebrates, which serve the functions of navigation,
410 detecting and sensing changes in the aquatic environment, intra-species communication
411 to assist in locomotor equilibrium, and predation, etc. Lateral line exists in fish,
412 primarily composed of the superficial and optic nerve mounds (Omura et al., 2014).

413 The functions of the lateral line in fishes include sensing changes in the water flow and
414 pressure (Mogdans, 2019), as well as prey detection, spatial localization, predator
415 avoidance, flocking behavior, intraspecific communication, and station keeping (Klein
416 and Bleckmann, 2015). Catfish have tentacles which are sensitive to changes in water
417 flow and help catfish navigate, sense and hunt in dark environments (Biedenbach, 1971;
418 Muka et al., 2010). Lateral line is also present in axolotls (Northcutt et al., 1994). We
419 found Anji salamander larvae were able to sense food beyond the distance of the
420 balance bar and slowly swim towards the prey, quickly biting it when the bar was about
421 to touch or touching. At the same time, if another salamander leaned into another
422 salamander, they were both able to quickly avoid each other without touching their
423 bodies.

424

425 *Patterns of limb development in the Anji salamander*

426 There are two patterns of vertebrate limb development. One pattern is preaxial
427 dominance, where structures close to the medial side of the body (i.e., radial or thumb
428 side) preferentially form or differentiate during the development of an animal's limbs,
429 such as the radius develops before the ulna in forelimb development, and the 2nd finger
430 develops before the 4th finger (Royle and Young, 2021). The other is postaxial
431 dominance, where structures close to the lateral side of the body (i.e., ulnar or pinky
432 side) preferentially form during the development of an animal's limbs (Shubin, 1986).
433 The limb development of Anji salamander follows a preaxial dominance developmental
434 pattern, similar to that of many other Caudata (Fröbisch et al., 2007). In terms of the

435 morphology of the limbs, except for the *Salamandrella keyserlingii* (Zhang et al., 1993c)
436 and *Batrachuperus pinchonii* (Jia et al., 2018) which have 4 fingers on the forelimbs
437 and 4 toes on the hindlimbs, most species of Hynobiidae have 4 fingers on the forelimbs
438 and 5 toes on the hindlimbs (Ma et al., 1994; Mi et al., 2007; Ning et al., 2021). The
439 preaxial dominance developmental pattern may reflect its long-term environmental
440 adaptation. It may also be closely related to the evolutionary process of 4-toed to 5-toed
441 hindlimbs in the Hynobiidae (Ledbetter et al., 2019; Jia et al., 2022; Urošević et al.,
442 2024). It has been shown that the four fingers of the forelimbs of amphibians with
443 Caudata are adapted to aquatic life, which helps to reduce the resistance when
444 swimming, and the forelimbs also have the role of maintaining the balance and stability
445 of the body, while the five toes of the hindlimbs are adapted to terrestrial life and used
446 to support the body and propulsion. These forelimbs and hindlimbs characteristics are
447 highly compatible with their amphibious lifestyle (Urošević et al., 2024).

448 We observed that the front four limbs of Anji salamander gathered to form a
449 shovel-like grubbing away soil or peat moss, similar to digging a burrow, a process in
450 which the back five fingers grasped the ground to support the body. We hypothesize
451 that the 4-fingered front and back salamanders and the 4-fingered front and 5-fingered
452 back salamanders evolved different numbers of fingers due to differences in their
453 lifestyles, such as some salamanders need to dig burrows, while others do not; some
454 live in static waters, while others inhabit dynamic waters. The corresponding number
455 of fingers allows them to better adapt to the resistance of water currents in their
456 environments. Further data are needed to validate or refute this hypothesis.

457 The limb function of *Hynobius amjiensis* may serve the same purposes of assisted
458 locomotion and maintenance of balance as many vertebrates (Molnar et al., 2021; Smith
459 et al., 2021; Chen et al., 2025b). In the larval stage, when there are individual small
460 salamanders with bitten limbs, their balance and locomotor ability will be reduced, and
461 their swimming speed will be slowed down or even flipped on its side, so the limbs in
462 the larval stage may play an important role in their locomotion and balance. The
463 development of forelimb buds in *Hynobius amjiensis* larvae prior to membrane the
464 emergence (Fig. 1 A12) may be an attempt to adapt as quickly as possible to body the
465 balance in the water after emergence from the membrane. Following the disappearance
466 of the balance bars, both hind limbs developed rapidly (Fig. 3 B4), which allowed the
467 body to continue to have “four symmetrical supports”, which may have been important
468 for the maintenance of the body's locomotor equilibrium. After the development of the
469 four limbs is completed, *Hynobius amjiensis* larvae often crawl underwater, probably
470 to develop the support and propulsion function of the hind limbs, which lays the
471 foundation for the metamorphosis to crawl long distances to the shore in search of food
472 and habitats. It has been suggested that the forelimbs of tetrapods mainly play a braking
473 role and the hindlimbs mainly play a propulsive role (Kawano and Blob, 2013).
474 Furthermore, we observed in the field that *Hynobius amjiensis* may have crawled out
475 of underwater burrows to pair-lay their eggs (Appendix 1). We hypothesized that their
476 more pointed heads and stout limbs may have the ability to excavate the burrows, but
477 we need further studies to confirm our speculations.

478

479 *Molting of Anji salamander*

480 This is the first report of the molting phenomenon in Anji salamander. Molting is
481 a natural process of growth and development in vertebrates, influenced by
482 environmental and hormonal factors (Jorgensen et al., 1960; Heimeier and Shi, 2010).
483 Extensive studies have shown that the molting process of amphibians is regulated by
484 thyroid hormones (Dowling et al., 1966; Tata, 2006). It has been found that the level of
485 thyroid hormone secretion in amphibians increases during metamorphosis (Denver,
486 2021; Tanizaki et al., 2021). The first molt occurred during the metamorphosis stage,
487 and it is possible that hormones were also involved in the development of molt and
488 metamorphosis in Anji salamander.

489 Amphibian molting behavior is necessary for their metamorphosis. The main
490 component of molting is the cuticle of the skin surface, and the presence of molting
491 indicates some keratinization of the skin (Heimeier and Shi, 2010; Akat et al., 2023).
492 The skin of amphibians serves as an important organ for their respiration and gas
493 exchange (Tattersall, 2007), and molting might regulate respiration and reduce water
494 loss to some extent (Torri et al., 2020). It has also been implied that amphibians in the
495 growth phase molt as a result of their body growth becoming increasingly tighter with
496 their cuticle, and the old cuticle skin needs to be shed to accommodate growth (Lu,
497 2018). Molting can also defend against pathogenic microorganisms on the old skin,
498 removing pathogenic microorganisms that have accumulated on the old skin over time
499 (Ohmer et al., 2017). In this study, we found that Anji salamander molted more
500 frequently after completing metamorphosis, so the molt of Anji salamander might be

501 more closely related to their growth, development, immunity and other physiological
502 processes.

503

504 *Risks faced during metamorphosis and development of Anji salamander*

505 Amphibian metamorphosis enables amphibians to cross the formidable ecological
506 barrier between aquatic and terrestrial environments by coordinating morphological
507 remodeling with physiological functional transitions, a process critically important for
508 their subsequent survival. Studies have indicated that during amphibian metamorphosis,
509 the degeneration of external gills may exhibit temporal mismatch with pulmonary
510 developmental processes. Furthermore, the gradual functional decline in gill respiratory
511 capacity throughout metamorphic progression can potentially lead to hypoxic stress and
512 associated mortality during gill resorption (Burggren, 1982; 1994). Amphibians use
513 their skin to assist respiration before and after gill shedding. However, after
514 metamorphosis, the use of skin for respiration needs to be carried out at a more suitable
515 humidity; otherwise, dry environments may cause respiratory distress, dehydration, and
516 disruption of skin microflora, increasing the risk of infection with pathogenic
517 microorganisms (Akat et al., 2023; Buttner et al., 2024; Riddell et al., 2024).
518 Amphibians can experience long period of cessation of feeding during metamorphosis
519 due to multiple reasons, including hypothalamic regulation and reorganization of the
520 digestive system (Kuzmin, 1997; Bender et al., 2018; Zhu et al., 2020).

521 Anji salamanders spend the vast majority of their metamorphosis to subadult in a
522 state of relative feeding cessation, and have to face threats such as hypoxia from

523 respiratory shifts after emerging onto land, significant energy expenditure from
524 crawling long distances, and elevated habitat temperatures, and even failing to survive
525 once they mistakenly enter a dry area with water-starved and wrinkled skin and frizzled
526 tails for 12-24 hours. Studies have also been shown that some small salamanders
527 maintain a larval form and are unable to complete metamorphosis (Okamiya et al.,
528 2021). Additionally, skin fusion of the head and neck following gill internalization
529 might greatly burden the immune and endocrine systems. Pathogen invasion at this
530 stage could pose a significant survival threat. Therefore, we believe that the
531 metamorphosis stage is a high-risk stage from fertilized eggs to subadults in Anji
532 salamander.

533

534

CONCLUSIONS

535 In this study, we delineated the six main developmental processes of Anji
536 salamander (Fig. 8) encompassing the whole developmental process, including 34
537 developmental nodes (Tables 1 and 2), and described the life habits of different
538 developmental periods, filling gaps in the life history of Anji salamander. It is worth
539 noting that Anji salamander is particularly vulnerable during the balance bar shedding
540 stage and throughout metamorphosis, which is informative for conservation. For
541 example, we in the reserve may consider the need to keep the habitat as free from
542 external disturbance as possible before and after the shedding of the balance bar and
543 during the process of metamorphosis. In addition, the subadult stage of Anji salamander
544 has a relatively high dependence on air humidity and water. Therefore, if there is an

545 extreme drought before or after the Anji salamander comes ashore, we can take the
546 necessary intervention to ensure adequate water supply in the reserve. In conclusion,
547 the knowledge of the life history of Anji salamander from larvae to subadults is useful
548 for its conservation and future captive breeding.

549

550

ACKNOWLEDGMENTS

551 We would like to express our gratitude to Anji Huangpu Jiangyuan Tourism
552 Development Co., Ltd. for their financial support to this public welfare research. We
553 thank the Animal Welfare Ethical Committee and Animal Experimental Ethics
554 Committee of the China Huzhou University Research Institute for approving our work.
555

REFERENCES

556

557 Akat Çömden, E., Yenmiş, M., Çakır, B. (2023): The Complex Bridge between Aquatic
558 and Terrestrial Life: Skin Changes during Development of Amphibians. *J. Dev.*
559 *Biol.* **1**: 6.

560 Biedenbach, M. A. (1971): Functional properties of barbel mechanoreceptors in
561 catfish. *Brain Res.* **27**: 360-364.

562 Burggren, W. W., West, N. H. (1982): Changing respiratory importance of gills, lungs
563 and skin during metamorphosis in the bullfrog *Rana catesbeiana*. *Respir. Physiol.*
564 **47**: 151-164.

565 Begon, M., Harper, J. L., Townsend, C. R. (1986): *Ecology: individuals, populations*
566 *and communities*, 130-330 pp. Wiley-Blackwell, Hoboken, New Jersey, USA.

567 Burggren, W. W., Infantino, R. L. (1994): The respiratory transition from water to air
568 breathing during amphibian metamorphosis. *Integr. Comp. Biol.* **34**: 238-246.

569 Bender, M. C., Hu, C., Pelletier, C., Denver, R. J. (2018): To eat or not to eat: ontogeny
570 of hypothalamic feeding controls and a role for leptin in modulating life-history
571 transition in amphibian tadpoles. *Proc. R. Soc. B Biol. Sci.* **285**: 20172784.

572 Buttmer, S., Moura-Campos, D., Greenspan, S. E., Neely, W. J., Ferrante, L., Toledo,
573 L. F., Becker, C. G. (2024): Skin microbiome disturbance linked to drought-
574 associated amphibian disease. *Ecol. Lett.* **27**: e14372.

575 Chen, C. S., Yang, J., Yu, L. P., Guo, R. (2018): *Reproductive Biology of the Critically*
576 *Endangered Anji Salamander (*Hynobius amjiensis*)*, 59 pp. Proceedings of the
577 Thirteenth Membership Congress and Academic Symposium of the Zoological

578 Society of Zhejiang Province, China, November 2018. Zhejiang Nature Museum,
579 Longwangshan Nature Reserve Administration, Qingliangfeng National Nature
580 Reserve Administration, Eds., China.

581 Cao, Z., Guo, R., Fang, Z., Wang, Z., Liu, Y., Lin, L., Ji, X. (2024): Normal table of
582 embryonic development in the Anji salamander *Hynobius amjiensis*
583 (*Hynobiidae*). *Dev. Biol.* **511**: 84-91.

584 Chen, C. S., Jia, J., Wang, X. T., Yang, J., Gao, K. Q. (2025): Anatomy of the Critically-
585 Endangered Anji Salamander (*Hynobius amjiensis*) Provides New Insights Into
586 Morphological Evolution of Salamanders. *J. Morphol.* **286**: e70028.

587 Dowling, J. T., Razevska, D. (1966): Thyroxine metabolism by amphibian skin during
588 metamorphosis and molting. *Gen. Comp. Endocrinol.* **6**: 162-169.

589 Dong, Y., Yao, Z. Y., L P P, Gao E H (2016): Conservation of the small salamander
590 (*Hynobius amjiensis*) in Anji. *Planting Raising*, (8), 218-219.

591 Denver, R. J. (2021): Stress hormones mediate developmental plasticity in vertebrates
592 with complex life cycles. *Neurobiol. Stress.* **14**: 100301.

593 Fröbisch, N. B., Carroll, R. L., Schoch, R. R. (2007): Limb ossification in the Paleozoic
594 branchiosaurid *Apaton* (Temnospondyli) and the early evolution of preaxial
595 dominance in tetrapod limb development. *Evol. Dev.* **9**: 69-75.

596 Fröbisch, N. B., Bickelmann, C., Olori, J. C., Witzmann, F. (2015): Deep-time evolution
597 of regeneration and preaxial polarity in tetrapod limb development. *Nature.* **527**:
598 231-234.

599 Gu, H. Q., Ma, X. M., Wang, J., Du, Z. H., Lou, X. Q. (1999): Population size and
600 population dynamics of the small Anji salamander (*Hynobius amjiensis*). Sichuan
601 Zool. **18**:104-106.

602 Heimeier, R. A., Shi Y B (2010): Amphibian metamorphosis as a model for studying
603 endocrine disruption on vertebrate development: effect of bisphenol A on thyroid
604 hormone action. Gen. Comp. Endocrinol. **168**: 181-189.

605 Iwasawa, H., Kera, Y. (1980): Normal Stages of Development of the Japanese Lungless
606 Salamander, *Onychodactylus japonicus* (Houttuyn). Jpn. J. Herpetol. **8**: 73-89.

607 Iwasawa, H., Yamashita, K. (1991): Normal Stages of Development of a Hynobiid
608 Salamander, *Hynobius nigrescens* Stejneger. Jpn. J. Herpetol. **14**: 39-62.

609 Jorgensen, C., Larsen, L. (1960): Hormonal control of moulting in
610 amphibians. Nature **185**: 244-245.

611 Jia, J., Zhang, M. H., Gao, K. Q., Jiang, J. P. (2018): Dataset of μ CT scan of the holotype
612 specimen of *Batrachuperus londongensis* Liu and Tian, 1978, an endemic
613 hynobiid (Amphibia, Urodela) from Mount Emei, Sichuan Province, China. Chin.
614 Sci. Data. **3**: 79-93.

615 Jia, J., Anderson, J. S., Jiang, J. P., Wu, W., Shubin, N. H., Gao, K. Q. (2022):
616 Ossification patterns of the carpus and tarsus in salamanders and impacts of
617 preaxial dominance on the fin-to-limb transition. Sci. Adv. **8**: eabq7669.

618 Kuzmin, S. L. (1997): Feeding of amphibians during metamorphosis. Amphib.-
619 Reptil. **18**: 121–131.

620 Kawano, S. M., Blob, R. W. (2013): Propulsive forces of mudskipper fins and

621 salamander limbs during terrestrial locomotion: implications for the invasion of
622 land. *Integr. Comp. Biol.* **53**: 283-294.

623 Klein, A., Bleckmann, H. (2015): Function of lateral line canal morphology. *Integr.*
624 *Zool.* **10**: 111-121.

625 Liu, B. Q. (2016): A new distribution site of the rare and endangered amphibian,
626 *Hynobius amjiensis*, discovered in our province. *Zhejiang For. Sci. Tech.* **36**: 28-
627 28.

628 Lu, X. B., Jiang, Q., Min, Y., Jiang, X., Yang, Z. P., Sun, L. S., Zhang, X. J. (2018):
629 Delineation of the molt cycle and the effect of molt frequency on growth of
630 *Rhodophorus rosenbergii*. *Freshw. Fish.* **48**: 88-93.

631 Ledbetter, N. M., Bonett, R. M. (2019): Terrestriality constrains salamander limb
632 diversification: Implications for the evolution of pentadactyly. *J. Evol. Biol.* **32**:
633 642-652.

634 Luedtke, J. A., Chanson, J., Neam, K., Hobin, L., Maciel, A. O., Catenazzi, A., Borzée,
635 A., Hamidy, A., Aowphol, A., Jean, A., Sosa-Bartuano, Á., Fong, G. A., de Silva,
636 A., Fouquet, A., Angulo, A., Kidov, A. A., Muñoz Saravia, A., Diesmos, A. C.,
637 Tominaga, A., Shrestha, B., Stuart, S. N. (2023): Ongoing declines for the world's
638 amphibians in the face of emerging threats. *Nature.* **622**: 308-314.

639 Ma, L. D., Ma, D. K., Gao, A. (1994): Early development of the small salamander
640 (*Hynobius amjiensis*) from Northeastern China. *J. Zool.* **232**: 46-51.

641 Mi, X. Q., Deng, X. J., Guo, K. Q., Niu, Y. D., Zhou, Y. (2007): Early Embryonic
642 Development of *Hynobius guabangshanensis*. *Sichuan Zool.* **26**: 377-378+239.

643 Mukai, Y., Tuzan, A.D., Lim, L. S., Yahaya, S. H. (2010): Feeding behavior under dark
644 conditions in larvae of sutchi catfish *Pangasianodon hypophthalmus*. *Fish. Sci.* **76**:
645 457-461

646 Mogdans, J. (2019): Sensory ecology of the fish lateral-line system: Morphological and
647 physiological adaptations for the perception of hydrodynamic stimuli. *J. Fish*
648 *Biol.* **95**: 53-72.

649 Molnar, J. L., Hutchinson, J. R., Diogo, R., Clack, J. A., Pierce, S. E. (2021) Evolution
650 of forelimb musculoskeletal function across the fish-to-tetrapod transition. *Sci.*
651 *Adv.* **7**: eabd7457.

652 Mousavi, S. E., Patil, J. G. (2022): Stages of embryonic development in the live-bearing
653 fish, *Gambusia holbrooki*. *Dev. Dyn.* **251**: 287-320.

654 Meletiadis, A., Di Nicola, M. R., Bovero, S., Favelli, M., Pezzolato, M., Grella, S.,
655 Rezza, G., Acutis, P. L. (2025): Prevalence of *Batrachochytrium dendrobatidis* in
656 Amphibians in Northwestern Italy's Protected Areas. *Animals.* **15**: 157.

657 Northcutt, R. G., Catania, K. C., Criley, B. B. (1994): Development of lateral line
658 organs in the axolotl. *J. Comp. Neurol.* **340**: 480-514.

659 Ning, M. H., Chi, H., Chen, Y., Yang, B., Wu, Z. J., Huang, H. Y. (2011): A Preliminary
660 Study on the Ontogenetic Characteristics of Captive-Bred *Hynobius*
661 *maoershanensis*. *Sichuan Zool.* **40**: 196-202.

662 Nettle, D., Frankenhuis, W. E. (2019): The evolution of life-history theory: a
663 bibliometric analysis of an interdisciplinary research area. *Proc. R. Soc. B Biol.*
664 *Sci.* **286**: 20190040.

665 Omura, A., Anzai, W., Endo, H. (2014): Functional and morphological variety in trunk
666 muscles of Urodela. *J. Vet. Med. Sci.* **76**: 159-167.

667 Ohmer, M. E. B., Cramp, R. L., Russo, C. J. M., White, C. R., Franklin, C. E. (2017):
668 Skin sloughing in susceptible and resistant amphibians regulates infection with a
669 fungal pathogen. *Sci. Rep.* **7**: 3529.

670 Okamiya, H., Sugime, R., Furusawa, C., Inoue, Y., Kishida, O. (2021): Paedomorphosis
671 in the Ezo salamander (*Hynobius retardatus*) rediscovered after almost 90
672 years. *Zool. Lett.* **7**: 14.

673 Pan, D. S., Pang, Y. W., Zhou, Y. Y., Huang, Y. X., Xu, J. F. (2020): Effects of Climate
674 Changes on the Habitat of *Hynobius amjiensis* Based on Meteorological and
675 Remote Sensing Data. *J. Hangzhou Norm. Univ. Nat. Sci. Ed.* **19**: 323-328.

676 Pottier, P., Kearney, M. R., Wu, N. C., Gunderson, A. R., Rej, J. E., Rivera-Villanueva,
677 A. N., Pollo, P., Burke, S., Drobniak, S. M., Nakagawa, S. (2025): Vulnerability
678 of amphibians to global warming. *Nature.* **639**: 954-961.

679 Qiu, Y., Chen, K., Mei, Y., Yang, J., Chen, C. (2024): Pre-Embryonic Period
680 Observation Shows a Unique Reproductive Strategy of the Critically Endangered
681 Anji Salamander (*Hynobius amjiensis*). *Animals.* **14**: 3007.

682 Royle, S. R., Young, J. J. (2021): Developmental biology: A 5'Hoxd-Gli3 balance
683 in tetrapod axial polarity. *Curr. Biol.* **31**: R1487-R1490.

684 Riddell, E. A., Burger, I. J., Muñoz, M. M., Weaver, S. J., Womack, M. C. (2024):
685 Amphibians Exhibit Extremely High Hydric Costs of Respiration. *Integr. Comp. Biol.*
686 **64**: 366-376.

687 Stearns, S. C. (1977): The Evolution of Life History Traits: A Critique of the Theory
688 and a Review of the Data. *Annu. Rev. Ecol. Syst.* **8**: 145-171.

689 Shubin, N. H., Alberch, P. (1986): A Morphogenetic Approach to the Origin and Basic
690 Organization of the Tetrapod Limb. In: *Evolutionary Biology*, 319-387 pp. Hecht,
691 M.K., Wallace, B., Prance, G.T., Eds., Springer, Boston, MA.

692 Smith, S. K., Hilliard Young, V. K. (2021): Balancing on a Limb: Effects of Gravity
693 on Locomotion in Arboreal, Limbed Vertebrates. *Integr. Comp. Biol.* **61**: 573-578.

694 Tata, J. R. (2006): Amphibian metamorphosis as a model for the developmental actions
695 of thyroid hormone. *Molecular and cellular endocrinology*, **246**: 10-20.

696 Tattersall, G. J. (2007): Skin Breathing in Amphibians. In: *Endothelial Biomedicine*,
697 85-91 pp. Aird, W. C., Ed, Cambridge: Cambridge University Press, United
698 Kingdom

699 Torri, C., Falini, G., Montroni, D., Fermani, S., Teta, R., Mangoni, A., Alibardi, L.
700 (2020): Cholesterol derivatives make large part of the lipids from epidermal molts
701 of the desert-adapted Gila monster lizard (*Heloderma suspectum*). *Sci. Rep.*, **10**:
702 17197.

703 Tanizaki, Y., Shibata, Y., Zhang, H., Shi, Y. B. (2021): Analysis of Thyroid Hormone
704 Receptor α -Knockout Tadpoles Reveals That the Activation of Cell Cycle Program
705 Is Involved in Thyroid Hormone-Induced Larval Epithelial Cell Death and Adult
706 Intestinal Stem Cell Development During *Xenopus*
707 *tropicalis* Metamorphosis. *Thyroid*. **31**: 128-142.

708 Tan, W. C., Herrel, A., Rödder, D. (2023): A global analysis of habitat fragmentation

709 research in reptiles and amphibians: what have we done so far?. *Biodivers.*
710 *Conserv.* **32**: 439-468

711 Urošević, A., Budečević, S., Ljubisavljević, K., Tomašević Kolarov, N., Ajduković, M.
712 (2024): Morphological variation, modularity and integration in the scapula and
713 humerus of *Lissotriton* newts. *J. Anat.* **245**: 97-108.

714 Williams, G. C. (1966): Natural Selection, the Costs of Reproduction, and a Refinement
715 of Lack's Principle. *Am. Nat.* **100**: 687-690.

716 Yang, J., Chen, C. S., Chen, S. H., Ding, P., Fan, Z. Y., Lu, Y. W., Yu, L. P., Lin, H.
717 (2016): Population genetic structure of critically endangered salamander in China:
718 recommendations for conservation. *Genet. Mol. Res.* **15**.

719 Zhang, L. S., Tang, G. Q. (1987): Some biological data on the Chinese small salamander
720 (*Hynobius chinensis*). *Chin. J. Zool.* **22**: 23-25.

721 Zhang, K. Q., Zhang, Z. H., Chen, Y. M. (1993): Research on the Reproductive Habits
722 of the Northern Salamander. *Chin. J. Zool.* **28**:9-12.

723 Zhang, K., Jiang, W. D. (2010): Demystifying the "Anji salamander". *Zhejiang For. Sci.*
724 *Technol.* **30**:16-17.

725 Zhu, W., Chang, L., Zhao, T., Wang, B., Jiang, J. (2020): Remarkable metabolic
726 reorganization and altered metabolic requirements in frog metamorphic
727 climax. *Front. Zool.* **17**: 30.

728

Table 1. Embryonic development of *Hynobius amjiensis*

Number	Developmental stage	Distinguishing Characteristics	Time of Emergence /h	Growth temperature/°C
1	Fertilized egg	The fertilized eggs are spherical, with a grayish-black color	0	6.5 ± 3.5
2	Multicellular stage	Cells continuously divide and the number of cells increases	16 ± 3	6.5 ± 3.5
3	Gastrula stage	The embryo is irregular and round, the embryo surface is smooth, and the blastoderm cells are involved into the embryo, with bulges or depressions on the edge	132±4	6.5 ± 3.5
4	Neural plate stage	Embryo exhibits slight folding, shallow ditch appears	164±4	6.5 ± 3.5
5	Neural fold stage	Embryonic folds became significantly opened	176±3	6.5 ± 3.5
6	Neural groove stage	Embryonic folds were significantly elevated and converged to the center	200±3	6.5 ± 3.5
7	Neural tube stage	Embryo is spindle shaped	260±5	6.5 ± 3.5
8	Early tail bud stage	Embryonic head rudiments appear	315±3	6.5 ± 3.5
9	Late tail bud stage	Embryonic eye sacs, gill arch, and tails are well characterized	355±5h	6.5 ± 3.5
10	Early external gill stage	Embryo appear with primitive outer gills and balance bars	420±5	6.5 ± 3.5
11	Late external gill stage	Embryonic eye formation with three outer gills and gill filament development	610±3	6.5 ± 3.5
12	Incubation stage	Embryonic development is basically complete, with a pair of balance rods and forelimb buds	672±3	6.5 ± 3.5
13	Hatching stage	The larvae lie on their side under the water with a small amount of yolk residue	694±48	6.5 ± 3.5

Table 2. Development of Larva to subadult of *Hynobius amjiensis*

Number	Developmental stage	Distinguishing Characteristics	Time of Emergence /Day	Growth temperature/°C
1	Forelimb forearm stage	Larval forelimbs become longer and there is no differentiation of fingers	8 ± 2	12 ± 3
2	Forelimb first finger stage	Larval forelimbs fingers form a triangle	13.5 ± 1.5	12 ± 3
3	Forelimb second finger stage	Larval forelimbs fingers are bifurcated, emergence of hindlimb buds and the shedding of balance bars	18.5 ± 1.5	12 ± 3
4	Hindlimb buds stage	A pair of transparent and spotless buds appeared in the hindlimb	18 ± 2	12 ± 3
5	Balance bar falling off stage	The balance bar on both sides of the mouth became thinner and disappeared in the form of falling off	19 ± 2	12 ± 3
6	Forelimb third finger stage	Larval forelimb fingers are trifurcat	19.5 ± 1.5	12 ± 3
7	Forelimb fourth finger stage	Larval forelimb fingers are basically formed , there are webbed connections between the fingers	20.5 ± 1.5	12 ± 3
8	Forelimb four - finger initial stage	Larval forelimbs with four fingers basically formed and webbed connections between fingers	21.5 ± 1.5	13 ± 3
9	Hind limb first finger stage	Larva hind limb bud is triangular	30 ± 1	13 ± 3
10	Hind limb second finger stage	Larval hind limbs toes are quad,but only two toes developed, and the excess was cutis	31 ± 1	13 ± 3
11	Hind limb third finger stage	Larval hind limbs toes are quad, but only three toes developed, and the excess was cutis	32 ± 1	13 ± 3
12	Hind limb fourth finger stage	Larval hind limbs are four to four forked	33 ± 1	13 ± 3
13	Forelimb four-finger mature stage	Larval forelimb fingers are fully developed, and there is no web connection between the fingers	36.5 ± 1.5	13 ± 3
14	Hind limb fifth finger stage	Larval hind limbs have pentamerous toes	40.5 ± 1.5	13 ± 3
15	Ventral costal fovea appearance stage	Larval appear as indistinct depressions on both sides of the abdomen near the head	42 ± 1	13 ± 3
16	Hindlimb five - toe initial stage	Larval five toes of the hind limbs of juveniles are largely formed, but are connected by webbing between the toes	43.5 ± 1.5	13 ± 3
17	Ventral costal fovea deepening stage	Larvae have more defined depressions on both sides of the abdomen and develop towards the sides of the abdomen near the hind limbs	44 ± 1	13 ± 3
18	Hindlimb five - toe mature stage	Five toes of the hind limb form but there are webbed connections between the toes	51.5 ± 1.5	13 ± 3
19	Pre metamorphosis stage	The bulge of the larva's eyeball, the body color becomes darker, and the caudal fin becomes smaller	58 ± 5	14 ± 1
20	Metamorphosis stage	The three outer gills of the larva gradually shorten, the body color deepens, and a small amount of white spots appear	67 ± 4	14 ± 1
21	Subadult stage	No outer gills, increased white spots, neck folds	106 ± 5	14 ± 1

733 **Figure legends**

734 **Figure 1.** Embryonic development in Anji salamander. (A1) Fertilized egg; (A2)
735 Multicellular stage; (A3) Gastrula stage; (A4) Neural plate stage; (A5) Neural fold stage;
736 (A6) Neural groove stage; (A7) Neural tube stage; (A8) Early tail bud stage; (A9) Late
737 tail bud stage; (A10) Early external gill stage; (A11) Late external gill stage; (A12)
738 Incubation stage. Red circles mark forelimb buds.

739

740 **Figure 2.** Development of the limbs in Anji salamander. Development of the limbs in
741 Anji salamander. (B1) Hatchlings of larva; (B2) Differentiation of forelimb forearm;
742 (B3) Differentiation of forelimb 1st finger; (B4) Differentiation of forelimb 2nd finger
743 and hindlimb buds; (B5) Differentiation of forelimb 3rd finger and hindlimb forearm;
744 (B6) Differentiation of forelimb 4th finger and hindlimb 1st toe; (B7) Webbing between
745 the fingers of the forelimbs and differentiation of the hindlimb 2nd toe; (B8)
746 Differentiation of hindlimb 3rd toe; (B9) Completion of forelimb development and
747 differentiation of hindlimb 4th toe; (B10) Differentiation of hindlimb 5th toe; (B11)
748 Webbing between the toes of the hindlimbs; (B12) No webbing of the toes of the
749 hindlimb. The numbers in the figure indicate the order of development of the different
750 fingers/toes from top to bottom.

751

752 **Figure 3.** Balance bar stage development in Anji salamander. (A10) Balance bar
753 emerges during embryonic development, indicated by the red arrow. (B1) Balancing
754 bars at the time of exiting the membrane; (B3) Balance bars get longer; (B3-1) Balance

755 bars falling off; (B4) Balance bars disappear.

756

757

758 **Figure 4.** Ventral costal fovea formation in Anji salamander. (B8) Day 32 post-hatching,
759 the ventral costal fovea have not yet formed; (B9) Day 34 post-hatching , the ventral
760 costal fovea begin to form; (B10) Day 39 post-hatching; (B11) Day 43 post-hatching;
761 (B16) Day 66 post-hatching, the ventral costal fovea is basically shaped; (B18) Day 70
762 post-hatching, the ventral costal fovea are obvious.

763

764 **Figure 5.** Metamorphosis in Anji salamander. (B12) Day 50 post-hatching; (B13) Day
765 60 post-hatching; (B14) Day 63 post-hatching; (B15) Day 65 post-hatching; (B16) Day
766 66 post-hatching; (B17) Day 68 post-hatching; (B18) Day 70 post-hatching.

767

768 **Figure 6.** Molting of Anji salamander. (C) Complete molted skin; (C-1) Molted skin
769 magnified 6.3 times; (C-2) Spots remaining on the skin, magnified 50 times.

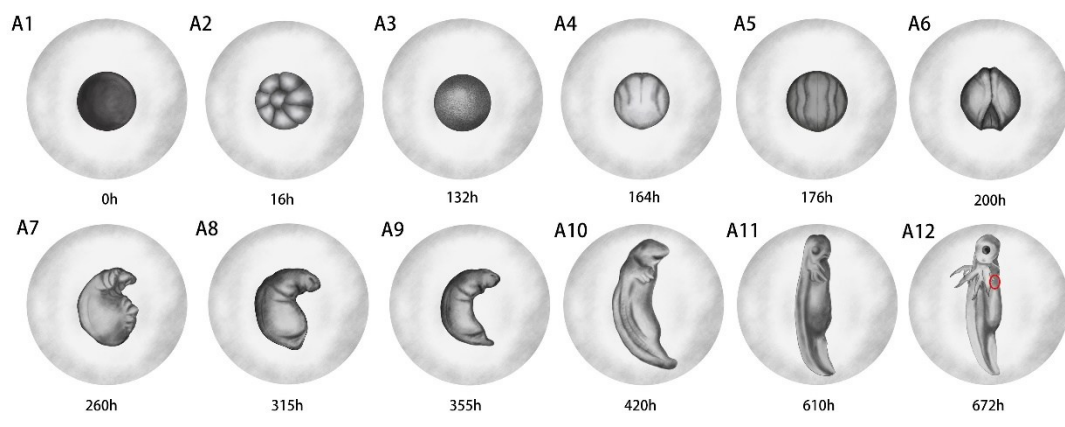
770

771 **Figure 7.** Column graphs comparing the development time of each stage of the egg
772 capsule of the Anji salamander in this study.

773

774 **Figure 8.** Developmental tree from fertilized egg to subadult in Anji salamander.
775 Complete developmental nodes from fertilized egg to subadult are illustrated.

776



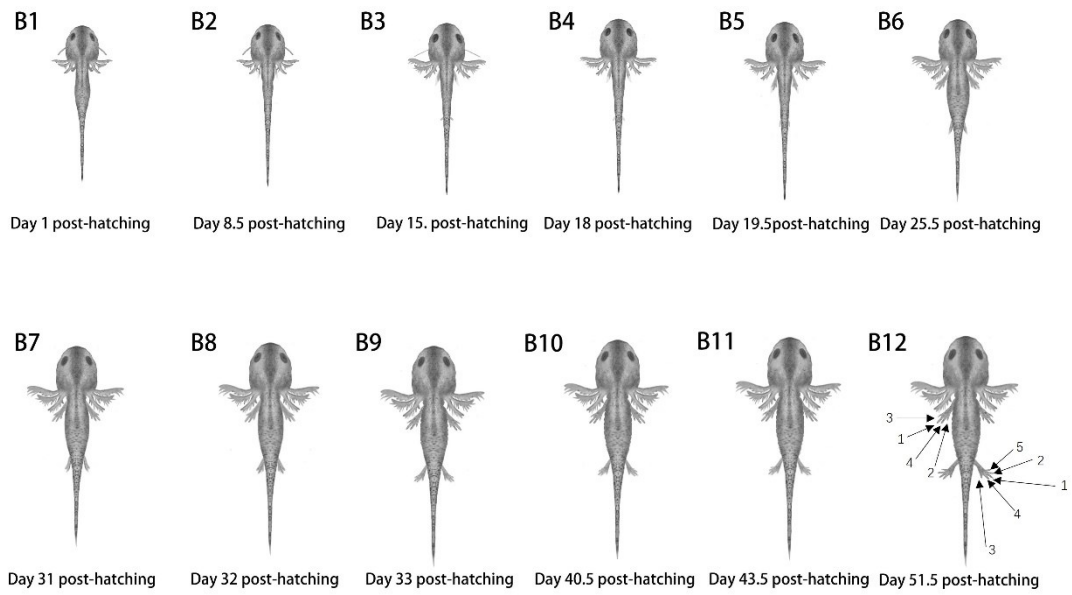
777

778 **Figure 1. Embryonic development in Anji salamander.**

779

accepted manuscript

780

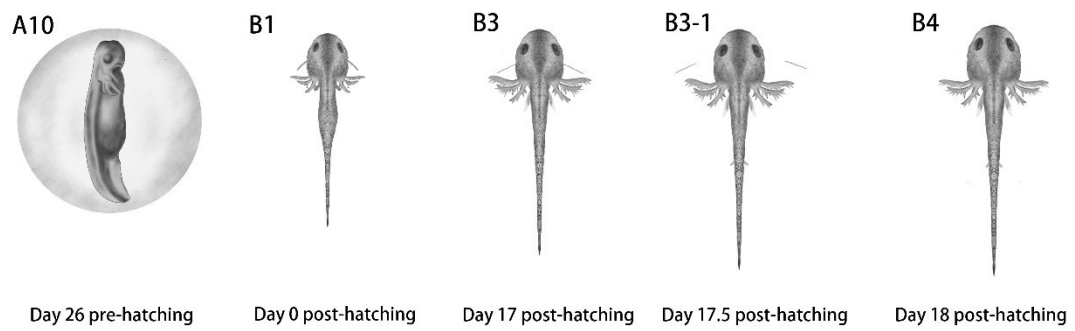


781

782 **Figure 2. Development of the limbs in Anji salamander. Development of the limbs**

783 **in Anji salamander.**

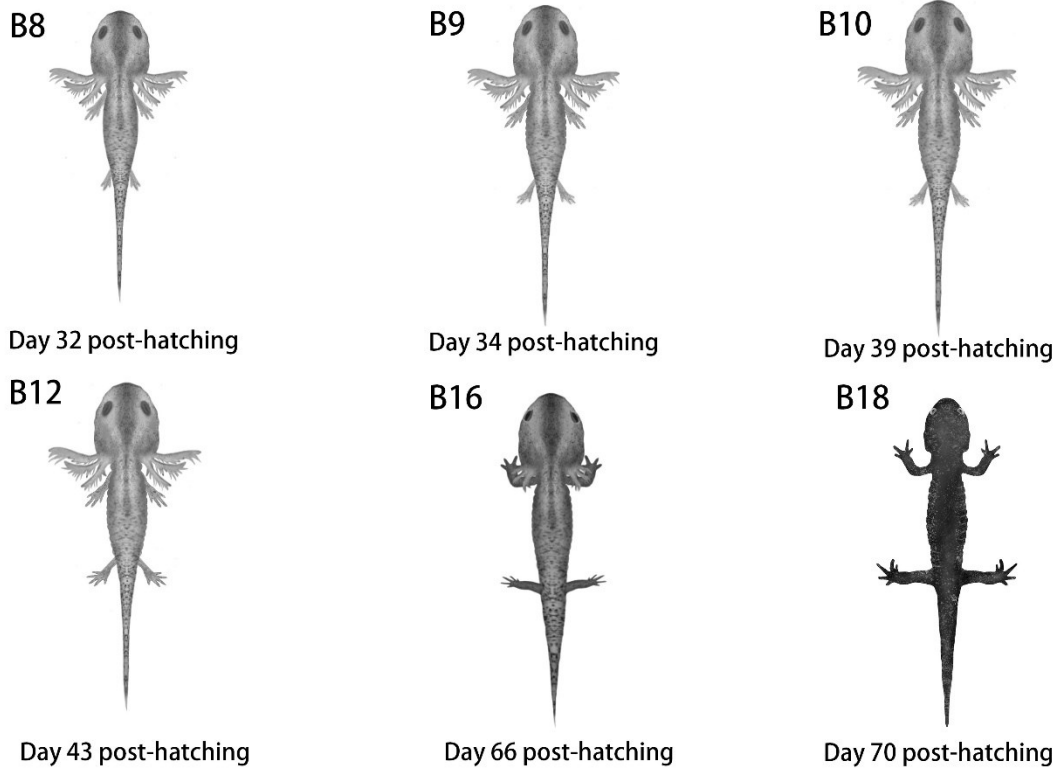
784



785

786 **Figure 3. Balance bar stage development in Anji salamander.**

787



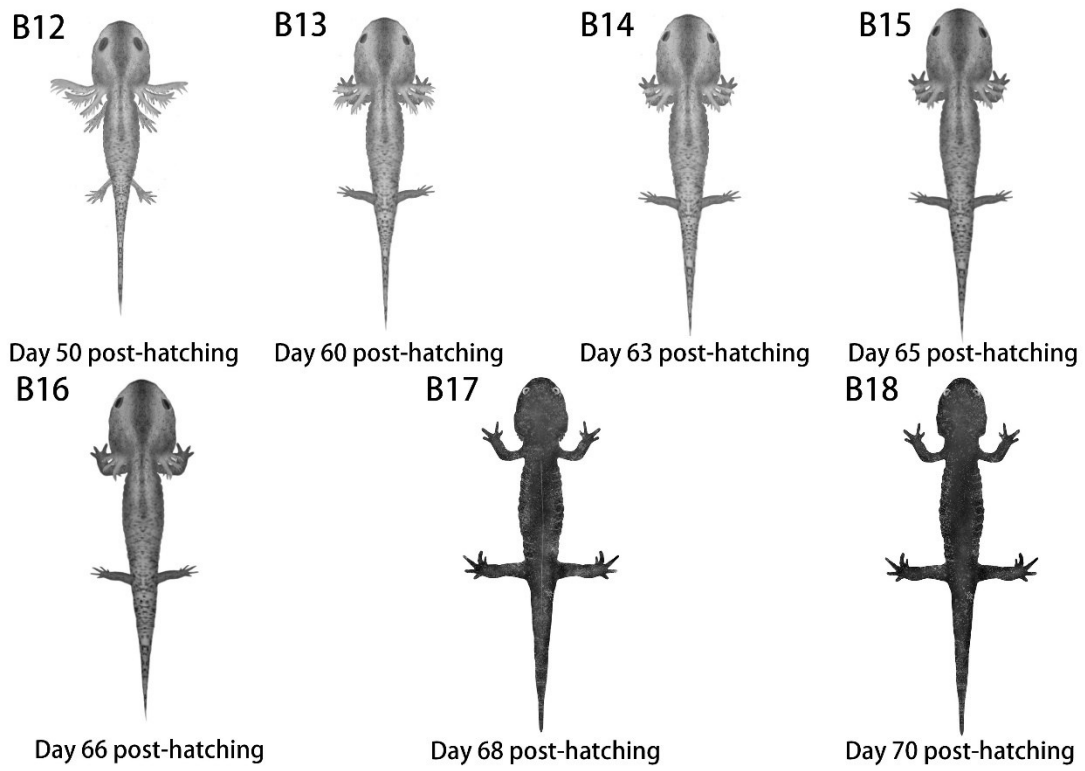
788

789

790

Figure 4. Ventral costal fovea formation in Anji salamander.

accepted manuscript



791

792

Figure 5. Metamorphosis in Anji salamander.

793

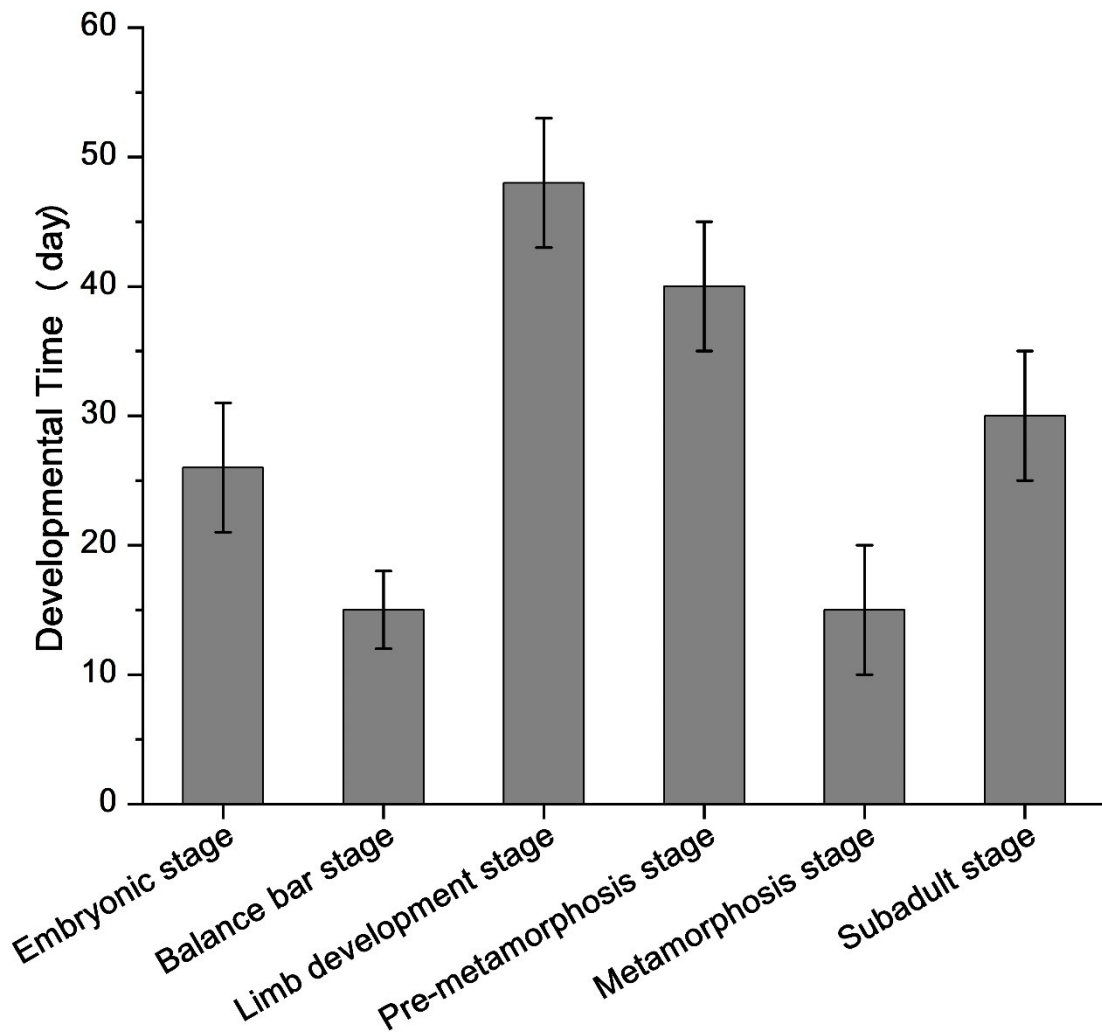


794

795 **Figure 6. Molting of Anji salamander.**

796

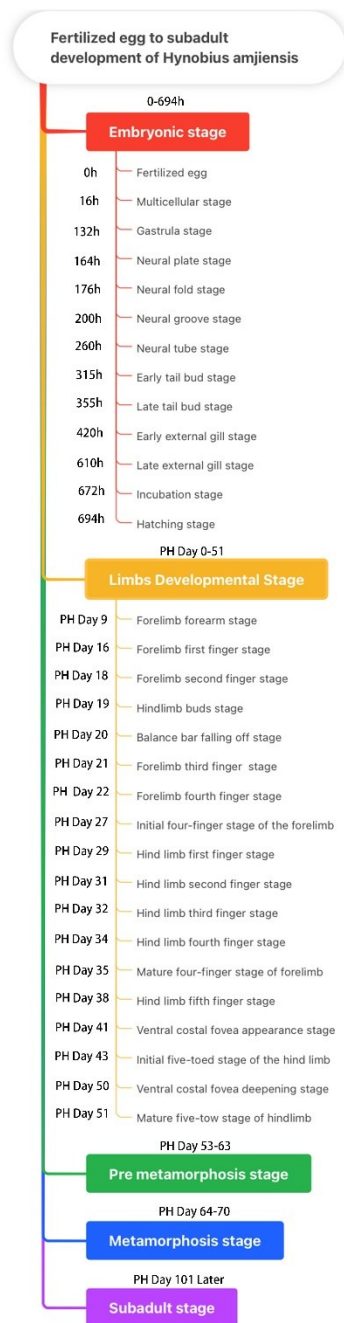
accepted manuscript



798

799 **Figure 7. Column graphs comparing the development time of each stage of the egg**800 **capsule of the Anji salamander in this study.**

801



802

803 **Figure 8. Developmental tree from fertilized egg to subadult in Anji salamander.**

804