Phenotypical expression of mobility reduction during limb ontogeny in frogs: the knee-joint case

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One of the most important epigenetic factors for the normal development of the muscle-skeletal system is movement, whose effect is particularly notable in the normal genesis and development of the joints. Studies about embryonic changes in mobility performed in chickens, mammals and anurans reported very similar phenotypes as a consequence of the reduction or absence of this stimulus. The time at which the stimulus acts, generating a phenotypic modification, has still not been elucidated. In this work we explore whether the effects of the severely abnormal mobility on development appear at any time during development or whether they become visible at particular phases of tadpole ontogeny. We conducted five experiments that showed that morphological abnormalities are visible only from Stages 40–42. The earlier stages retain a normal morphology probably because the bones/muscles/tendons have still not developed and therefore might not have been affected by movement/immobilization. These results suggest the existence of a period of phenotypical expression during which normal limb movement seems to be a key factor in the assembly of the tissue integrating the joint framework.

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Short running page heading: development of immobilized limb

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ABSTRACT

One of the most important epigenetic factors for the normal development of the muscle-skeletal system is movement, whose effect is particularly notable in the normal genesis and development of the joints. Studies about embryonic changes in mobility performed in chickens, mammals and anurans reported very similar phenotypes as a consequence of the reduction or absence of this stimulus. The time at which the stimulus acts, generating a phenotypic modification, has still not been elucidated. In this work we explore whether the effects of the severely abnormal mobility on development appear at any time during development or whether they become visible at particular phases of tadpole ontogeny. We conducted five experiments that showed that morphological abnormalities are visible only from Stages 40–42. The earlier stages retain a normal morphology probably because the bones/muscles/tendons have still not developed and therefore might not have been affected by movement/immobilization. These results suggest the existence of a period of phenotypical expression during which normal limb movement seems to be a key factor in the assembly of the tissue integrating the joint framework.

Keywords phenotypical expression; reduced mobility; knee-joint; anurans; development.

INTRODUCTION

One of the most important environmental factors for the normal development of skeletal structures in tetrapods is movement, whose effect is particularly notable in the normal genesis and development of the joints (Abdala & Ponssa, 2012; Pitsillides, 2006; Nowlan, 2015). Most studies dealing with embryonic responses to changes in mobility have been performed in chickens, mammals or zebrafish (Sullivan, 1966; Hall, 1975; Hosseini & Hogg, 1991; Müller, 2003; Pitsillides, 2006; Kahn et al., 2009; Shwartz et al., 2012; Nowlan et al., 2010ab; Nowlan, 2015). Conducting such studies in vertebrates that undergo metamorphosis totally independently of maternal influence may help us to elucidate the role and effects of external environmental factors on mobility patterns, and gain new insights into the lack of maternal effect on limb bud development. Abdala and Ponssa (2012) reported that the larvae of organisms that are free-living during development exhibit the same morphological responses to reduced mobility as embryos that undergo development in controlled environments, such as a uterus or a shelled egg, which indicates a considerable degree of self-sufficiency in developing individuals. One possibility is that intrinsic, genetic factors may be responsible for initiating organogenesis, and those extrinsic, epigenetic factors, including movement, may have a strong effect on this process only at later stages of development (Pitsillides, 2006). Another possibility relies on the concept of 'critical periods', e.g., a period of the development during which the tissues, joints, etc., are sensitive to epigenetic factors (Hall, 1977; Pitsillides, 2006). Although the effects of immobilization on the development of vertebrates are relatively well known (Pitsillides, 2006; Abdala & Ponssa, 2012; Nowlan, 2015), the time at which the stimulus acts generating a phenotypic modification has still not been fully elucidated. In hip joint of chicken embryo was stressed that the timing of fetal movements on joint shape is correlated with the event of cavitation in the chick embryo, with

fetal movements not having an effect on the joint until after cavitation should have occurred (Nowlan et al., 2014a). In experiments analyzing mobility reduction in tadpoles, Abdala and Ponssa (2012) found that anomalies in experimental tadpoles began at Stages 41–42, with no observable morphological consequences of mobility reduction during the initial developmental stages of experimental tadpoles. In this work we focus on the timing at which the phenotypical expression of mobility reduction during limb development of frogs is evident. Our experimental design was aimed to evaluate 1) whether severely abnormal mobility affects the development of the limb tissues in the knee-joints; if it is the case, we hypothesize that the effects of the stimulus would appear from the beginning of ontogeny; or 2) whether mobility reduction affects only the assembly of the limb tissues in the knee-joints; in this case, we hypothesize that the phenotypical effects would appear only from Stage 40–42, because about this ontogenetic phase tadpole limb tissues are mature and ready to act (Manzano et al., 2012). Thus, we aim to understand the importance of the movement (or its lack) in the development of the limb bud tissues after and before they assemble in the knee-joint.

MATERIAL AND METHODS

One hundred and fifty tadpoles of *Pleurodema borellii* were collected from temporary ponds in Lules and Yerba Buena (Tucumán, Argentina) (Field permit Res. 21/2012, Ministerio de Turismo), and maintained under laboratory conditions. Experiments were conducted in summer 2011 and 2014. We performed five experiments with *P. borellii* tadpoles. Each experiment consisted of three containers with 1 Li of water; in two of them, 9 gr of agar were added (Abdala & Ponssa, 2012). In order to evaluate the role of movement in knee-joint development, it is

necessary to employ an experimental design which decreased movement of the limb tadpoles, without producing other conditions adverse to growth and development. The tadpoles were fed with fish pellets *ad libitum*, which were located achievable. Both, control and experimental tadpoles feed normally. The dissolved oxygen, measured with an oxygen meter (Hach sensION6), was 6.02 mgr/Li in the agar solution and 7.22 mgr/Li in the water control tanks. We made successive measurements to confirm that difference in dissolved oxygen between the agar and the water was never below 2 mgr/Li. In a typical fishpond, the critical oxygen concentration threshold is about 2 mgr/Li (Heargreaves & Tucker, 2002); therefore, the agar solution was a normally oxygenated medium in physiological terms. Density of the medium was measured with a float-type densitometer. The agar solution had a density of 1.0 gr/cm^3, because water-colloids have the density of water. At 25 ºC, water has a viscosity of 0.008 Pa/sec. By adding agar, medium viscosity was increased to 0.06 Pa/sec, thereby imposing resistance to larval movement. Viscosity was measured with an Ostwald viscometer. To estimate tadpole mobility, a 1-min digital video was recorded for 10 experimental and 5 control containers selected at random (Abdala & Ponssa, 2012). Tadpole of each container was selected randomly from the videos. The time tadpoles spent moving in each container was quantified and used as a measure of mobility. The videos were edited and analyzed using the program Windows Movie Maker (2006) version 6.0. The frequency and duration of mobility of the experimental tadpoles was significantly lower than that of control tadpoles. Of the 1 min recorded, the selected experimental tadpoles moved 5.63 ±8.539 sec (N = 10); meanwhile, controls moved for 33.2± 18.06 sec (N = 10) ($F = 4.48$; $p < 0.05$). To avoid contamination, excessive solidification and a drastic decrease of dissolved oxygen in the agar medium, the colloid was renewed three times a week. Ten *Pleurodema borellii* tadpoles at Stage 34 were placed in each container. In experiment A, 20 tadpoles were

105 reared in agar medium; when they reached Stage 40–42, the agar solution was replaced by water,
106 where the development continued until the juvenile stage. In experiment B, 20 tadpoles were
107 reared in agar medium until the juvenile stage. In experiment C, 20 tadpoles were reared in water;
108 water was then replaced by agar medium at Stage 40–42 of tadpoles, where they reached the
109 juvenile stage. In experiment D, 20 tadpoles were reared in water, when they reached Stage 40
110 the water was replaced by agar. When they reached Stage 42 the agar solution was replaced by
111 water where the development continued until the juvenile stage. In experiment E, 20 tadpoles
112 were reared in agar, when they reached Stage 40 the agar medium was replaced by water. When
113 they reached Stage 42 the water was replaced by agar where the development continued until the
114 juvenile stage. In addition, 50 larvae placed in plain water were used as controls to assess normal
115 tadpole anatomy and development. Tadpole development involves growth and development of
116 larval structures and metamorphic changes. The Stage 40 is identifiable because the total length
117 begins to diminish through resorption of the tail; the larval mouth parts begin to break down
118 (Gosner, 1960). The beginning of metamorphosis (at Stage 42; Gosner, 1960) is externally
119 evident when the forelimbs emerge, angle of mouth is anterior to nostril, labial denticles are lost
120 and horny beaks disappear. The end of the metamorphic stages (Stage 46; Gosner, 1960) and
121 beginning of the juvenile period is recognizable by the development of the mouth and total tail
122 resorption (Gosner, 1960).

123 To assess the mechanical effect of the agar medium on tissue differentiation, we
124 compared histological sections of the skeletons (knee-joint) of control tadpoles with those of
125 experimental tadpoles showing clear phenotypic modifications. Formalin-fixed specimens were
126 treated with 10% neutral buffered formalin and dehydrated with graded alcohols. Serial sections
127 (6 μm thick) were cut on an MSE sledge microtome, along the long axis of the limbs, and at right

and sagittal angles of the bone. Six sections were made every 1 mm and stained with Harris's picrosirius haematoxylin (Totty, 2002). Histological sections of 21 experimental specimens and of five control individuals of *P. borellii* were prepared. Histological samples of *Pleurodema borellii* described in Abdala and Ponssa (2012) were used to compare the tadpole anatomy. Histological data from the experimental tadpoles in water through the first developmental stages are not shown because they exhibit no variation with respect to the control tadpoles.

Health status of the tadpoles was checked throughout the experiment by examining the skin, oral disc, and limbs (Richards, 1962). In all cases, tadpoles showed a good health status, and no malformations associated with parasites or chemical compounds were observed (Meteyer, 2000). All the experiments were approved by the Bioethics Committee at the Facultad de Medicina, Universidad Nacional de Tucumán, Argentina (Res. Nº1206 2010).

RESULTS

The control individuals showed histological features of normal tissue developmental (Fig. 1). Of each experiment some individuals of the most representative stage were selected for detailed display.

Experiment A included specimens reared in agar medium between Stages 34 and 42; when they reached Stage 42, the agar solution was replaced by water, and the tadpoles raised in water until juvenile stages. The external aspect of the specimens was highly modified from Stage 42 (Fig. 1 and 3). Their limb joints exhibited abnormal angles and descended hindlimbs not supported by the pelvic girdle joint; the volume and tone of limb muscles evidenced flaccidity. Tadpoles reached Stage 42 without any evidence of malformation or abnormality in their histological

150 structures (Fig. 2). At Stages 42–43, tadpoles exhibited clear signals of morphological pathology
151 (Fig. 3). The histological samples showed that the knee-joint of the experimental tadpoles had an
152 articular cartilage with irregular boundaries, and that the lateral articular cartilages were thinner
153 than in the control specimens (Fig. 1 and 3). The chondrocytes of hypertrophic zone of the long
154 bones were highly malformed and presented extremely irregular borders (Fig. 3); many of them
155 were flattened (Fig. 3). Once metamorphosis was completed, the articular regions of the juvenile
156 specimens exhibited irregular borders and the typical curved-shape of the articular area of the
157 epiphyses was not observed; the area of lateral articular cartilage was severely flattened (Fig. 3).

158 Experiment B included tadpoles reared in agar medium from Stage 34 to juvenile stage (Fig. 4).
159 The phenotype showed modifications similar to those observed in experiment A. In experimental
160 tadpoles at Stages 41– 42 the articulation areas of the long bones of the hindlimb appeared
161 deformed. The shape of the epiphyses was entirely modified. The cells of the osteochondral
162 ligaments were not distinguishable and all the articular cartilages were deformed. Ligaments and
163 tendons of the knee-joint were not recognizable, with only a mass of pathological tissue between
164 femur and tibiafibula epiphyses being distinguishable (Fig. 4). Deformation was remarkable in
165 the menisci, which were composed of hypertrophied cells with irregular boundaries (Fig. 4). The
166 chondrocytes of the resting, proliferating and hypertrophic zones were highly irregular and
167 flattened compared to chondrocytes of normal specimens (Fig. 4). For a more complete
168 description of the effect of the agar medium on *Pleurodema borellii* tadpole development, see
169 Abdala and Ponssa (2012).

170 Experiment C included tadpoles reared in water between Stages 34–42 and in agar from Stage 42
171 onwards (to juvenile stages). Tadpoles of Stages 34–42 were entirely normal (data not shown).
172 After Stage 42 deformations became evident (Fig. 5). Tadpoles of Stage 44 showed the long

bones of the hindlimb entirely composed of hypertrophied cartilage. This cartilage consisted almost completely of large lacunae that were highly irregular compared to the cartilage of control specimens (Fig. 5). The amount of interlacunar matrix was very small, forming a very thin boundary line between the lacunae that resulted in a characteristic net-like appearance. The typical zones of differentiated chondrocytes present in anuran long bones (reserve, hypertrophic and proliferation zones) were not distinguishable. No clear articulation area in the knee-joint was differentiated (Fig. 5). The cells of the osteochondral ligaments and the borders of the epiphyses were highly malformed. All articular cartilages were malformed, showing irregular borders.

Experiment D included tadpoles reared in water until Stage 40 and in agar between Stages 40–42, and in water from Stage 42 onwards (until juvenile stages). Tadpoles of Stages 34–40 were entirely normal (data not shown). After Stage 40 deformations became evident, with the phenotypical modifications described above (Fig. 6).

Experiment E included tadpoles reared in agar until Stage 40 and in water between Stages 40–42, and in agar from Stage 42 onwards (to juvenile stages). Tadpoles of Stages 34–40 were entirely normal (data not shown). After Stage 40 deformations became evident, with the phenotypical modifications described above (Fig. 7).

Pathological anatomical features in the joint:

We recognized two or more body parts abnormally angled, bent or contractured. By example, extended and pronated elbow, all digits positioned in the palm, flexed wrist and extended knees, rotated shoulders. These defects severely modify the normal locomotory position of fore and hind limbs, and their ability to jump (Fig. 8 and Appendix 1). Also, we recognize a dermopathy in all the body (Fig. 3 and 8).

DISCUSSION

The experimental design showed a clear effect on tadpole mobility. The experiments allow us to postulate a phenotypical expression period that starts between the stages 40–42 (Gosner, 1960), when abnormalities caused by immobilization are observed in the knee-joint. Knowledge of this phenotypical expression period provides greater understanding of the importance of the range of developmental time points in which the stimulus occur. The provoked limb abnormalities are irreversible if the tadpoles are switched back to normal water after this critical phase. Our results show that although limb tissues of the knee-joint were under the stimulus (reduced mobility) from the beginning of ontogenetic development, they exhibit a normal phenotype until Stage 40–42 (Fig. 9), when the elements of the knee-joint, cavity and shape of the epiphyses, are formed since Stage 38–39 (Manzano et al., 2012). The most parsimonious explanation would be that the bones/muscles/tendons are not mature until these stages, and because of that, mobility reduction has no effect on their development. In other words, once bones, muscles and tendons are formed (Stage 41) (Manzano et al., 2012), movement is critical for maintaining healthy tissues and the proper knee-joint assembling and functioning in juveniles. These are counter-intuitive results because a synchronicity between reduced mobility (stimulus) and tissue malformation (phenotypical expression) would be expected (i.e. modifications in the tissue might be expected from Stage 34 in experiment A). From our data we infer a new insight into the effect of reduced mobility on knee-joint formation: limb tissue development seems to be insensitive to mechanical stimuli engendered by movement, at least until the Stage 40. This stage is critical to the assemblage, coordination and integration of the components of the knee-joint framework (Manzano et al., 2012; this work). Limb tissue differentiation might be under strict genetic

control, and driven by the embryo movement produced by the ischiadic nerve action (Manzano et al., 2012), whereas the coordinate activity and normal development of the tissue is achieved through an epigenetic stimulus, i.e. movement. Likely, embryonic movements are necessary to correctly shape the form of the bone ends by friction of the coupled surfaces (Murray & Selby, 1930; Hamburger & Waugh, 1940; Drachman & Sokoloff, 1966). Nowlan et al. (2014a, 2015) showed that the shape morphogenesis is advanced prior to cavitation of the hip joint raise, and that hip joint shape morphogenesis was dramatically affected by the absence of movements only after the time point at which joint cavitation should have occurred, both aspects (time of cavitation and phenotypes modifications) coincide with our data: the joint shape is already formed before the cavitation (Stage 39), and is affected in the posterior stages. Apparently, the early morphogenesis could be influenced by the bending at the joint that occur before to cavitation joint; or by the stresses and strains induced by the differential growth of developing tissues (Henderson & Carter, 2002; Nowlan et al., 2014a). Alternatively, the morphogenetic events prior to cavitation are intrinsically determined by cellular and genetic programs (Nowland, 2015).

Muntz (1975) proposed four stages in limb development relative to muscular maturity and movement possibilities: non-motile, pre-motile, motile, and fully functional stages. Considering that the start of our experimental work coincided with the end of the pre-motile stage, we infer that the ischiadic nerve has completed its role of inducing mobility of the limb bud (Muntz, 1975; Manzano et al., 2012), and this movement would be induce the normal knee-joint formation and hindlimb ossification in the earliest stages. Our experimental design prevented the movement characteristic of the motile stage, and its absence dramatically affected further development of the limb and its associated locomotory function. During the earliest

241 stages of the motile phase (Stages 37–40), anurans showed an apparent insensitivity to external
242 mechanical stimuli. We consider that this insensitive phase is a reasonable consequence of the
243 absence or maturity of the elements involved in joint function: bones, muscles and tendons
244 (Dunlap, 1967; Manzano et al., 2012). Thus, we propose that the severe latent problem
245 originated by the lack of movement since the early stages is dramatically manifested from Stages
246 40–42 (Gosner, 1960), because all the alterations in the morphology of the experimental tadpoles
247 were visible only from Stage 40. This is in accordance with the relatively late acquisition of the
248 mechano-sensitivity of knee-joint tissues, and the insensitive to mechanical effects of the early
249 stages (Pitsillides, 2006). The Stage 42 has been indicated as the beginning of the metamorphic
250 stages (Gosner, 1960). During this critical phase, tadpoles lose their larval characteristics and
251 present adult structures; the tail begins to degenerate, larval feeding elements are replaced by
252 adult jaw and tongue, and forelimbs and hindlimbs become functional. This period typically
253 implies the passage from the aquatic to the terrestrial environment (McDiarmid & Altig, 1999).
254 Thus, mobility reduction seriously affects the locomotor capacity of the froglet and its adaptation
255 to the terrestrial habitat. Interestingly, damages produced were irreversible because tadpoles
256 reared in agar medium until Stage 42 showed morphological alterations even when they were
257 transferred to water until the froglet stage (Fig. 4). Likewise, tadpoles reared in agar medium
258 until metamorphosis and then transferred to water developed into juveniles with anomalies in
259 their locomotor abilities (Online Resource 1). Hence, our data allow us to propose the existence
260 of a phenocritical phase that begins between the Stages 40–42. During this phase the acquisition
261 of the limb joint mechanosensitivity is produced and its alterations will have lasting
262 consequences. This demonstrates that defined periods of the early development of frogs may
263 impact upon the accurate arrangement of the individual components of the musculoskeletal

system of the limb joint. Similar effect was described by Drachman and Coulombre (1962) who found that immobilization of fetal chicks through a brief period of the development provoked deformities, with the more advances degrees of deformities being associated with older ages for initiation of treatment. The authors highlight the significance that brief period of immobilization caused a permanent deformity.

It is surprising that the response to this particular stimulus (reduced mobility) is independent of the ontogenetic environment of the individuals, which suggests a small influence of the controlled environment such us the uterus or shell in mitigating the effects of reduced mobility. Kahn et al. (2009) showed that movement-induced mechanical stimuli play a key role in the regulation of organ progenitor cells during joint development. They also showed that failure in joint formation was observed in some joints of mouse mutant embryos. They consider that in some joints the lack of movement was offset by the other components in the genetic program that regulates joint development. Our data also suggest that normal limb movement does not seem to be a key factor in limb tissue development until the joint framework is assembled, although after that, the lack of movement can produce a phenotype of an osteoarthritic joint. Alterations in metamorphic phenotype arising from hostile larval environment can limit ecologically relevant performance capacity such as locomotion, and consequently influence food acquisition, predator avoidance, and dispersal capacities (Charbonier & Vonesh, 2015). The dramatic consequences of the reduced mobility in the joint movement capacities strongly indicate that this is a relevant avenue to explore its consequences in the survival possibilities of frogs.

286 *Comparison of pathological features in our experimental frogs with abnormalities provoked by*
287 *fetal akinesia*

288 The anatomical modifications observed in limbs of experimental frogs follow a pathological
289 pattern coincident with the arthrogryposis condition, which describes congenital joint
290 contractures in two or more areas of the body. The affected individuals are unable to do passive
291 extension and flexion in the affected joint (Nowlan, 2015). The syndrome can be caused by
292 neurogenic, myogenic, or connective tissue pathologies, or by environmental factors, such as
293 decreased intrauterine movement (Kalampokas et al., 2012). Its etiology still remains unclear but
294 generally any cause that leads to reduced fetal movement may lead to congenital contractures
295 and in severe cases to fetal akinesia deformation sequence (Kalampokas et al., 2012). As detailed
296 in our results sections, experimental specimens exhibit all the typical pathologies of
297 arthrogryposis. The similar pathological phenotypes effects of the reduced mobility on the
298 development of the skeleton of chicken, mouse and frogs, allow us to suggest that frogs could be
299 also considered suitable animal model system to use in the research of the effect of the altered
300 mechanical environment on the development of the skeletal system of vertebrates (Nowlan et al.,
301 2010b). This research in frogs could be a useful avenue to study how to prevent, diagnosis and
302 treat these different syndromes included in the definition of arthrogryposis. Finally, this research
303 could in turn serve to validate idealized geometries of joint configurations created through
304 computational programs (Roddy et al., 2009; Nowlan et al., 2014b).

305

306 *Validity of experimental method*

The inferences of our work depend upon the validity of our hypothesis that mobility reduction is responsible of the alterations of knee-joint development instead of some no considered feature. We would like to highlight that oxygen levels, feed consume and health status related to parasites or chemical compounds were appropriate for a normal development along all the experimental phase. With regard to the tissues, one of the consequences observed was a severe flattening of the hypertrophic chondrocytes. Quinn et al. (1998) stressed that mechanical loading through an increased static compression is associated with a decreased cell radius in the direction of compression, and this would be the case in the cartilaginous cells of the experimental tadpoles. The deformation illustrated by Quinn et al. (1998: Fig. 2Cd) resembles the present observations and the results reported in a previous work (Abdala & Ponssa, 2012). This cell deformation suggests that the agar compresses the entire tadpole. However, although compression is a collateral effect of life in agar medium, other damages in the cartilage are similar to those present in animals immobilized by drugs or denervation, which did not suffer any mechanical load (Kim et al., 2009; Hosseini & Hogg, 1991). Likewise, the zones of epiphyseal proliferation were the most affected areas of the long bones and the pathologies were similar to those previously described in embryos of tetrapods, such as free-living tadpoles (Abdala & Ponssa, 2012), mouse, rat (Coutinho et al., 2002; Kahn, 2009) and chicken (Sullivan, 1966; Murray & Drachman, 1969; Hall, 1975; Hall & Herring, 1990; Quinn et al., 1998; Pitsillides, 2006). This uniform response can be expected because in all cases the affected material was the same: connective tissue (e.g., cartilages, bone, muscle, etc.).

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REFERENCES

Abdala V, Ponssa ML. 2012. Life in the slow lane: the effect of reduced mobility on tadpole limb development. *Anatomical Record* **295**:5–17.

Charbonier J, Vonesh JR. 2015. Consequences of life history switch point plasticity for juvenile morphology and locomotion in the Túngara frog. *PeerJ* DOI 10.7717/peerj.1268

Coutinho EL, Gomes ARS, Franca CN, Salvini TF. 2002. A new model for the immobilization of the rat hind limb. *Brazilian Journal of Medical Biological Research* **35**:1329–1332.

Dunlap DG. 1967. The development of the musculature of the hindlimb in the frog, *Rana pipiens*. Journal of Morphology **119**:241–258.

Drachman DB, Coulombre AJ. 1962. Experimental clubfoot and arthrogryposis multiplex congenital. *Lancet* **280**: 523–526.

Drachman DB, Sokoloff L. 1966. The role of movement in embryonic joint development. *Developmental Biology* **14**: 401–420.

Gosner KL. 1960. A simplified table for staging anurans embryos and larvae with notes on identification. *Herpetologica* **16**:183–190.

348 **Hall BK**. 1975. A simple, single-injection method for inducing longterm paralysis in embryonic
349 chicks, and preliminary observations of growth of the tibia. *The Anatomical Record* **181**:767–
350 778.

351 **Hall BK**. 1977. Thallium-induced achondroplasia in chicken embryos and the concept of critical
352 periods during development. *Teratology* **15**:1–16.

353 **Hall BK, Herring S**. 1990. Paralysis and growth of the musculoskeletal system in the embryonic
354 chick. *Journal of Morphology* **206**:45–56.

355 **Hamburguer V, Balaban M, Oppenheim R, Wenger E**. 1965. Periodic motility of normal and
356 spinal chick embryos between 8 and 17 days of incubation. *Journal of Experimental Zoology* **159**:
357 l–14.

358 **Heargreaves JA, Tucker CS**. 2002. Measuring dissolved oxygen concentration in aquaculture.
359 *South Regional Aquaculture Center* **4601**:1–6.

360 **Henderson J, Carter D**. 2002. Mechanical induction in limb morphogenesis: the role of
361 growth-generated strains and pressures. *Bone* **31**:645–653.

362 **Hosseini A, Hogg DA**. 1991. The effect of paralysis on skeletal development in the chick
363 embryo. II. Effects on the histogenesis of the tibia. *Journal of Anatomy* **177**:169–178.

364 **Kalampokas E, Kalampokas T, Sofoudis**. 2012. Diagnosing arthrogryposis multiplex
365 congenital: a review. *International Scholarly Research Network* **2012**: 1–6.

366 **Kahn J, Shwartz Y, Blitz E, Krief S, Sharir A, Breitel DA, Rattenbach R, Relaix F, Maire P,**
367 **Rountre RB, Kingsley DM, Zelzer E**. 2009. Muscle contraction is necessary to maintain joint
368 progenitor cell fate. *Developmental Cell* **16**:734–743.

Kim HT, Olson WM, Hall BK. 2009. Effects of hind limb denervation on the development of appendicular ossicles in the dwarf African clawed frog, *Hymenochirus boettgeri* (Anura: Pipidae). *Acta Zoologica* (*Stockholm*) **90**:352–358.

Manzano A, Abdala V, Ponssa ML, Soliz M. 2012. Ontogeny and tissue diferentiation of the pelvic girdle and hind limbs of anurans. *Acta Zool (Stockholm)* **94**:420–436.

McDiarmid RW, Altig R. 1999. Introduction. The tadpole arena. In: McDiarmid RW, Altig R, eds. *Tadpoles. The Biology of Anuran Larvae*. The University of Chicago, 1–23.

Meteyer CU. 2000. Field guide to malformations of frogs and toads. *Biological Science Report USGS.*

Müller G. 2003. Embryonic mobility: environmental influence and evolutionary innovation. *Evolution and Development* **5**:56–60.

Muntz L.1975. Myogenesis in the trunk and leg during development of the tadpole of *Xenopus laevis* (Daudin 1802). *Journal of Embryology and Experimental Morphology* **33**:757–774.

Murray PD, Drachman DB. 1969. The role of movement in the development of joints and related structures: the head and neck in the chick embryo. *Journal of Embryology and Experimental Morphology* **22**:349–371.

Murray P LF, Selby D. 1930. Intrinsic and extrinsic factors in the primary development of the skeleeton. Wilhelm Roux'Arch. Entwicklungsmech. *Organ* **122**: 629–662.

Nowlan NC, Bourdon C, Dumas G, Tajbakhsh S, Prendergast PJ, Murphy P. 2010a. Devolping bones are differentially affected by compromised skeletal muscle formation. *Bone* **46**: 1275–1285.

Nowlan NC, Sharpe J, Roddy KA, Prendergast PJ, Murphy P. 2010b. Mechanobiology of Embryonic Skeletal Development: Insights from Animal Models. *Birth Defects Research* **90**: 203–213.

Nowlan NC, Sharpe J. 2014a. Joint shape morphogenesis precedes cavitation of the developing hip joint. *Journal of Anatomy* **224**:482–489.

Nowlan NC, Chandaria V, Sharpe J. 2014b. Immobilized chicks as a model system for early-onset developmental dysplasia of hip. *Journal of Orhopaedic Research* **32**: 777–785.

Nowlan NC. 2015. Biomechanical of foetal movement. *European Cells and Materials* **29**: 1–21.

Pitsillides A. 2006. Early effects of embryonic movement: "A shot out of the dark". *Journal of Anatomy* **208**:417–431.

Quinn TM, Grodzinsky AJ, Buschmann MD, Kim YJ, Hunziker EB. 1998. Mechanical compression alters proteoglycan deposition and matrix deformation around individual cells in cartilage explants. *Journal of Cell Science* **111**:573–583.

Richards CM. 1962. The control of tadpole growth by algae-like cells. *Physiological Zoology* **35**:285–296.

Rody KA, Nowlan NC, Prendergast PJ, Murphy P. 2009. 3D representation of the developing chick knee joint: a novel approach integrating multiple components. Journal of Anatomy **214**: 374–387.

Shwartz Y, Farkas Z, Stern T, Aszo´di A, Zelzer Elazar. 2012. Muscle contraction controls skeletal morphogenesis through regulation of chondrocyte convergent extension. *Developmental Biology* **370**:154–163.

411 **Sullivan GE**. 1966. Prolonged paralysis of the chick embryo, with special reference to
412 effect on the vertebral column. *Australian Journal of Zoology* **14**:1–17.

413 **Totty BA**. 2002. Mucins. In: Bancroft JD, Gamble M, eds. *Theory and practice of histological*
414 *techniques*. Edinburgh: Churchill Livingston, 163–200.

415 **Windows Movie Maker**. 2006. Window Movie Maker, Version 6.0.

416 http://windows.microsoft.com/en-us/windows-vista/products/home.

Figure Captions

Fig. 1 Control specimens (left) and histological samples (right) showing the knee-joint area in successive developmental stages. Stage 37–38; in the histological sample muscle tissue still not completely differentiated is observed; in the femur and tibiofibular epiphyses hyaline cartilage is evident, consisting of chondrocytes immersed in a basophile matrix composed of fibers and ground substance. Mesenchymal tissue surrounds the long bone epiphyses, forming a regular tissue in the presumptive knee articulation area, where the long-bone articular surfaces begin differentiation. Joint formation is evidenced by the interzone, a close package of mesenchymal cells. The graciella sesamoid is embedded in a condensation of dense connective tissue or tendon anlage (future tendinous tissue). Stage 39; in the histological sample the cavitation process in the knee-joint led to a physical separation between the articular surfaces. In the cartilage of the long bones the resting, proliferating and hypertrophic zone can be distinguished. The osteochondral ligament and the articular lateral cartilage are differentiated in the epiphyses of femur and tibiafibula. The future tendinous tissue still shows appearance of undifferentiated connective tissue. Stage 42; the elements of the knee-joint, cavity and shape of the epiphyses, are already formed. Stage 45; the histological sample was stained with Mallory trichome, which allows us to observe the mature tendon in blue. The tissues (cartilage, muscle and tendon) are completely mature at this stage. ct: connective tissue; e: epiphysis; F: femur; fmgm: future gracilis major muscle; HZ: hypertrophic zone; i: interzone; lac: lateral articular cartilage; ocl: ostechondral ligament; ta: tendon anlage; PZ: proliferating zone; RZ: resting zone; TF: tibiafibula

Fig. 2 Histological section of knee and ankle joint of tadpoles of *Pleurodema borellii*. Experiment A. Stage 38 and Stage 39. No evidence of malformation or abnormality is observed in the tissues of the hindlimb in the first stages studied. F: femur; Fe: feet; g: graciella sesamoid;

lac: lateral articular cartilage; plm: plantaris longus muscle; ta: tendon anlage; TF: tibiafibula; T-F: tibiale and fibulare

Fig. 3 External morphology (left) and knee-joint histological section (right) of tadpoles of *Pleurodema borellii*. Experiment A. Stage 42 and Stage 43, the articular areas of the epiphyses are malformed, and the hyaline cartilage shows flattened cells with irregular borders. Juvenile specimen, note the severely damaged lateral articular cartilage in the detail indicated by the curved arrow. F: femur; TF: tibiafibula; lac: lateral articular cartilage; ocl: osteochondral ligament; g: graciella sesamoid

Fig. 4 External morphology (left) and knee-joint histological section (right) of tadpoles of *Pleurodema borellii*. Experiment B. Stage 36–37, joint area without abnormality. Stage 41, the articular cartilages are deformed; the chondrocytes are irregular and flattened. Stage 44, pathologic menisci composed of hypertrophied and irregular cells. F: femur; lac: lateral articular cartilage; me: menisci; TF: tibiafibula

Fig. 5 External morphology (left) and knee-joint histological section (middle and right) of tadpoles of *Pleurodema borellii*. Experiment C. Stage 44, note the hyaline cartilage with large lacunae and irregular borders, poorly organized, almost without interlacunar matrix. The joint tissue is unrecognizable due to the high level of damage and there are no differentiated elements of the articulation. F: femur; TF: tibiafibula

Fig. 6 Histological section of femur epiphyses of tadpoles of *Pleurodema borellii*. Experiment D. Stages 43 to 46. Note the damage in the articular area and in the lateral articular cartilage of the epiphysis of femur. F: femur; lac: lateral articular cartilage; m: muscle

Fig. 7 Histological section of femur epiphyses of tadpoles of *Pleurodema borellii*. Experiment E: Stage 40 (left), Stage 44 (right). Note the completely irregular articular area and the injury in the lateral articular cartilage of the epiphysis of femur. F: femur; lac: lateral articular cartilage; m: muscle; ocl: osteochondral ligament.

Fig. 8 Experimental juvenile and metamorphic individuals showing anatomical consequences of mobility reduction. Fingers-fixed flexion, finger in palm, pronation of elbow, rotation of shoulder, abnormal extension of knee

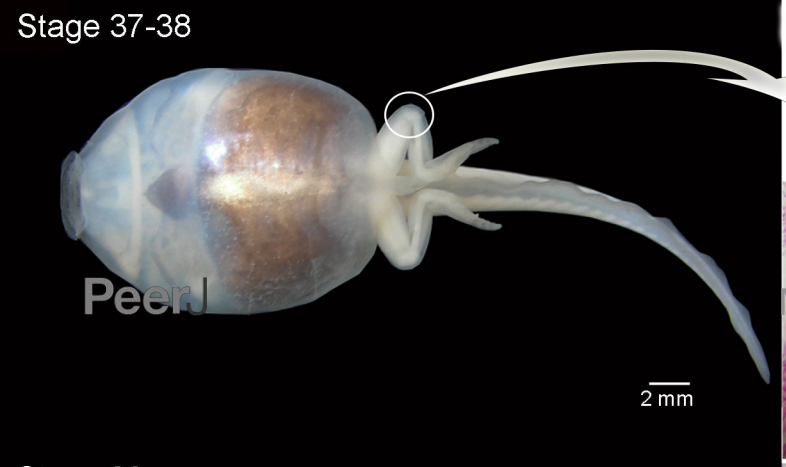
Fig. 9 Scheme showing the results of the three experiments. The red line indicates the appearance of abnormalities at Stage 40–42 in the five experiments

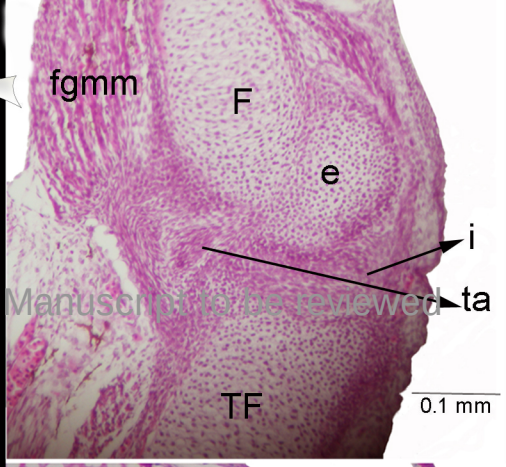
Online Resource 1. Video showing juvenile specimen reared in agar medium until metamorphosis and then transferred to water, with difficulty in its normal locomotion

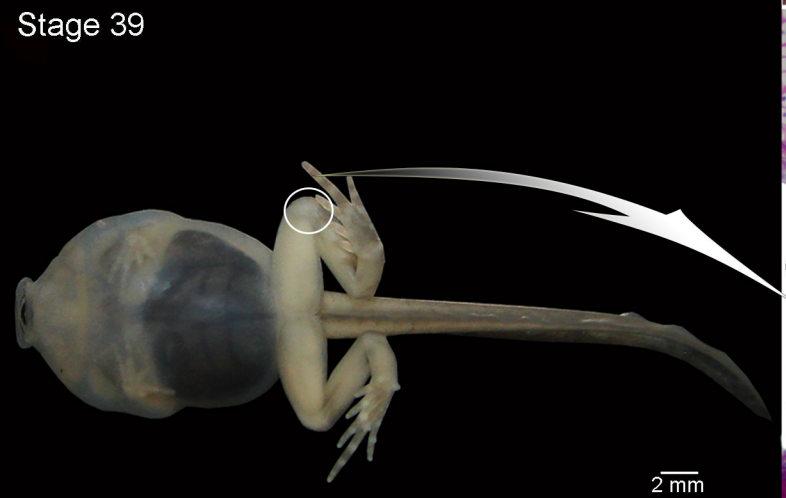
Figure 1(on next page)

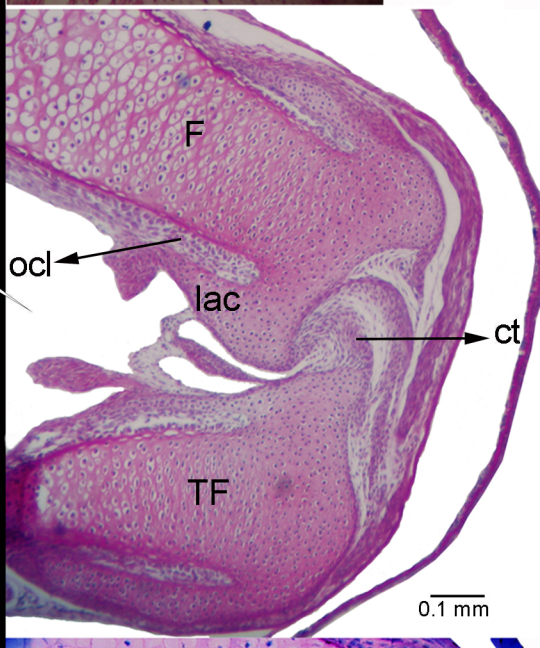
Fig. 1 Control specimens (left) and histological samples (right) showing the knee-joint area in successive developmental stages.

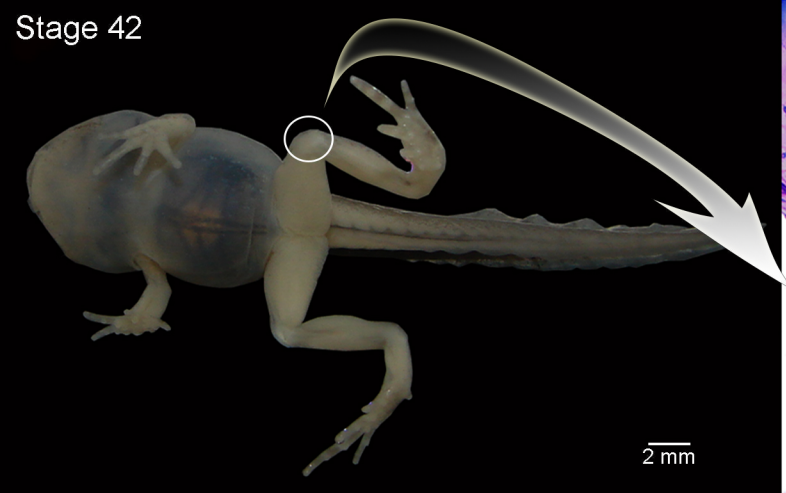
Stage 37–38; in the histological sample muscle tissue still not completely differentiated is observed; in the femur and tibiofibular epiphyses hyaline cartilage is evident, consisting of chondrocytes immersed in a basophile matrix composed of fibers and ground substance. Mesenchymal tissue surrounds the long bone epiphyses, forming a regular tissue in the presumptive knee articulation area, where the long-bone articular surfaces begin differentiation. Joint formation is evidenced by the interzone, a close package of mesenchymal cells. The graciella sesamoid is embedded in a condensation of dense connective tissue or tendon anlage (future tendinous tissue). Stage 39; in the histological sample the cavitation process in the knee-joint led to a physical separation between the articular surfaces. In the cartilage of the long bones the resting, proliferating and hypertrophic zone can be distinguished. The osteochondral ligament and the articular lateral cartilage are differentiated in the epiphyses of femur and tibiafibula. The future tendinous tissue still shows appearance of undifferentiated connective tissue. Stage 42; the elements of the knee-joint, cavity and shape of the epiphyses, are already formed. Stage 45; the histological sample was stained with Mallory trichome, which allows us to observe the mature tendon in blue. The tissues (cartilage, muscle and tendon) are completely mature at this stage. ct: connective tissue; e: epiphysis; F: femur; fmgm: future gracilis major muscle; HZ: hypertrophic zone; i: interzone; lac: lateral articular cartilage; ocl: ostechondral ligament; ta: tendon anlage; PZ: proliferating zone; RZ: resting zone; TF: tibiafibula

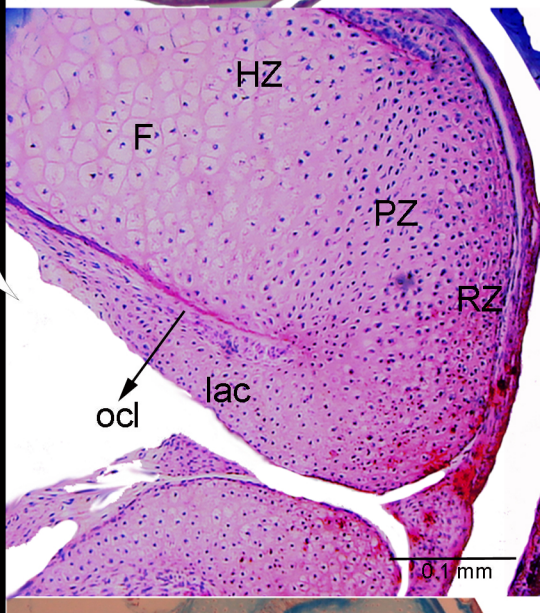


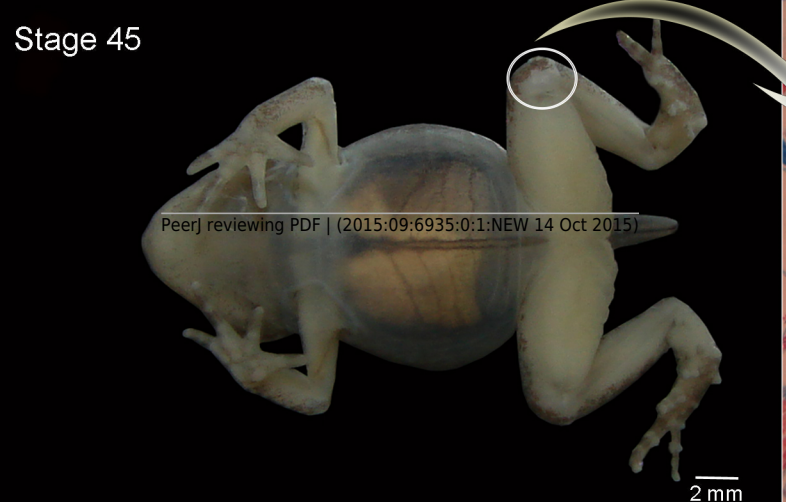












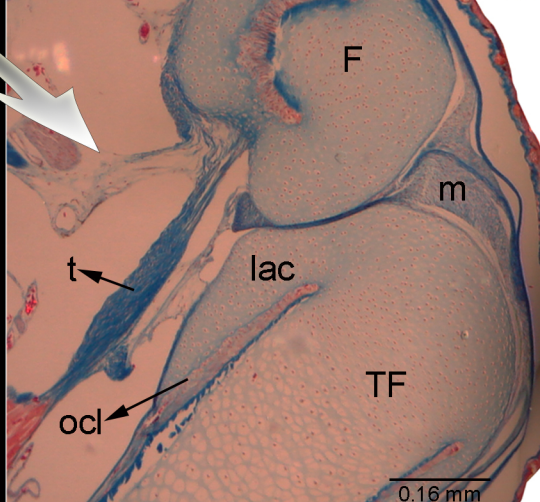


Figure 2(on next page)

Fig. 2 Histological section of knee and ankle joint of tadpoles of *Pleurodema borellii*.

Experiment A. Stage 38 and Stage 39. No evidence of malformation or abnormality is observed in the tissues of the hindlimb in the first stages studied. F: femur; Fe: feet; g: graciella sesamoid; lac: lateral articular cartilage; plm: plantaris longus muscle; ta: tendon anlage; TF: tibiafibula; T-F: tibiale and fibulare

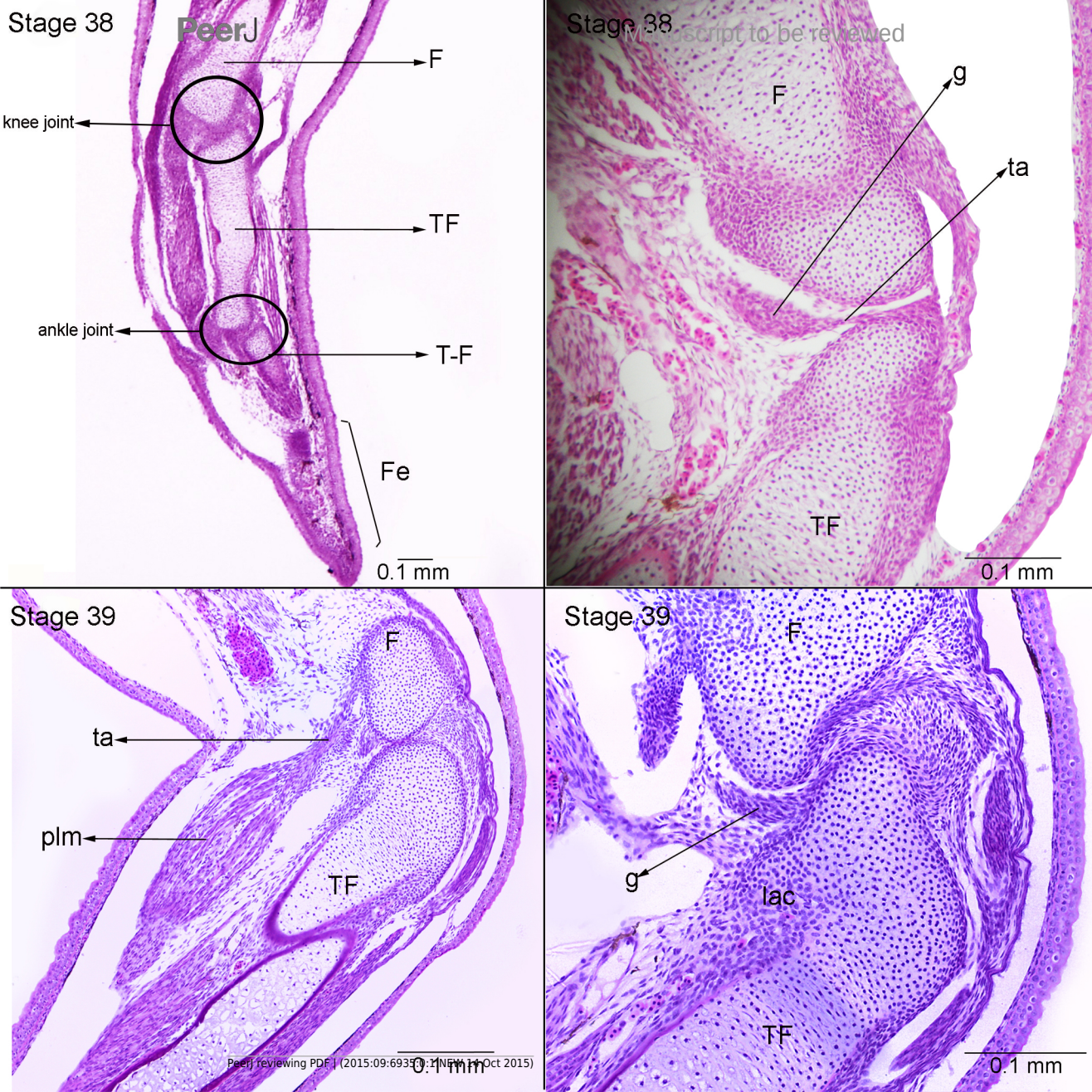


Figure 3(on next page)

Fig. 3 External morphology (left) and knee-joint histological section (right) of tadpoles of *Pleurodema borellii*.

Experiment A. Stage 42 and Stage 43, the articular areas of the epiphyses are malformed, and the hyaline cartilage shows flattened cells with irregular borders. Juvenile specimen, note the severely damaged lateral articular cartilage in the detail indicated by the curved arrow. F: femur; TF: tibiafibula; lac: lateral articular cartilage; ocl: osteochondral ligament; g: graciella sesamoid

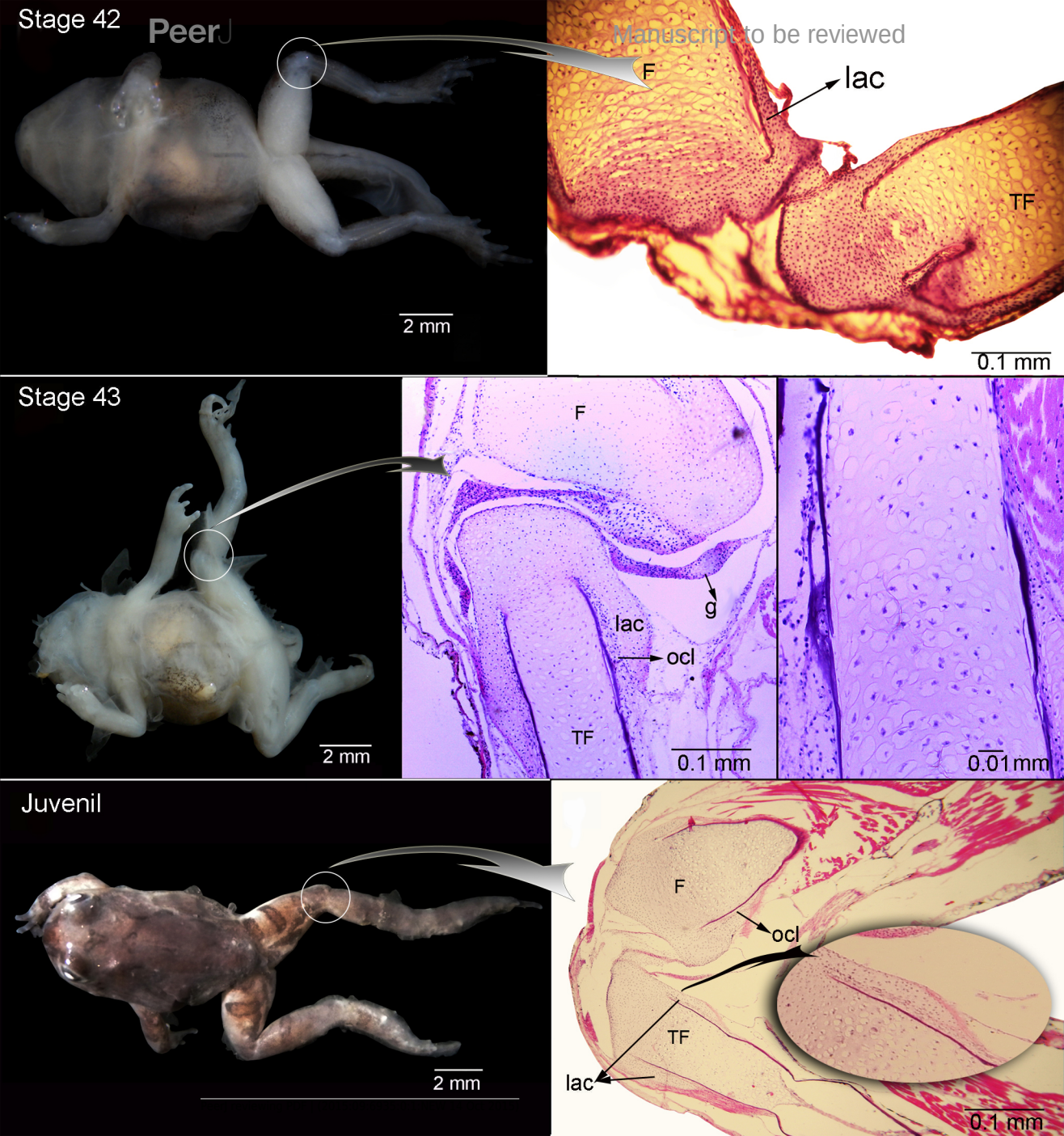
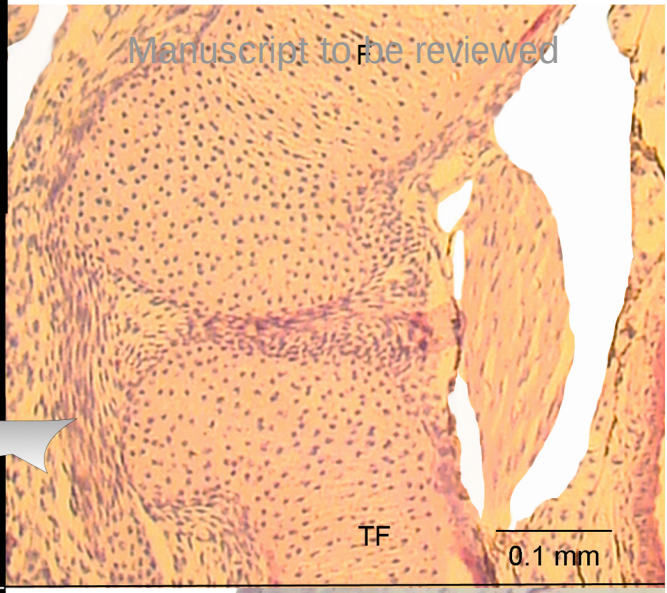
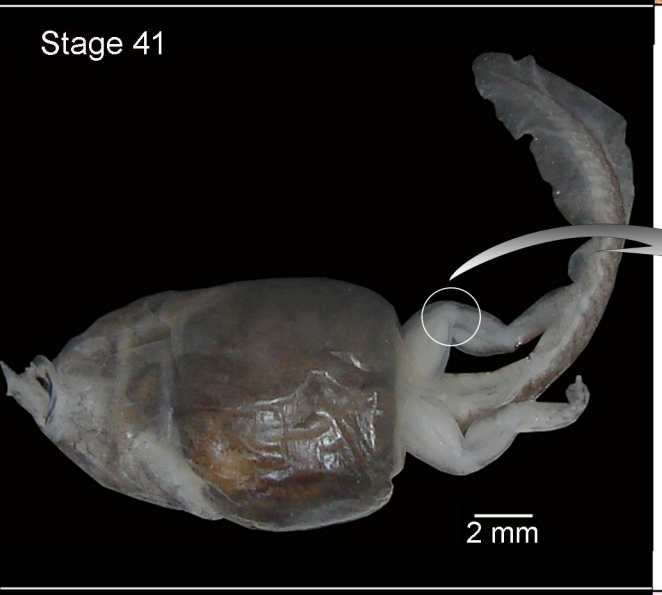
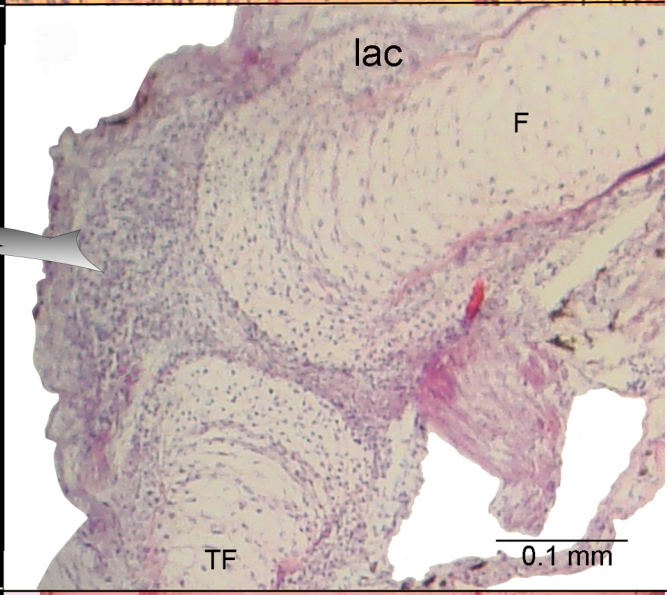


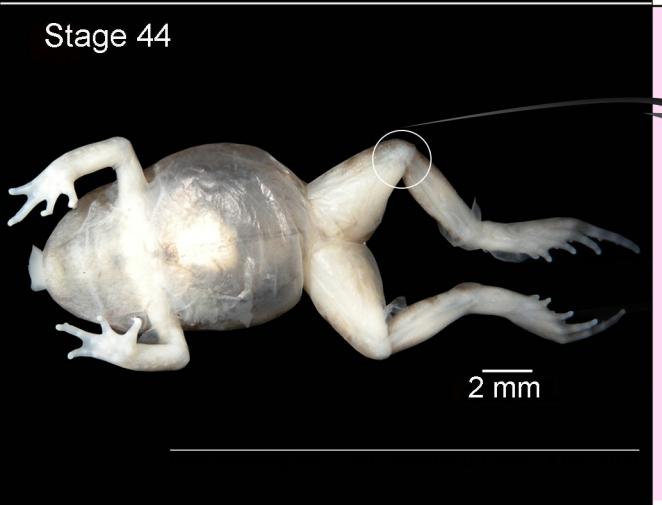
Figure 4(on next page)

Fig. 4 External morphology (left) and knee-joint histological section (right) of tadpoles of *Pleurodema borellii*.









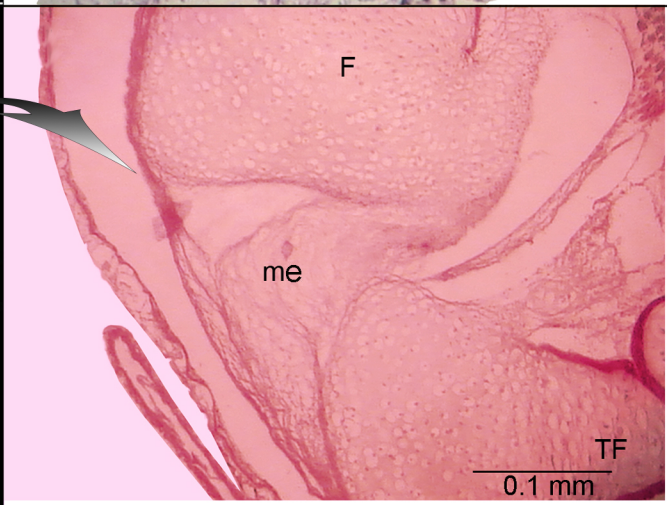
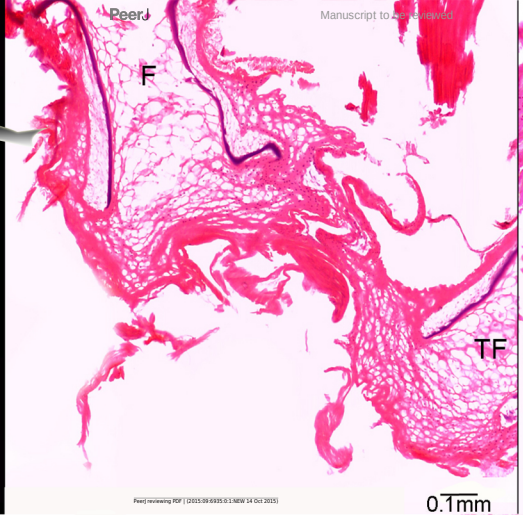


Figure 5(on next page)

Fig. 5 External morphology (left) and knee-joint histological section (middle and right) of tadpoles of *Pleurodema borellii*.

Experiment C. Stage 44, note the hyaline cartilage with large lacunae and irregular borders, poorly organized, almost without interlacunar matrix. The joint tissue is unrecognizable due to the high level of damage and there are no differentiated elements of the articulation. F: femur; TF: tibiafibula



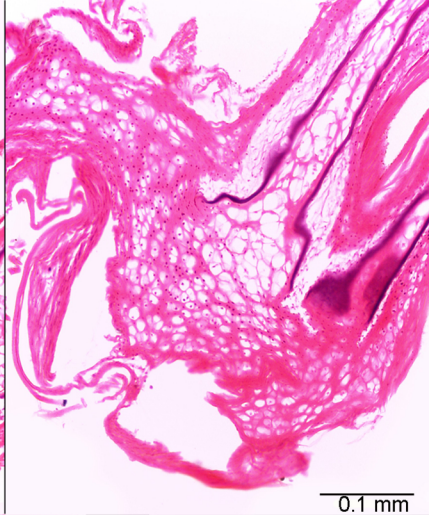
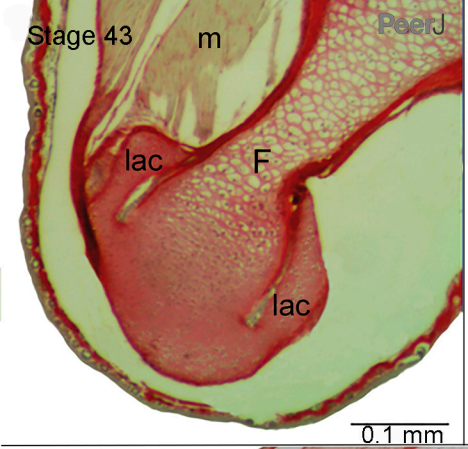
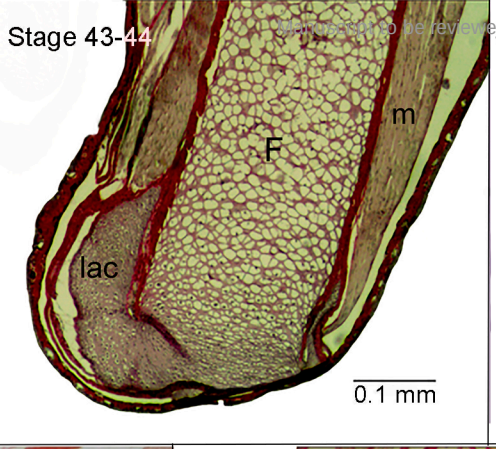


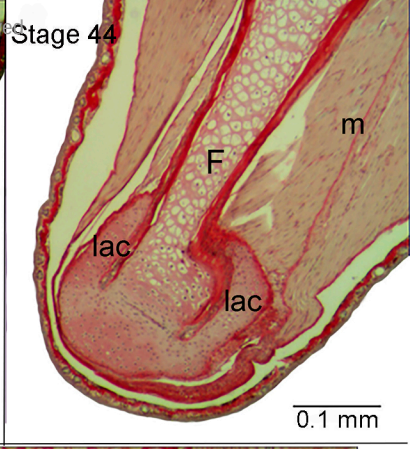
Figure 6(on next page)

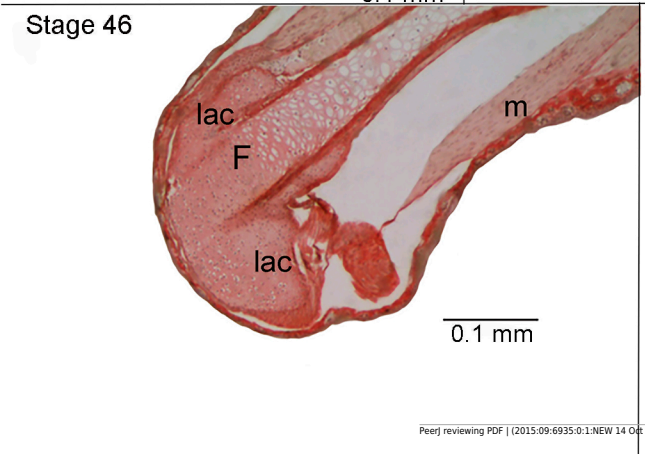
Fig. 6 Histological section of femur epiphyses of tadpoles of *Pleurodema borellii*.

Stages 43 to 46. Note the damage in the articular area and in the lateral articular cartilage of the epiphysis of femur. F: femur; lac: lateral articular cartilage; m: muscle









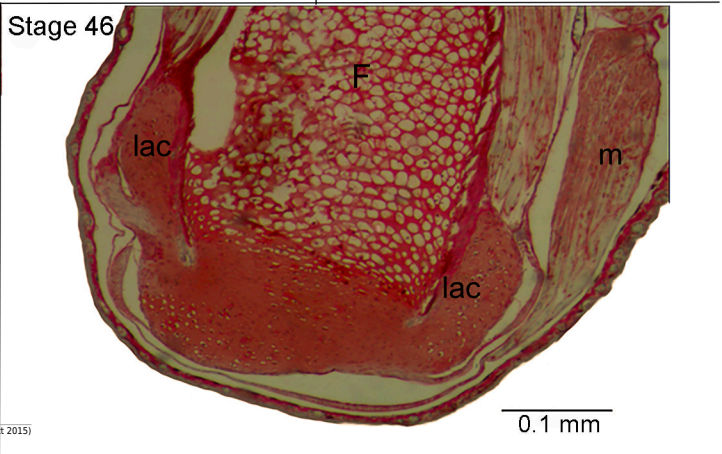
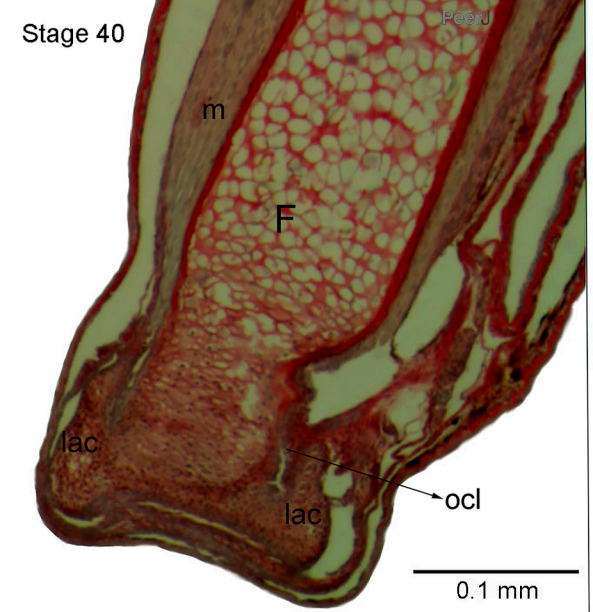


Figure 7 (on next page)

Fig. 7 Histological section of femur epiphyses of tadpoles of *Pleurodema borellii*.

Experiment E: Stage 40 (left), Stage 44 (right). Note the completely irregular articular area and the injury in the lateral articular cartilage of the epiphysis of femur. F: femur; lac: lateral articular cartilage; m: muscle; ocl: osteochondral ligament.



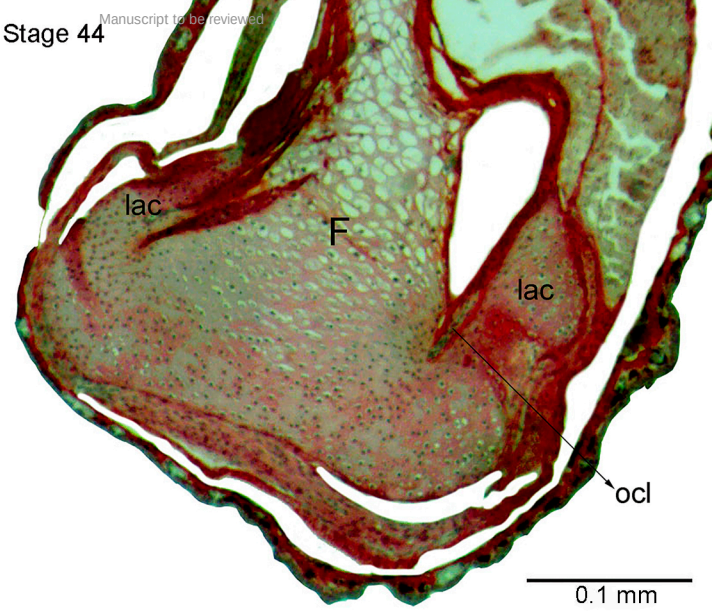


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Fig. 8 Experimental juvenile and metamorphic individuals showing anatomical consequences of mobility reduction.

Fingers-fixed flexion, finger in palm, pronation of elbow, rotation of shoulder, abnormal extension of knee

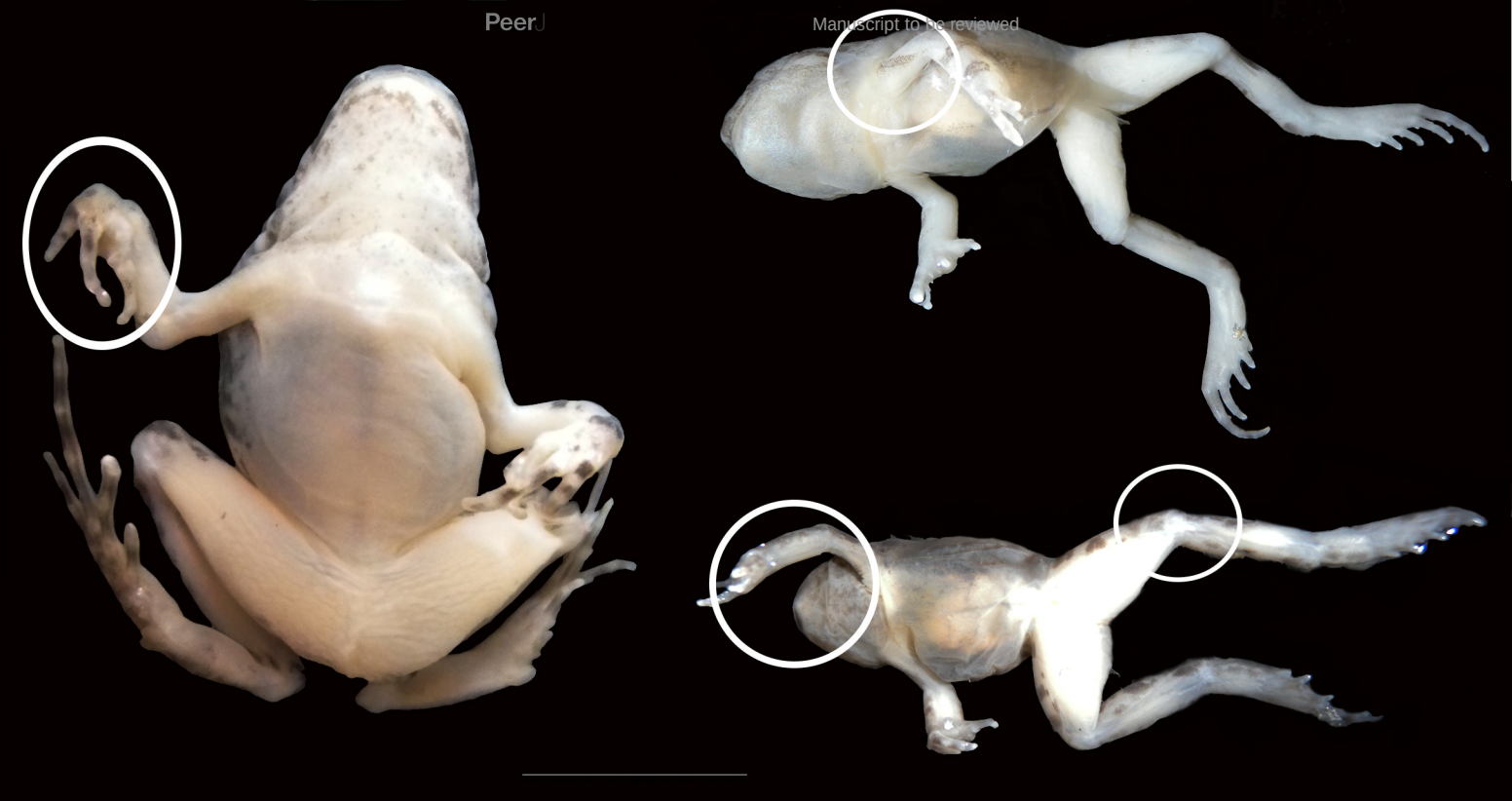

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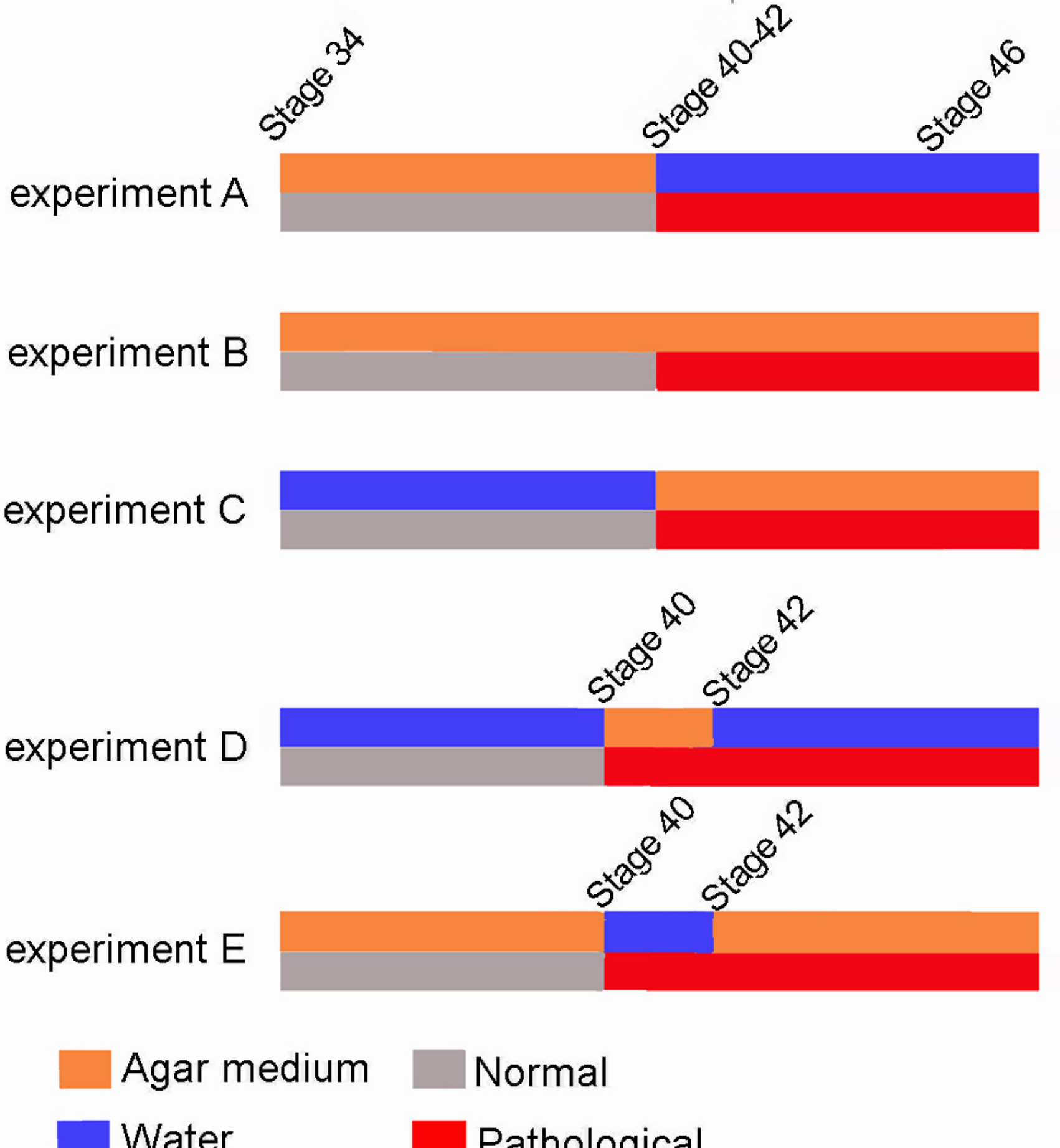
Fig. 9 Scheme showing the results of the three experiments.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Stage 40-42
Stage 46
experiment A
experiment B
experiment C
Stage 40
Stage 42
experiment D
Stage 40
Stage 42
experiment E
Agar medium
Normal
Water
Pathological