Intraspecific variation in *Pelophylax ridibunda (Rana ridibunda*) in Southern Iran: life history and developmental patterns

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ABSTRACT

This study describes the influences of variations in life history traits and developmental patterns of *Pelophylax ridibunda*. The causes of this variation were investigated by comparing observed patterns of interpopulational differences from geographic variables. The aim of the study was to investigate whether embryos and larvae from different populations of *P. ridibunda* show the same pattern of development when they are reared in the same conditions in the laboratory and to find out further whether if any differences appearing in the pattern among populations is environmentaly or geneticaly based. Egg masses of *P. ridibunda* were collected from four sites in Fars Province, Iran and reared in the same conditions. Samples from most of the developmental stages were fixed and clutch parameters were measured at early developmental stages. Morphological characters in embryos and larvae including egg diameter, growing size of embryos and larvae at different stages, external gill, cement gland and mouth parts structures were examined with light and scanning electron microscopy. Statistical analysis showed that the mean diameter of eggs and jelly coats were significantly different ($p<0.05$) from each other in all four populations. In addition, variation among sites in developmental stage at age of embryos and larvae were found. The results also showed at least three different types of dental formula, two main branches of external gill on each side, and type A cement gland developmental pattern among the populations. Our data suggest that local adaptation may be responsible for life history, and morphometric and morphological variations among eggs, embryos and larvae of *P. ridibunda*. Further study is needed to quantify the relative contributions of the genotype and the environment to embryo and tadpole morphology and to assess the adaptive significance of morphological differences.

KEYWORDS: *Pelophylax ridibunda* , Fars Province, Iran, developmental patterns, local adaptation, intraspecific variation

INTRODUCTION

Intra-specific variation in life history traits provides important information for the consideration of environmental constraints or local genetic adaptations and the evolution of life history (Berven, 1982a, b; Berven and Gill, 1983; Miaud et al., 1999; Palo et al., 2004; Richter-Boix, et al., 2011).

Environmental variation during development mostly has long-term impacts on the phenotypes of organisms. In some organisms, such impacts may transfer from one life stage into the next stages, Influencing the success of reproduction and survival of the population. Although there has been extensive research reporting the effect of environmental variations during the larval stage transition to adult stage of amphibians (e.g. Hillis, 1982; Travis, 1983; Jennings and Scott, 1993; Laurila, Pakkasmaa, and Merila, 2001), limited research has followed how environmental variation during the egg stage impacts embryonic or larval development, metamorphosis or adult phenotype (Broomhall, 2004).

Numerous biotic and abiotic factors have been reported as influencing intra-specific differences in development and growth of amphibian embryos (e.g. Licht, 1975; Ryser, 1996; Voss, 1993). Temperature is amongst the most important factors affecting intra-specific variation in the developmental rate of embryo, and is most influenced by geographical differences (e.g. Frisbie, Costanzo, and Lee, 2000; Sanuy, Oromí, and Galofré, 2008).

Higher developmental rates and shorter hatching times with increased temperature have been reported in a number of amphibian species including *Lithobates pipiens*, *L. sylvatica* and *L. palustris* (Moore, 1939), *Ambystoma maculatum* (Voss, 1993), and *Rana temporaria* (Laurila, Pakkasmaa and Merila, 2001; Laugen, et al., 2003). Moreover, some intra-specific studies have also shown that the rate of embryonic development is influenced by altitude and /or latitude (Licht, 1975; Ryser, 1996; Laugen, et al., 2003). For example those species and individuals at high altitudes were reported to hatch later as the higher altitude areas have colder water temperatures (e.g. Berven, 1982b; Howard and Wallace, 1985; Morrison and Hero, 2003).

Among other water indicators, spawn could be sensitive to acidity. Anurans are highly sensitive to low pH during the fertilization and embryonic development stages (e.g. Pierce, Hoskins, and Epstein 1984; Clark and Hall, 1985; Beattie,Tyler-Jones and Baxter,1992). Low pH causes developmental abnormalities (e.g. Pierce, 1985; Portnoy, 1990), impacting perivitelline space to shrink and therefore affecting on embryos to become curled within the

limited space. Prevention of embryo hatching is another effect of low pH (Freda and Dunson, 1985).

On the other hand, high levels of pH can reduce the size and developmental stage at age of embryos, causing a delay on hatching time, producing abnormal hatchlings, and arrest development in its early stages (Tyler, 1994).

Although there are some reports on morphological differences among the adult populations of the green frog *Pelophylax ridibunda*, Pallas 1771 (e.g. Frost, 2010; Gül, et al., 2011), important aspects of intra-specific life history variation in *P. ridibunda* including embryonic and larval development have been poorly documented. In addition, there have been no studies specifically investigating the possible differences in developmental patterns that may occur among different populations of the same species when they are examined in the same conditions within the lab.

P. ridibunda is a species found in open landscapes and develops in both still and running water. This species has several spawning periods from late winter, throughout spring and into early summer in Iran (Nokhbatolfoghahai, 2009).

Green frogs are distributed throughout most parts of Europe and Asia: from Russia to Afghanistan, Iran, and Pakistan and to Far East countries such as China.

In Iran, the green frog is reported as having been observed in all Provinces except Sistan & Balouchestan Province, from below sea level to 2100 m (Baloutch and Kami, 2007).

The aim of the present study is to examine whether embryonic and larval development of four Iranian populations of *P. ridibunda* indicate the same pattern when they are reared in the same conditions. In order to exclude environmental effects and predation pressure, an experimental design in the laboratory was chosen.

In this paper, we examine and summarize the life-history characteristics of *P. ridibunda* populations living in different habitats in one of the main provinces (Fars) in Iran. The characteristics examined are: embryonic and larval development; size and developmental stage at age of egg, and clutch/egg size.

MATERIALS AND METHODS

Sites of collections

P. ridibunda spawn was collected from four different ecological sites in Fars province: Polberenji, Mehkoyeh, Kohmare, and Ghadamgah. Based on previous records, these sites are among the most suitable and accessible breeding sites. The four studied sites were located at different altitude and latitude, as well as differing in habitat quality. From the four localities examined, Mehkoyeh and Kohmare belong to undisturbed natural habitat. Polberenji and Ghadamgah are placed in the boundary of the agricultural fields (within a radius of 1 km). In all habitats except Polberenji the frogs and the spawns were available in permanent wetland. Egg clutches were found in Mehkoyeh and Kohmareh in slow current streams and in Ghadamgah in a permanent pool. In Polberenji the spawns were found in temporary pools.

A map of Fars Province **(Fig.1)** shows localities from which the specimens were collected. The distance between the populations studied here varies from 12 km to 150 km.
Field and laboratory studies were carried out over a two-year period (2007-2008). Environmental measurements, including water analysis made during each collection, as well as other times of field work were taken of pH, conductivity, salinity, and temperature at each ecological site using a multimeter water analyzer, USA Hack. Water temperatures were measured at a depth of 20 cm at the time when the egg clutches were collected and the average of temperatures was obtained during the period between April to July in each year when we were sampling.
Observations of egg deposition sites, including counts of numbers of eggs per clutch and attachment characteristics, were recorded at each site. The clutches used in the experiments are the same as those recorded in **table 2**.

Egg collection, incubation, and fixation

Spawn was collected from a pond in Polberenji, from streams in Mehkoyeh and Kohmare, and from a spring/pond in Ghadamgah between late April and early July in each year.
Then the spawn was transferred to the laboratory within 1-4 hours, depending on the site of collection. Spawn in the laboratory were maintained in aluminum containers (25 cm W. × 45 cm L. × 5 cm H.) in identical conditions of food, light (12 hrs light & 12 hrs darkness), temperature (25 °C ± 1.0), pH (8.32), salinity (0.1) and the number of samples per liter of water. The spawn collected from each area were subdivided and incubated in three tanks,

each tank holding approximately three liters of water and incubating 100 eggs. The containers were filled with dechlorinated and aerated tap water. Once the eggs hatched, the tadpoles at the feeding stage were fed with fresh lettuce. The amount of food was about 15g for each container for the first week, and about 30g for the second week and thereafter.

At different stages of development, samples were taken for fixation in order to examine the morphological and biometrical patterns of most Gosner (1960) stages, from approximately early cleavage (stage 2-3) to a larva at stage 25. Specimens for light microscopic examination were fixed in buffered neutral formalin or Bouin's fluid and for scanning electron microscopy (SEM) were fixed in 2.5% glutaraldehyde in phosphate buffer.

The stage and the time at which hatching occurred in each clutch of different populations were also noted. Hatching time was counted when approximately 50% of total eggs were hatched. Hatching stage was assessed by randomly choosing 30-40 of the newly hatched larvae from each clutch.

The mortality rates were also measured by the number of dead samples divided by the total number of samples.

Clutch parameters

Some clutch parameters, including egg numbers in each clutch and egg diameters, were investigated. Thirty – three fixed eggs from each clutch were chosen from the first day of development at Gosner stage 10 to measure the mean diameter of the eggs and the thickness of their jelly coats. To estimate developmental stage at time, we assumed the eggs had been fertilized at midnight of the night prior to the collection of fresh spawn.

Specimen staging and examination

The jelly coat and fertilization membrane were removed from fixed specimens using forceps or filter paper. They were then staged using Gosner's (1960) developmental table. Specimens for microscopic examination were fixed in Bouin's fluid or 2.5% glutaraldehyde in phosphate buffer. Measurements of fixed embryos were made using a dissecting microscope with calibrated eyepiece graticule.

Size and developmental stage at time

Body measurements (body length, total length, tail length) were taken of a series of embryos and larvae; these measurements were taken from specimens fixed in Bouin's fixative solution and stored in 70 percent alcohol.

Growth parameters including body length, total length, and developmental stage according to Gosner (1960) were measured daily from fixed samples(5 to 30 tadpoles based on availability) until early feeding tadpole stage and analyzed by the one way ANOVA test using SPSS version 11 looking for any developmental pattern differences between clutches from different sites. Size at time of embryos and larvae were calculated by measuring the different length of larvae at different times Developmental stage at time of embryos and larvae were also calculated by measuring the different developmental stages at different times.

Biometry and morphological analyses of larvae

Biometry of 15 larvae at stage 25 from each population was carried out, based on Altig and McDiarmid (1999). We measured tadpole total length (TL), body length (BL), inter-orbital distance (IOD), inter-narial distance (IND), tail length (TAL), tail muscle width at the base of the tail (TMW), tail muscle height(TMH) and maximum tail height (MTH).

Differences in tadpole morphology among habitats were evaluated considering the variable of natural population at each site.

Scanning Electron Microscopy (SEM) preparation and examination

Glutaraldehyde-fixed specimens for scanning electron microscopy were post-fixed in 1% osmium tetroxide, stained in 0.5% aqueous uranyl acetate, dehydrated in an acetone series, then critical-point dried, and coated with gold with a Polaron SC 515. They were then examined with a JSEM 6400 scanning electron microscope. Morphological characters of embryo including cement gland and external gill were examined, from different views over a magnification range of 24X– 400X and recorded by Image-slave for Windows (Meeco

Holdings, Australia). Because of time and budget limitations, SEM photographs were taken only from one of the populations (Kohmareh).

Morphological assessment of mouth parts

In order to investigate any differences in dental formula, 15 samples from Mehkoyeh and 15 samples from Polberenji and 6 samples from Kohmare at Gosner stage 25 (the stage when the tooth rows are well developed) were randomly chosen from lab reared tadpoles and analysed comparatively. No sample from Ghadamgah was available at this stage.

Statistical Analysis

The data obtained from the numbers and percentages of the eggs, embryos and larvae were analyzed by the one way ANOVA test using SPSS version 11. Growth parameters such as embryonic and larval length, and biometric characters of larvae were analyzed through multiple comparisons and Tukey's procedure. Linear regression test was used to analyse any correlation between clutch size and altitude amongst different populations. All tests were done at 5% level significance.

RESULTS

Collection site characteristics

Geographical characteristics and water analysis for all four ecological sites are shown in **Table 1**. Analysis of the water in four ecological sites during late April to early July showed that the highest pH (10.68 ± 1.07) was found in the Polberengi site.

Water temperature ranged from 11.6 ± 1.4 ºC in Kohmare to 22.7 ± 3.2 ºC in Polberenji at same time.

The highest level of salinity was found in Polberenji (2.1 ± 0.12 mg/lit) and the lowest in Ghadamgah (0.1 ± 0.005 mg/lit). The altitude varied from 1400 m in Polberenji to 2500 m in Kohmare.

Clutch parameters

Table 2 shows the clutch parameters and hatching times in different populations. The mean number of eggs per clutch varied from 900 in Polberenji to 2500 in Ghadamgah . The mean diameter of eggs of all clutches collected from each site was calculated and was highest (2.22 mm ± 0.28) in Mehkoyeh and lowest (1.55 ± 0.16) in Ghadamgah. The mean thickness of jelly coat varied from (1.01 ± 0.32) in Kohmare to (0.12 ± 0.08) in Ghadamgah.
One-way ANOVA showed that the mean diameter of eggs and jelly coats were significantly different ($P<0.05$) in all four populations. The hatching time varies as embryo hatch after 41 hours in Ghadamgah, and after 50 hours in Mehkoyeh. Hatching stage also varies between stage 18 (in Polberenji and Ghadamgah) and stage 19 (in Mehkoyeh and Kohmareh).
Tukey test showed the thickness of jelly coats were significantly different from each other among the four populations. The egg diameter was significantly different among populations except between Mehkoyeh and Polberengi. The total egg size (i.e. egg diameter + jelly coat) was significantly different between each other among the four populations.

Developmental stage and size at time

Developmental patterns for all populations were described stage by stage.
Figure 2 shows the developmental stage at time in the four populations. Developmental stage at time did not differ greatly among the three populations (Ghadamgah، Mehkoyeh، Polberenji). The progression in developmental stages was directly correlated to the time at least up to stage 25 and the graph shows a linear pattern. However the graph of Kohmare shows tadpoles have fast growth in early stages then a slow growth in mid stage and again a fast growth in late stage. Tadpoles in Ghadamgah showed a slightly lower rate of development compared to the three other populations. The reason for the slowest and the quickest rate of development respectively between Kohmareh and Ghadamgah populations may be due to differences in salinity and temperature as well as differences in altitudes. Fig. 3 shows size at time based on total length of embryos and larvae in all populations (the lack of data for later stages of larvae in Kohmareh and Ghadamgahdidn’t allow us to consider growth patterns completely in these two populations. Therefore, the interpretation given bellow is only for Mehkoyeh and Polberenji.

For size at time, total body length increases as the larval stage grows up, but the rate of growth is different among the twopopulations. The relationships between time and body length were shown in a linear pattern in general.Figure 3 shows the rate of growth in size are almost similar at early and mid stages of development, but there is faster growth in the larvae of Mehkoyeh compared to the larvae of Polberenji. Different growth patterns may be causes by differences in some abiotic factors in the nature of these populations including pH, salinity and temperature, which are all higher in Polbrenji than Mehkoyeh habitat. The significant bigger length of larva in Mehkoyeh than Polberengi is due to the faster growth of the tail and developed longer tail at stage 25 in Mehkoyeh population compared to the Polberenji population (Table 3.).

High mortality rate (22.2 %) was found from Polberenji samples. The mortality rate for Ghadamgah and Mehkoyeh was 20% and 10.9 % respectively. No mortality was found among the samples from Kohmareh collected samples. Mortality stage for the all three sites was found in cleavage stages.

Morphological study of oral discs

Figure 4 shows diagrams of the oral disc of *P. ridibunda* from the populations of three different ecological sites. The dental formulae of tadpoles at stage 25 were determined and revealed differences in the number and situation of dental rows among populations. The dental formula for the samples of Mehkoyeh was 1/3(1), Polberenji was 1[1]/2[2] and Kohmare was 1/2. The dental formula of a tadpole depicts the number and arrangement of tooth rows on its oral disc. In this case, the number on the left and right of "/" indicates the number of tooth rows on anterior labium and posterior labium respectively. The number in parentheses is the number of interrupted rows (gap) in order of arrangement on the labium as a stable condition. But the number in [ ] is the number of interrupted rows (gap) in order of arrangement on the labium where there is variability, which was found in Polberenji samples in the first of anterior labium and second tooth row of posterior labium.

Morphological studies of external gill and cement gland

Figure 5 shows SEM photographs of Mehkoyeh samples from stage 22 to 24. The figures revealed two main branches of external gill on each side and 6 filaments oN main branches

on each side. The external gills in *P. ridibunda* are relatively poorly developed with intermediate ciliated cell density.The figures also show Type A of cement gland developmental pattern base on the Nokhbatolfoghahai & Downie (2005) reported. In this type the V-shaped groove flattens to a U, with the whole gland being M-shaped; then the two arms separate to form almost oval-shaped structures, each with a central anterior–posterior groove, so that each has a horseshoe shape overall (figure 5 b). The groove then slowly disappears and each structure becomes nearly circular before disappearing (figure 5c).

Morphometrical analysis of larva

Among the 15 characteristics of larva (n=30) of Mehkoyeh and (n=15) of Polberenji, the Tukey test showed significant differences ($P < 0.05$) in the following characteristics among the two populations:

Total length (TL); Tail length (TAL); Maximum tail height (MTH) and Tail length to TL (TAL.TL); were significantly bigger in Mehkoyeh compared to Polberenji. Body length to TL (BL.TL); Inter-orbital distance to TL (IOD.TL); Tail muscle width to TL (TMW.TL); Inter-narial distance to TL (IOD.TL) and tail muscle height to TL (TMH.TL) were significantly bigger in Polberenji compared to Mehkoyeh (**Table 3**). Results show that although the BL.TL is significantly bigger in the tadpoles of Polberenji than Mehkoyeh, the TL and the TAL.TL are significantly larger in the tadpoles of Mehkoyeh than Polberenji, which means the bigger tadpoles in Mehkoyeh is related to the bigger tail and not to the size of body trunk. Longer tails along with deeper tail (MTH) are suggested to be better adjusted to the stream habitat (the habitat of Mehkoyeh population).

DISCUSSION

Species often show high variation in life-history traits amongst populations (Mayr, 1963; Endler, 1977; Berven, Gill and Smith-Gill, 1979; Laugen *et al*., 2003). A single genotype may produce various phenotypes when they are in different environmental conditions. A characteristic is canalized when it adapts well to an environment, but it may be plastic when a characteristic is well-adapted to different environmental conditions (Pigliucci, 2001).

This paper investigated differences in some life history features, including egg development established by uniform laboratory culture, among four populations of *Pelophylax ridibunda*,

and attempted to account for these differences in terms of selective variation among the habitats of these populations.

Apart from certain life history characteristics such as clutch size, egg diameter and egg jelly thickness, which are directly correlated to natural habitat conditions, other characteristics, in particular egg development, were expected to show similar behaviour among different populations due to being treated in the same conditions in the lab. Significant differences in some aspects of clutch parameters such as egg diameter as well as differences in developmental and growth rates of the populations and many characteristics of larva including TL; TAL.TL; IOD.TL show that geographical effects in these eggs still being expressed in the controlled conditions, which could be suggested that at least in some small scale, genetic differences are seen in populations at different sites. The differences in the dental formula of larvae among the populations could also support some sort of genetic variations among the populations.

Water qualities at different ecological sites

Some studies show that anurans with aquatic eggs can be highly sensitive when exposed to aquatic contaminants (Holcombe et al., 1987; Hall and Henry, 1992). Anurans will also only develop in a species specific pH and temperature threshold.

In the water that we analyzed from four different ecological sites, there were variations in salinity, temperature and pH of water. Temperature is believed to influence the thickness of egg jelly. Kluge (1981) reported that most anuran species are more likely to spawn eggs with thicker egg jelly in a higher temperature than in a lower temperature. Although this finding is contradictory to our findings where the thickest egg coat is from the site with the lowest temperature, we suggest that in both the cold and warm waters the thicker jelly coat surrounding the eggs act as an effective isolator and protect the growing embryos from the harmful effects of low and high environmental temperature, Hassinger (1970) studied the effect of surrounding water temperature on the eggs of two species (*Rana sylvatica* and *R. pipiens*) and pointed out the role of jelly coat as an isolator and protector of eggs from environmental variable temperatures.

Among other water indicators, eggs could be sensitive to high level of pH, that can reduce the length and developmental stage at time of embryos and larvae (Tyler, 1994). Reduction of size in the larvae of Plberenji may cuse by high level of water pH (10.68) in this area.

Gomez-Mestre and Tejedo (2003) showed that adaptation to salinity stress increases when embryos and larvae of some anuran species such as *Bufo calamita* are treated to high concentrations of saline. It has also been shown that increasing water salinity will reduce the life of embryos and larvae. In our experiment, the spawn were collected from different water salinities (0.1, 0.3, 0.4 and 2.1 mg/lit), then they were transferred to the same water salinity (0.5 mg/lit) in the lab. Of the four ecological sites, the salinity of the Polberenji samples was the highest (2.1 mg/lit). The high mortality (22.2 %) found in the population of Polberenji could have resulted from a high salt concentration as well as high pH and temperature predisposed naturally in this breeding site.
It could also suggest that tadpoles from this site would be genitically predisposed to survive better in high salinity and pH and not do as well in lab conditions because they were being raised in lower salinity and pH.

Clutch size and egg size

Studies show that in most of the anuran species the size of a clutch (number of eggs in each clutch) and the diameter of the egg are correlated to the food availability, size and age of the mother, female reproductive activities and the spawning site (Crump, 1974; Duellman and Trueb, 1994). Although we had no information about the age and size of the adult female *P. ridibunda*, our examination of the clutches from different sites showed that the larger clutches had smaller egg size compared to the smaller clutches, probably due to the mother saving energy for her growth and survival as well as survival chances of resultant offspring based on r/K strategy trade-off (Castellano et al., 2004).

Egg size is determined by the amount of energy available for reproduction (i.e. size and body conditions of the female) as well as external factors such as habitat variation (Salthe and Duellman 1973). Egg size affects offspring survival by influencing the size, development and growth rates of embryo during embryonic development (Kaplan and King 1997). For example larger eggs produce larger embryo and larva as well as longer duration of development. Our results showed that there are significant differences ($P < 0.05$) in egg diameter and jelly coat thickness amongst the four populations. Mean clutch size in the Ghadamgah samples was lower than that in the other samples but higher in egg size

compared to the other three sites. Data analysis for each single clutch also showed this association. For example, Kuramoto (1978) studied 12 amphibian speciesmostly from two families Ranidae and Hylidae in Japan. He found that the mean diameter of eggs in smaller clutches are bigger both inter specifically and intraspecifically. The correlation between altitude and clutch size in amphibians are often contradictory in that there is no clear overall trend (Kozlowska 1971, Berven, 1982; Ryser, 1996; Morrison, and Hero, 2003;). Some studies reported that higher altitude with larger clutches (Berven, 1982 in *Rana sylvatica*); Ryser (1996) reported no difference in clutch size in populations of *R. temporaria* from different altitudes in Switzerland. Another study has reported larger clutch sizes at low altitudes (Kozlowska 1971 in *R. temporaria*). However Morrison, and Hero (2003) found in their literature review that most of this variation in clutch size, , can be related to variation in female body size.

The pattern of increasing egg size with altitude is well-documented, with no significant difference has been reported between egg sizes found in high- and low-altitude populations. For example, Ryser (1996) found no relationship between egg size and high and low elevation populations of *Rana temporaria*. Morrison and Hero (2002) investigated altitudinal variation in the size of egg and clutch size in five anuran species in mid-eastern Australia. They found no significant difference in the size of the eggs produced by any of the species in different altitudes.

Our limited data also suggests that there is no relationship between altitudes, clutch size and egg size. To confirm the relationship between egg size and altitude, the analysis of a considerable number of individual clutches is required, which was not possible for the present study. Most of these variations in clutch size can be also explained in terms of variation in female body size, age of female which the data is also not available.

We did not find any relation between hatching time and egg size. Thumm and Mahony (2005) suggested that hatching time has very little relation to egg size and is controlled by specific genetic factors as well as yolk quality, which is independent from egg size. This finding could be supports our results.

The size and developmental stage at time

As we reviewed in the introduction, the growth and developmental stage at time of anurans are affected by several biotic factors such as egg size, body size and yolk quantity and quality (Berven, Gill, and Smith-Gill 1979; Duellman and Trueb 1994) as well as by abiotic factors

including temperature, pH, density and competition (Volpe 1957; Licht 1975; Berven, Gill, and Smith-Gill 1979; Kaplan 1980; Andrén et al., 1988; Pahkala et al., 2001, 2002). In our experiment, egg size was the only biotic factor we considered, and the temperature and pH were among abiotic factors in the breeding habitat.

Thumm and Mahony (2005) concluded that egg size is independent from the duration of development but our experiment showed that there was a relationship between egg diameter and the developmental stage at time of embryos and larvae.

The slight differences at the early and mid stages of development are likely to be due to the presence of other variables such as pH and salinity, which the eggs experienced at the earliest stages within the breeding site. For example, the low growth rate of embryos and larvae of the spawn collected from the Polberenji site raised in the lab (see fig. 3) may is due to the high salinity and pH in the Polberenji site. The size at age (total length and time) from different sites is also in a linear pattern and in spite of the same lab conditions for all spawn during growth in the lab,indicated some size at age differences among the different populations. The total length of the samples was shown to be almost the same in different populations, but the differences in the size at age indicated that growth increased in the middle stages of development, which may be related to the differences of trunk or tail growth.

Our experiment also showed that, although the eggs in the Mehkoyeh samples presented as a larger size, there were no significant differences in the size at age of the larvae between this population and the others. Therefore, we can conclude that there is no correlation between egg size and larval growth in *P.ridibunda*.

Morphological study in dental formula of larvae of different populations

Dental formulae are variable among different species of anurans (Jennings and Scott,1993; Khan and Mofti, 1994), but variation among the populations of a single species is controversial. Badpayman (2008) found no differences in dental formulae among four populations of *Pseudepidalea viridis* she studied in Fars Province in Iran. We found in our study three types of dental formulae among the three populations of *Pelophylax ridibunda*. This variation along with other variations we found could potentially contributed to speciation. These differences in dental formula were not due to differences in food quantity or quality because in our study variations in dental formulae were independent from the type of food availability.

Biometrical study of larvae

The biometrical study of larvae at stages G 25 showed significant differences in all larval characteristics between the two populations - Mehkoyeh and Polberenji (table 3).
These two populations are most dissimilar in their environmental conditions (one - natural and the other placed within an agricultural field). However, in the case of existence in these conditions over extended periods their differences can have some genetic base.

Environmental influence

Differences in morphological characteristics observed here may represent, to a large degree, responses to environmental cues during embryo and larval development. The growth and developmental rates showed a direct correlation with the degrees of temperature of nature spawns collected. The size of embryos and larvae in exothermic animals will be where the increased with increasing time in the colder water and in each developmental stage (Zuo et al., 2011). We found significant differences in the total size at stage 25 between larvae that preexposed their eggs in cold water (Mehkoyeh, 18.2) than the larvae which their eggs preexposed to the warm water (Polberenji, 22.7 °C). Further study is needed to quantify the relative contributions of the genotype and the environment to tadpole morphology, and to assess the adaptive significance of morphological differences. To find more information on the natural variation that occurs with these tadpoles in the wild, we need to compare egg development in wild conditions.

In conclusion, there is significant differences in egg diameter (ovum diameter + Jelly coat thickness) among all four populations which could be influenced by local biotic/abiotic effects from the different habitats. There is also negative correlation between Jelly thickness and temperature. There is direct correlation between egg diameter and size of larvae. Therefore, bigger egg diameter in Mehkoyeh population produced larger tadpoles compared to smaller egg diameter in Polberenji that produced smaller tadpole. Data collected from lab examinations where the biotic and abiotic variation were reduced still showed some variation between growing eggs among different populations including total length, tail length, inter orbital and inter nasal distances. There is also some differences in dental formula (mouth

parts) among the larvae from different populations confirming the possibility of genetic variation. Our data suggest that local adaptation may be responsible for life history, morphometric and morphological variations among populations of ***Pelophylax ridibunda*** embryos and larvae.

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Legends

Fig. 1. Map of Fars province showing localities from which the specimens were collected.

Fig. 2. Comparison of developmental stage at time of the four populations collected from the four breeding sites.

Fig. 3. Comparison of mean total length at time of the four populations collected from the four breeding sites.

Fig. 4. General organization of *P. ridibunda* oral apparatus, showing different patterns of tooth rows in tadpoles stage 25 examined from the population of three different ecological sites; (A) Mehkoyeh, (B) Polberenji, (C) Kohmare . A = anterior tooth rows; A gap = medial gap in anterior tooth row; P1, P2 and p3 = first , second and third posterior tooth rows. P1gap= medial gap in first anterior tooth row, P2 gap = medial gap in second anterior tooth row

Fig. 5. *P. ridibunda* in Mehkoyeh population at Stage 22 from lateral view (a); stage 23 from ventral view (b); stage 24 from ventral view (c). Figures show external gill regression and covering by operculum from ventral view at Stage 23, (b) and at Stage 24 (c). The pattern of cement gland development (type A) shows in figure b (horseshoe shape) and figure c (conical shape). Cg = Cement gland; Eg = External gill; M = Mouth; N = Nostril