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Breeding Ecology and Larval Development of Marsh Frogs (*Pelophylax ridibundus* s.l.) from East Mediterranean, Türkiye

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Abstract: Reproductive biology of Marsh frogs (*Pelophylax ridibundus* s.l.) was investigated in the Eastern Mediterranean Region of Türkiye in terms of reproductive phenology, fecundity, clutch size, reproductive effort, and mate choice. Frogs are active for approximately ten months throughout the year and hibernate between November and January. Breeding occurs from mid-January until early June and metamorphosis took 45-65 days, with juveniles observed from the end of March. The tadpole reaches its largest size at stage 38, with a mean total length of 60.61 mm and juveniles have an average snout-vent length of 20 mm at the end of metamorphosis. Secondary sexual characters can be observed on individuals reaching 45 mm snout-vent length and we could not detect any amplexed pairs below this length. The clutch size of spawned egg masses ranged between 144 – 645 and the mean egg size was calculated as 1.73 mm. The mean fecundity was 3853 (ranging between 940 and 6000) eggs and the reproductive effort was 0.094 in females. Females were approximately 16% larger than males and the snout–vent length of males and females of amplexed pairs is significantly different but not correlated. These findings signify random mating instead of size-assortative mating. However, the preference of 6.8% larger females than single ones to mate indicates a size-dependent mating for Western Mediterranean population.

Keywords: Reproductive phenology, reproductive effort, fecundity, clutch size, mate selection, interspecific amplexus.

Doğu Akdeniz, Türkiye'deki Su Kurbağalarının (*Pelophylax ridibundus*-complex) Üreme Ekolojisi ve Larval Gelişimi

Öz: Anadolu su kurbağalarının (*Pelophylax ridibundus* s.l.) üreme biyolojisi, üreme fenolojisi, fekundite, yumurta kümesi büyüklüğü, üreme çabası ve eş seçimi açısından Türkiye'nin Doğu Akdeniz Bölgesi'nde incelenmiştir. Kurbağalar yıl boyunca yaklaşık on ay aktiftir, Kasım ve Ocak ayları arasında kış uykusuna yatar. Üreme Ocak ortasından Haziran başına kadar gerçekleşir. Metamorfoz süresi 45 – 65 gün olarak belirlenmiş ve Mart ayı sonundan itibaren metamorfozunu tamamlamış yavrular gözlenmiştir. İribaşlar, ortalama 60,61 mm total uzunluk ile 38. aşamada en büyük boyutuna ulaşır. Yavrular metamorfoz sonunda ortalama 20 mm burun ucu-kloak uzunluğuna sahiptir. 45 mm burun ucu-kloak uzunluğuna sahip bireylerde ikincil eşeysel karakterler gözlenebilir ve bu uzunluğun altında herhangi bir ampleksus çifti tespit edilmemiştir. Bırakılan yumurta kümelerinin yumurta sayısı 144 - 645 arasında değişmektedir ve ortalama yumurta boyutu 1.73 mm olarak hesaplanmıştır. Dişilerde ortalama fekundite 3853 (940 ile 6000 arasında) yumurta ve üreme çabası 0.094 olarak hesaplanmıştır. Dişiler erkeklerden yaklaşık %16 daha büyüktür ve ampleksustaki erkek ve dişilerin burun ucu-kloak uzunluğu önemli ölçüde farklıdır ancak aralarında bir korelasyon yoktur. Bu bulgular büyüklük-sınıflamalı çiftleşme yerine rastgele çiftleşmenin gerçekleştiğini, bunun yanında, çiftleşmek için çiftleşmeyen dişilere oranla %6.8 daha büyük dişilerin tercih edilmesi Batı Akdeniz popülasyonu için büyüklük-bağımlı çiftleşmenin gerçekleştiğini işaret etmektedir.

Anahtar kelimeler: Üreme fenolojisi, üreme çabası, fekundite, yumurta kümesi, eş seçimi, türlerarası ampleksus.

1. Introduction

Various factors threaten many amphibian populations around the world and they are gradually facing extinction (Beebee & Griffiths, 2005). To prevent extinction or lessen its impact, it is important to implement effective and sustainable management plans urgently (AmphibiaWeb, 2023). To understand the causes of this extinction and disappearance, it is very important to investigate how the diversity of amphibian populations has changed and to implement long-term action plans and urgently needed solutions (Gascon et al., 2007). Understanding the phenology of species is a crucial first step in developing effective management strategies for the conservation of amphibian diversity (Paton & Crouch, 2002).

Marsh frogs are generally common amphibians of wetlands from sea level to high altitudes in Türkiye. The number of Marsh frogs living in rivers in southern Europe can reach more than a thousand individuals per kilometer of the riverbank (AmphibiaWeb, 2023). Reproductive activities begin a few days or a month after the emergence from hibernation. Males display advertisement calls especially intense at the peak of the breeding season. Adult females lay nearly 670-13.000 eggs, depending on their size. The timing of metamorphosis depends on weather conditions, habitat characteristics, and latitude but is usually completed in April (Özeti & Yılmaz, 1994; AmphibiaWeb, 2023).

Sexual size dimorphism (SSD), the difference in size between males and females of the same species, is a common phenomenon among many taxa. One hypothesis that has been proposed to explain SSD in amphibians is sexual selection. Sexual selection has long been recognized to influence morphological evolution in sexually reproducing species (Darwin, 1871). For

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example, in some species, males may compete for females and larger males may have a competitive advantage in mating. Alternatively, females may choose larger males as mates because they may have better genetic quality or offer better resources for the offspring (Neff & Pitcher, 2005).

Assortative mating can be assessed by measuring the correlation between the values of a phenotypic or genotypic character that are shared between individuals within mated pairs (Lipsey & Wilson, 2001; Redden & Allison, 2006). One of the most extensively researched traits related to assortative mating is body size (e.g., Crespi, 1989; Taborsky et al. 2009; Chajma & Vojar, 2016), since both sexes supposably can enhance their reproductive success by selecting the largest partner available (Andersson, 1994; Green, 2019).

Türkiye is an important source of frog trade and Marsh frogs have been harvested for approximately 50 years (Çiçek et al., 2021). Understanding the life history traits of frogs is essential for establishing effective harvesting regulations that can have a crucial impact on the sustainability of populations. Although many studies on Marsh frogs have been made, there are almost no studies on the reproductive ecology of the species. The aim of this study is to provide information on the reproductive ecology (phenology, fecundity, clutch size, reproductive phenology, reproductive effort, larval morphology, and mate choice) of Marsh frogs in the eastern Mediterranean Region (Mersin and Adana provinces, Türkiye).

2. Material and Methods

In order to determine the reproductive phenology of the species, 5 stations were selected in Mersin (1) and Adana provinces (4) between 2013 and 2015 and fieldwork was carried out all year round intensely in the breeding season from the beginning of January to the end of June (Table 1, Fig. 1). The sampling stations were visited frequently (at least once a week) in between February and April to determine the clutch size and to observe the emergence of individuals from hibernation and the development of tadpoles.

The phenology is considered as the initiation and duration of the courtship, breeding, and completion of metamorphosis. The first and the last observation day of each phenological event were determined by the presence of calling males (courtship), amplexed pairs and egg masses (breeding), and the presence of juveniles (completion of metamorphosis).

Table 1. Geographic position and habitat type of the stations sampled.

Station	Location	Coordinates (Lat., Lon.)	Altitude (m)	Habitat
Işıklı	Kozan/Adana	37.368040, 35.799450	74	Stream
Çatalan HES	Sarıçam/Adana	37.136640, 35.335870	81	Pond
Mercimek	Ceyhan/Adana	37.058639, 35.769007	25	Pond
Yeniköy	Yüreğir/Adana	36.772263, 35.210784	4	Irrigation channel
Akgöl	Silifke/Mersin	36.318740, 33.951380	3	Lake

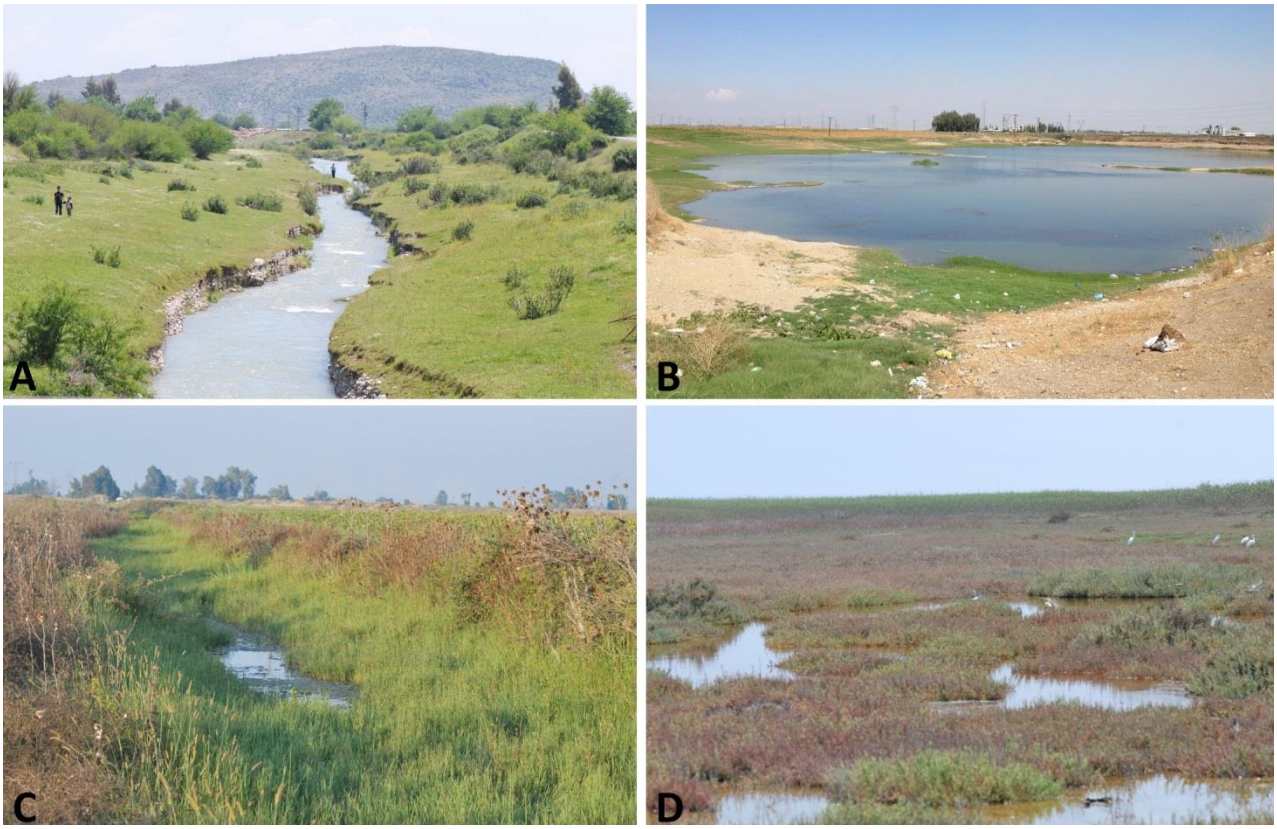

Figure 1. General view of the studied stations. A: Işıklı, B: Mercimek, C: Yeniköy (Adana), D: Akgöl (Mersin).

Sampling sites were divided into 0.5 × 0.5 m grids which were sampled randomly using a scoop net to determine the average number of tadpole (Heyer et al., 1994; Skelly and Richardson, 2009). We also used quadrat surveys to calculate the abundance of newly transformed young frogs at Gosner stage 46 (Heyer et al., 1994).

The number of eggs in some masses was counted both in the field and in the laboratory via stored in 5% formalin and; thus, the average number of eggs was determined. Besides, to reduce the possible damage to the eggs, some clutches were counted by the volumetric method (Surova & Cherdantsev, 1987; Cherdantsev et al., 1997). The volumetric and counted methods were used together than the average calculated.

The 34 amplexed pairs were placed in the laboratory for a brief period and some other 20 females were dissected to determine the clutch size. As females can lay eggs 2 to 7 times during the reproductive period (e.g., Tarkhnishvili & Gokhelashvili, 1999), amplexed pairs were temporarily kept in specially designed aquariums under constant room temperature throughout the study and the average fecundity was calculated by counting the total number of eggs laid. We both weighted the spawned and dissected females to determine the weight loss by egg laying. Reproductive effort (E) in females was calculated with the formula $E= (F*R^3)/SVL^3$. [F: fecundity, number of eggs in a clutch, R: egg diameter, SVL: snout-vent length (Cherdantsev et al., 1997)].

Eggs laid by the females of amplexed pairs or recently laid clutches collected from the field were kept in captivity in the laboratory in order to obtain data on some parameters such as hatching success, metamorphosis, and growth rate. The diameter of the egg and its sheath at stage 10 (Gosner, 1960) was measured with a micrometric eyepiece under a stereomicroscope. The development of the embryo was examined and the hatching time and stage were determined. After the embryos were hatched, samples were taken at different stages (stages 20-46) and preserved in 5% formalin. At every stage, determined measurements as snout-vent length (SVL), total length (TL), tail length (TaL), tail width (TW), the distance between eyes (DBE), and the distance between nostrils (DBN) were taken according to Altig (2007). We measured the body weight and SVL of amplexed pairs. We used the sexual dimorphism index (SDI) [(mean SVL of the larger sex / mean SVL of the smaller sex) ± 1 (SDI = 0 when both sexes are of similar SVL, defined as positive when females are larger)] proposed by Lovich and Gibbons (1992) to describe sexual size dimorphism.

We used the student's t-test to evaluate differences in the body size between mated males and females (dataset has normal distribution, Kolmogorov Smirnov test, $p>0.05$); and Pearson correlation analysis to test the presence of size-assortative mating in amplexed pairs. To determine whether mate choice of sexes was affected by their size (size-dependent mating), we also measured and evaluated the captured single males (n = 286) and females (n = 148) during the same sampling period. Mean SVL of mated and unmated sexes were compared using a student's t-test. The effect sizes (Cohen's *d*) for t-test were calculated according to Cohen (1988). All the statistical analyses were performed with the program PAST (Hammer et al., 2001). The study protocol was approved by the Laboratory Animals Ethical Committee at Ege University, Türkiye (Decision No: 2012/29).

3. Results

3.1. Breeding phenology and larval development

Marsh frogs are in hibernation between November and January in Mersin and Adana provinces and the period of reproductive activity occurs between mid-January and early June. Advertisement calls were detected from 1-3 males at night with 7-10 ^oC air temperature in February. We observed that 5-10 males made advertisement calls at night when the air temperature was between 14-16 ^oC whereas the water temperature was in the range of 10-12 ^oC in March. Meanwhile, individuals of other frog species (*Bufotes viridis* and *Hyla savignyi*) which inhabit the same habitat have a higher number of advertisement calls and perform amplexus. Besides, an unusual interspecific amplexus were observed during the field studies. A male *B. viridis* amplexed with a female *P. ridibundus* (see Fig. 2 –B). Amplexed pairs were more frequently observed between March and April and newly metamorphosed froglets emerged since the end of March. During the whole field surveys, no amplexed pairs were observed after June. Although four amplexed pairs were observed on a survey carried out in August, there were no egg masses detected.

Depending on the temperature, the embryos hatch within 2 – 4 days in March in the wild and the tadpole are released at the 21st stage (Fig. 3). The completion of metamorphosis took 66-95 days at ambient temperature under laboratory conditions. Almost all embryos were released at the 21st stage (98%) and the survival of the tadpoles was quite high (85%) in the laboratory. In the field surveys, juveniles that had just completed metamorphosis were observed since the end of March; therefore, we conclude that the metamorphosis was completed in a much shorter time (45-65 days) under natural conditions in contrast to captivity.

10 - 20 froglets just completed metamorphosis were observed per square meter in August while 5 - 10 froglets in March. Therefore, it is determined that the survival rate of juveniles in their first year is approximately 50%. Froglets complete their metamorphosis when they reach approximately 20 - 25 mm in length (Fig. 4). By the end of August, juveniles reach an average length of 37.6 mm (28 - 45 mm). After their first hibernation, they reach a length of 26 - 47 mm.

3.2. Mate choice, morphometry, and fecundity

Mean SVL was calculated as 68.15 mm (SD= 5.54, range: 57.17-82.03) for amplexed females, 59.03 mm (SD= 5.64, 45.72-70.96) for amplexed males. Mean body weight was calculated as 33.13 g (SD= 10.11, 17.90-60.5) for amplexed females and 20.56 g (SD= 5.7, 9.20-32.40) for amplexed males. The difference in mean SVL between males and females is statistically significant (*t-test*= 8.35, *df*= 39, $p< 0.001$) with a large effect size (Cohen's $d = 1.63$). Out of 40 amplexed pairs, the majority (37 pairs or 92.5%) consist of females that are larger than males in terms of body size. Mean SDI is calculated as 0.16 (-0.09 - 0.54).

The morphometric dataset of amplexed pairs was distributed normally according to Kolmogorov-Smirnov

test ($p > 0.05$); thus, we used the parametric Pearson test for correlation analysis. There was no correlation between male and female regarding SVL (*Pearson correlation*, r = 0.237, p = 0.14) and body weight (r = -0.16, p = 0.92), indicating the random mating instead of size-assortative.

SVL of amplexed females (68.15 mm, ± 5.54) was significantly larger than that of single females (63.83 mm, ± 10.78) by 6.8% (t = -3.46, df = 125, $p < 0.001$) with a medium-small effect size (Cohen's d = 0.44), thereby indicating size-dependent mating. The difference of SVL among amplexed males (59,03 mm, ± 5.64) and single males (58.71 mm, ± 7.9) is statistically insignificant (t = -0.32, df = 62.7, p = 0.751).

The number of eggs counted in the spawned egg masses was between 144 and 645 (n = 37). Egg masses were spawned in temporary ponds at the shallow edges of wetlands which were 25 - 40 cm deep (see Fig. 2 – E, F). Moreover, fecundity was detected as between 940 and 5400 eggs from 20 females dissected in the laboratory. Spawning females lose approximately 4-6% of their body weight. Moreover, we calculated the total number of eggs in the clutches laid by 34 females captivated in the laboratory. Thus, mean fecundity was calculated as 3853 (SD = 1133.34, 940–6000) eggs and reproductive effort (E) of females was calculated as 0.094 (SD = 0.009, 0.07 – 0.11). The mean egg size was calculated as 1.73 mm (SD = 0.08, 1.7-2.1).

Figure 2. Amplexed pair of male *Bufotes viridis* and female *P. ridibundus* (A, B); amplexed pairs of Marsh frog *P. ridibundus* s.l. (C, D); spawned egg masses (E, F).

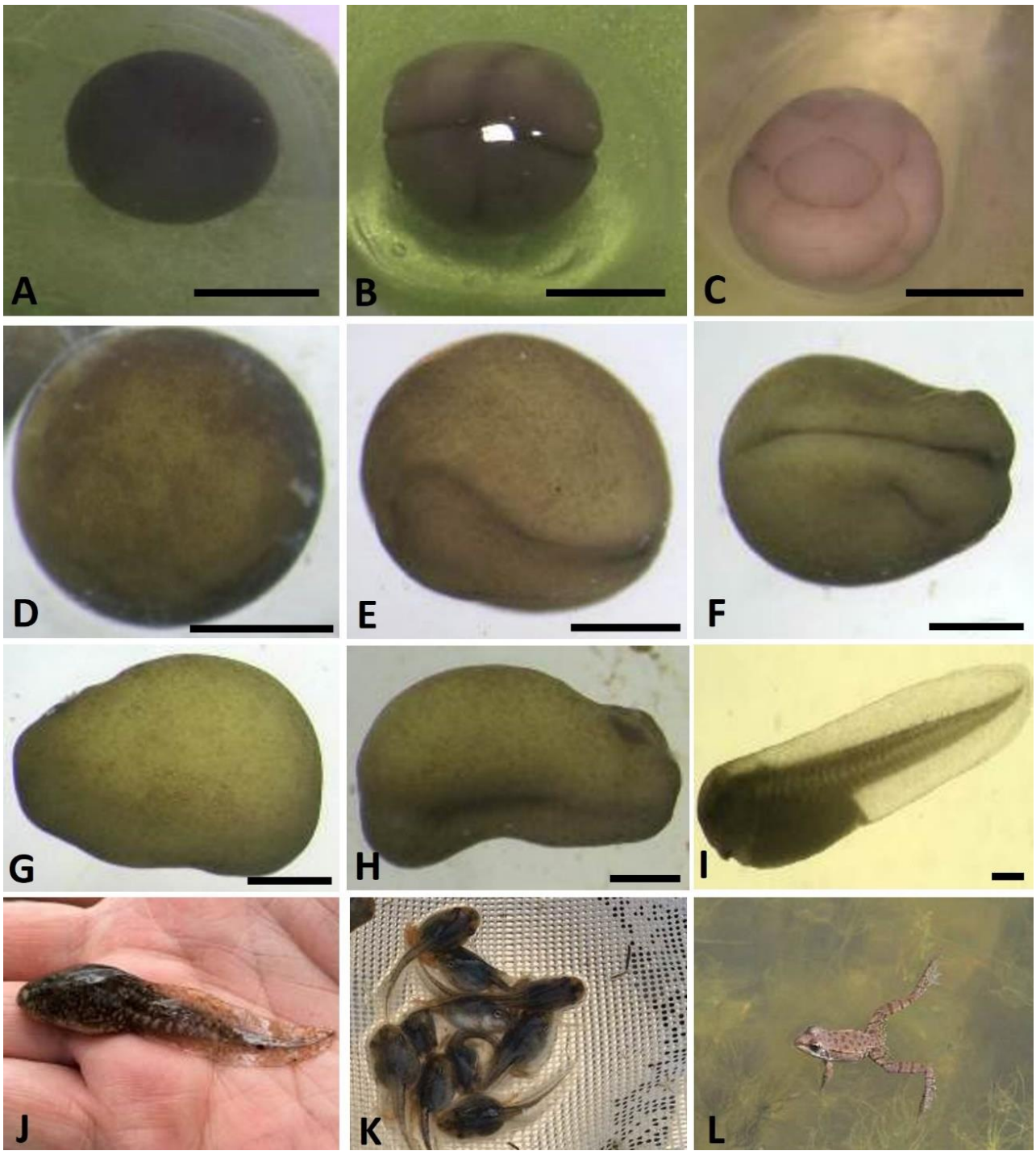

Figure 3. Stages of embryo and tadpoles according to Gosner (1960) (A: stage 1 - fertilization, B: stage 4 – 4-cell, C: stage 6 – 16-cell, D: stage 10, E: stage 14 – neural folds, F: stage 15 – rotation, G: stage 16 – neural tube formation, H: stage 17 – tail bud, I: stage 19 – heartbeat, J: stage 38, K: Tadpole at stage 38- 41, L: juvenile completed metamorphosis (bars represent 1 mm in length)

4. Discussion

The duration and timing of the breeding period are primarily dependent on abiotic factors such as temperature, precipitation, and hydroperiod at aquatic breeding sites and there is considerable interspecific and intraspecific variation in egg-deposition time; thus, determining breeding phenology in the local area is critical (Paton & Harris, 2009).

Species in temperate zones typically breed in warmer months, with many ranids and bufonids breeding explosively in early spring. Generally, Marsh frogs breed when the water temperature is above 18°C (Tarkhnishvili & Gokhelashvili, 1999). Males begin their advertising calls towards the end of March while egg laying begins in the second half of April at lower altitudes of Tbilisi (Georgia) (Tarkhnishvili & Gokhelashvili, 1999). The breeding season in the Caucasus is between May and June and it extends until mid-September, depending on the altitude (Tarkhnishvili & Gokhelashvili, 1999).

Rarely, interspecific amplexus can be seen in anurans, even in between anurans and salamanders (Oswald et al., 2022). Similar cases have been reported

between *Pelophylax* and *Bufo* from Türkiye (Gül et al., 2018). Breeding activities of sympatric *Bufotes viridis* and *Hyla savignyi* species start just before *P. ridibundus* in the sampling stations. Fewer numbers of females of the same species may cause males of other species to interspecific amplexus with relatively abundant females of *P. ridibundus*.

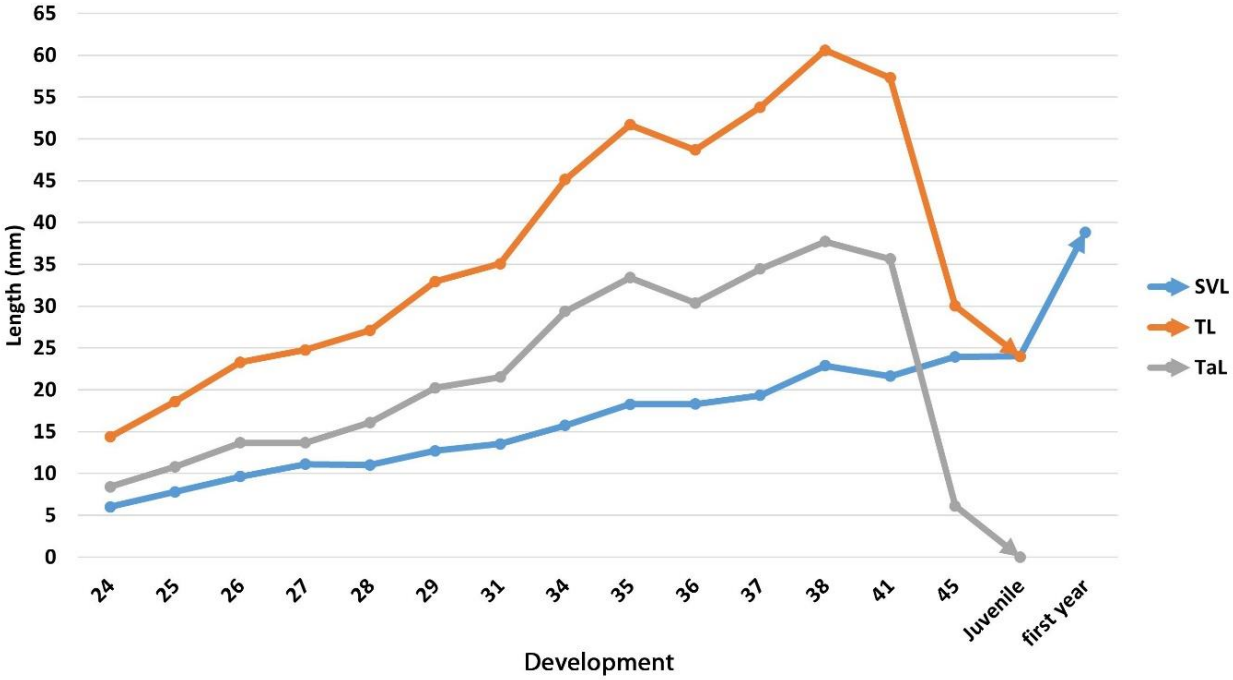

Figure 4. Changes in average SVL, TL, and TaL during larval development and in the first year.

Nearly 80% of anurans deposit eggs in standing water (Wells, 2007). Species that breed in smaller wetlands (e.g., Ranidae, Microhylidae) are probably best suited for egg-mass counts (Paton & Harris, 2009). Marsh frogs lay between 1000 – 16400 eggs in a breeding season (Tarkhnishvili & Gokhelashvili, 1999) and this number can be higher in some toads (e.g., 23.227 for *Rhinella arenarum*, Bionda et al., 2011). As Marsh frogs can lay eggs 2 to 7 times during the breeding period (Tarkhnishvili & Gokhelashvili 1999), the number of eggs in the cluster does not give the total fecundity of the female. For example, females of *R. arvalis* lay one clutch of 300-1500 eggs (Räsänen et al., 2008) and of *R. temporaria* lay 131-1897 eggs per year (Gibbons & McCarthy, 1986). The number of eggs counted in the egg clusters found in the wild is between 144 and 645 (n= 37) and we conclude from the results of dissections (fecundity was detected as between 940 and 5400 eggs) that the females should spawn multiple clutches.

According to Tarkhnishvili and Gokhelashvili (1999), in low-altitude populations of *P. ridibundus*, the tadpoles are released 3 to 6 days after fertilization, indicating a correspondence with our results. In parallel with our findings, Shchupak and Ivanova (1990) also reported that the larval development took about 81 days to complete their metamorphosis under laboratory conditions. The completion larval development of Marsh frog in Anatolia was reported as 90-120 days (Özeti & Yılmaz, 1994) which is considerably longer than we observed. Özeti and Yılmaz (1994) drew a general conclusion and there are no local studies regarding the issue. Obviously, temperature and altitude affect the duration of metamorphosis; for example, in higher altitudes with cool temperatures, larval development would be slower. Considering that our study area is at the sea level and in a mild Mediterranean climate zone, a shorter metamorphose period would be an expected result.

Egg diameters reported from Anatolia are between 1.5-2 mm (Özeti & Yılmaz, 1994; Erişmiş, 2011) and from Caucasian populations ranged from 1.6 to 1.9 mm (Tarkhnishvili & Gokhelashvili 1999). Özeti and Yılmaz (1994) stated that the average total length of the tadpoles is 40-50 mm. Tadpoles of Marsh frog can reach 70-80 mm in total and during the 38-39th larval stage has the greatest overall length (Tarkhnishvili & Gokhelashvili 1999).

Reproductive effort is usually known as the total amount of energy spent in reproduction during a reproductive season. Presuming that energy is limited, a given reproductive effort can be used up either by the production of a few large offspring or a great number of small ones (Stearns, 1992). Most of the anurans, such as ranids and bufonids, produce large numbers of small eggs. Reproductive efforts can be measured as counts of calling males, sperms, egg masses, fecundity, and amplexed pairs. It is expected that larger individuals produce more egg/sperm and; thus, contribute to reproduction further. Cherdantsev et al. (1997) formulated this effort for female frogs and calculated the reproductive effort in relation to size. According to this formulation reproductive effort of *Rana arvalis* is between 0.026-0.037 (Cherdantsev et al., 1997) and *Rana macrocnemis* is 0.068 (Çiçek et al., 2011). We calculated the reproductive effort as 0.094 for *P. ridibundus* in Anatolia, indicating that they produce more eggs than previously reported species in relation to body size.

Assortative mating can be categorized as either positive, indicating a preference for mating with individuals who share similar phenotypic traits or negative (also known as disassortative), indicating a preference for the opposite (e.g., Hooper & Miller, 2008; Jiang et al., 2013). The body size is a frequently examined trait related to assortative mating, particularly in anurans where larger individuals of both sexes may provide reproductive advantages (Green, 2019). Pairs of anurans may be formed randomly or nonrandomly and it could be a large variation in the amplexed male-female size correlations among species and populations (Hartel et al., 2007). For example, some studies reported that males do not show preference for larger females (e.g., Hetteyi et al., 2005; Liu et al., 2012) while others do (e.g., Chajma & Vojar, 2016). In our study, amplexed females was significantly larger than single females by 6.8%, indicating size-dependent mating. However, in contrast to Erişmiş (2011), we did not find any correlation between the amplexed males and females, indicating that a random mating between sexes, not size-assortative. Larger females were more preferred to mate by males; on the other hand, body size of males was not related with females.

Generally, females are larger than males in 90% of amphibians (Shine, 1979); however, in some species, it is the opposite. For example, in *Limnonectes kuhlii*, females are generally smaller than males (Tsuji & Lue, 2000). Within this study, female-biased sexual size dimorphism was observed (37 of 40 pairs, 92.5%) among the amplexed pairs.

As well as body size, factors such as activity, reproductive calls or coloration may play an important role in mate choice. In fact, studies have shown that in some sympatric *P. esculentus* and *P. lessonae* populations, *P. esculentus* females tend to mate with *P. lessonae* males regardless of body size and activity and genetic factors are also involved in mate selection (Abt & Reyer, 1993).

In conclusion, since the winter season is not harsh in the Mediterranean region, Marsh frogs are active for almost 10 months. According to the weather conditions, reproductive activities occur from January to June. Advertisement calls can be detected in February. Amplexed pairs are more frequently observed between March and April and newly metamorphosed froglets can be observed since the end of March. The metamorphosis was completed in a much shorter time (45-65 days) under natural conditions in contrast to captivity. The mean fecundity was calculated as 3853 eggs and the mean egg diameter was calculated as 1.73 mm. Within the amplexed pairs, differences in SVL are statistically significant which points out a female-biased sexual size dimorphism. SVL of the amplexed pairs is not correlated, indicating that species prefer random mating instead of size-assortative mating. Besides, with larger mean body size of the amplexed females than single ones by 6.8%, we conclude that the size-dependent mating occurred in Western Mediterranean population of *Pelophylax ridibundus* s.l.. We hope that our results will be useful for regulating the harvest and ensuring the sustainability of Marsh frog populations.

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Ethics committee approval: This study was conducted in accordance with ethical standards for animal experimentation. The necessary research ethics committee approvals were obtained from the Ege University Animal Ethics Committee (No: 2012/29).

Conflict of interest: The authors declare that there is no conflict of interest.

Author Contributions: Conception – K.Ç.; Design – Y.B.; Supervision: K.Ç.; Materials – Y.B., K.Ç.; Data Collection or Processing: Y.B., K.Ç.; Analysis Interpretation: Y.B.; Writing – Y.B.; Crtical Review – K.Ç.

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