World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

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Age of sexual maturity of *Hoplobatrachus occipitalis* frogs in a rearing environment

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World Journal of Advanced Research and Reviews, 2024, 24(03), 2960-2971

Publication history: Received on 09 November 2024; revised on 24 December 2024; accepted on 27 December 2024

Article DOI: https://doi.org/10.30574/wjarr.2024.24.3.3882

Abstract

The aim of this study was to determine the age of sexual maturity of *Hoplobatrachus occipitalis*, the most commonly consumed frog in Côte d'Ivoire, under rearing conditions. Histological analyses were carried out on gonads taken from farmed male and female frogs aged between 06 and 12 months. The results showed that the gonado-somatic ratios of males (0.045 to 0.73%) are much lower than those of females (0.22 to 17.03%). Histological sections indicate that the age of full sexual maturity of males and females under rearing conditions is 10.5 months. At this age there is a massive presence of spermatozoa in the lumen of the seminiferous tubules in males and a high number of mature oocytes (V and VI) in females. Determining the age at which *Hoplobatrachus occipitalis* frogs reach sexual maturity will enable future frog breeders to build up stocks of broodstock and thus plan breeding periods.

Keywords: Frog ; Histological; sections; Sexual maturity; Breeding

1. Introduction

Frogs are poikilotherms [12] and are excellent indicators of stress in their living environments [5] [1]. They are also essential to the balance of wetland habitats [4]. In economic terms, certain species of edible frogs generate income and are an important source of animal protein for humans [13] [14] . These include *Hoplobatrachus occipitalis*, which is one of the most widely consumed species in Africa, particularly in Côte d'Ivoire [8]. Given the importance of frogs in agricultural, ecological and nutritional terms, and the world, frog farming or raniculture is proving to be an unavoidable alternative to alternative for reducing pressure on wild populations [13]. In order to achieve this objective, one of the most important functions is to control reproduction. However, data concerning the period of sexual maturity of this species is patchy in the wild and virtually non-existent in farmed environments, hence the importance of this study. To overcome these shortcomings, it is necessary to accurately determine the period of sexual maturity of male and female *Hoplobatrachus occipitalis* frogs in the breeding environment. This can be done by taking histological sections and by analysing the sexual maturity of the frogs.

2. Material and methods

2.1. Study site

This study was carried out on the fish farm of the Association for Fish Farming and Rural Development in Humid Tropical Africa (APDRACI) located in the southern part of the town of Daloa (Côte d'Ivoire). The farm has nineteen fish ponds, a water reservoir, three large concrete basins, six covered hatcheries, a water tower and other facilities required for fish farming. The geographical coordinates of the APDRACI farm are: latitude 6°51'30 north and longitude 6°27'50 west (Figure 1).

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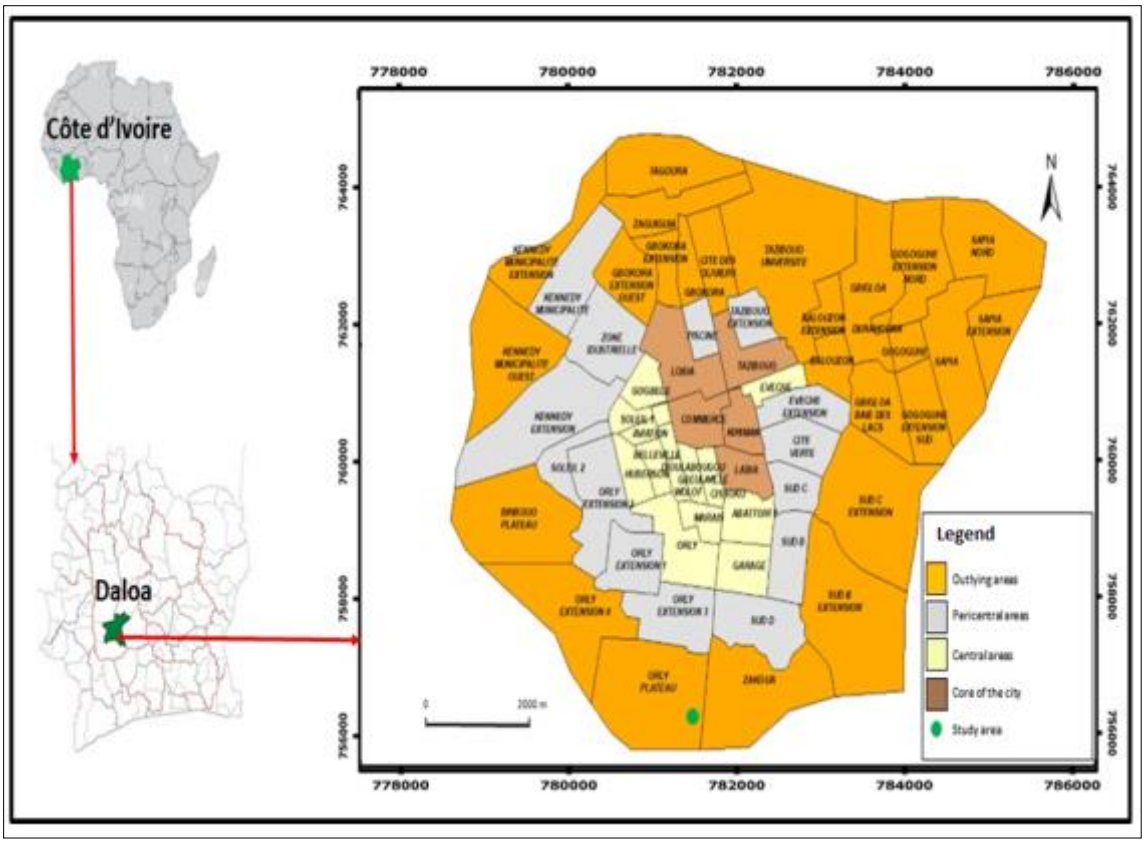


Figure 1 Location of the study site

2.2. Methodological approach

2.2.1. Dissection

Hoplobatrachus occipitalis frogs reared in concrete basins and ponds (Figure 2) were sampled at different stages of development. Thus, 02 males and 02 females were captured from the 6th month of age in ponds and concrete basins. The dissection operations began by measuring the size and weight of the frogs. The frogs were then decerebrated and demedulated in order to remove the gonads. The gonads were weighed and placed in a plastic tube containing 10% formaldehyde. The tube was labelled and stored in an appropriate medium. This operation was repeated every 45 days until the 12th month.

Figure 2 Male specimen of *Hoplobatrachus occipitalis* at 9 months old

2.2.2. Histological sections

Histological sections of the sampled gonads were taken in the pathological anatomy and cytology laboratory of the Treichville University Hospital (Abidjan, Côte d'Ivoire), using a protocol comprising the following steps :

Sample sectioning

First, the samples were taken out of the tubes and a different section (transverse and longitudinal) was made on each gonad of a specimen using a scalpel. The two fragments from each section were then placed in a labelled cassette. A total of 40 cassettes were obtained for the 20 individuals and preserved in 10% formaldehyde.

Dehydration

The machine used for this stage was the SLEE MTP automatic dehydration machine (Figure 3A). Its role is to dehydrate the fragments in the cassettes using 12 chemical baths. These baths are in the following order:

- 2 baths of 10% formalin to complete the fixation of the fragments (total removal of blood from the sample);
- 5 alcohol baths with increasing concentrations (01 bath of 75% alcohol, 01 bath of 85% alcohol, 01 bath of 95% alcohol and 02 baths of absolute alcohol or 100% alcohol). This treatment allows the gradual and total elimination of water from the fragments;
- 02 baths of 99.8% toluene to lighten the fragments and remove all impurities;
- 03 liquefied granulated paraffin baths for histology. These baths were used to harden the fragments after the water had been removed. The time taken for the fragments to harden in each formalin, alcohol and toluene bath was 01 hour. This time was reduced to 45 minutes for each paraffin bath.

Embedding

The TEC 2900 Embedding Center (Figure 3B) was used for this stage. Its role is to encapsulate the sample. This involved combining three elements (fragment, cassette and paraffin). First of all, the sample was placed in the liquid paraffin apparatus, then placed in the mould on the paraffin, respecting the cutting plane (transverse and longitudinal). The whole was covered by the cassette without its lid. These 3 elements were then placed on a cooling plate of the same machine to fix everything together. At the end of the process, the mould is detached from the rest.

Fine cuts of 03 microns

This stage required the use of 03 pieces of equipment: the Rotary Microtome, the Cryo Console and the Thermal Console. The sample and cassette in solid paraffin were cut using the Rotary Microtome (Figure 3C) with its microtome blade. The first step was to remove the paraffin covering the fragment. To do this, 25-micron sections were cut progressively until the fragment appeared. Next, the sample that had been heated following the various 25-micron sections was refrozen on the Cryo Console (Figure 3D). Next, 3-micron sections of the fragments were cut to form 9 ribbons. The ribbons were then placed on the Thermal Console bain-marie (Figure 3E). The slide was labelled and a ribbon from the fragment was cut.

Heating the blade with the ribbon

This stage consists of heating the blade and ribbon assembly in a Memmert oven at 55°C for 01 hour to ensure that the ribbon adheres well to the blade.

Colouring the sections

This stage involves the gradual rehydration of the sections. This process is the reverse of dehydration. Rehydration began with the slides in 02 successive Toluene baths for 05 min each. The purpose of these baths was to remove paraffin residues from the slide. Rehydration then followed 05 successive alcohol baths, but this time in decreasing order of concentration (02 100% alcohol baths, 01 95% alcohol bath, 01 85% alcohol bath and 75% alcohol bath). Each bath lasted 05 min. To complete the rehydration of the sections, they were rinsed in distilled water for 05 min. Staining was carried out using 02 successive dye baths. First, a Hematoxylin bath for 05 min, which stained the nucleus blue or violet, then rinsed to bring out the colour.to bring out the colour. The slides were then immersed in an eosin bath for a maximum of 03 min. At the end of this process, the slides were dehydrated by the corresponding process.

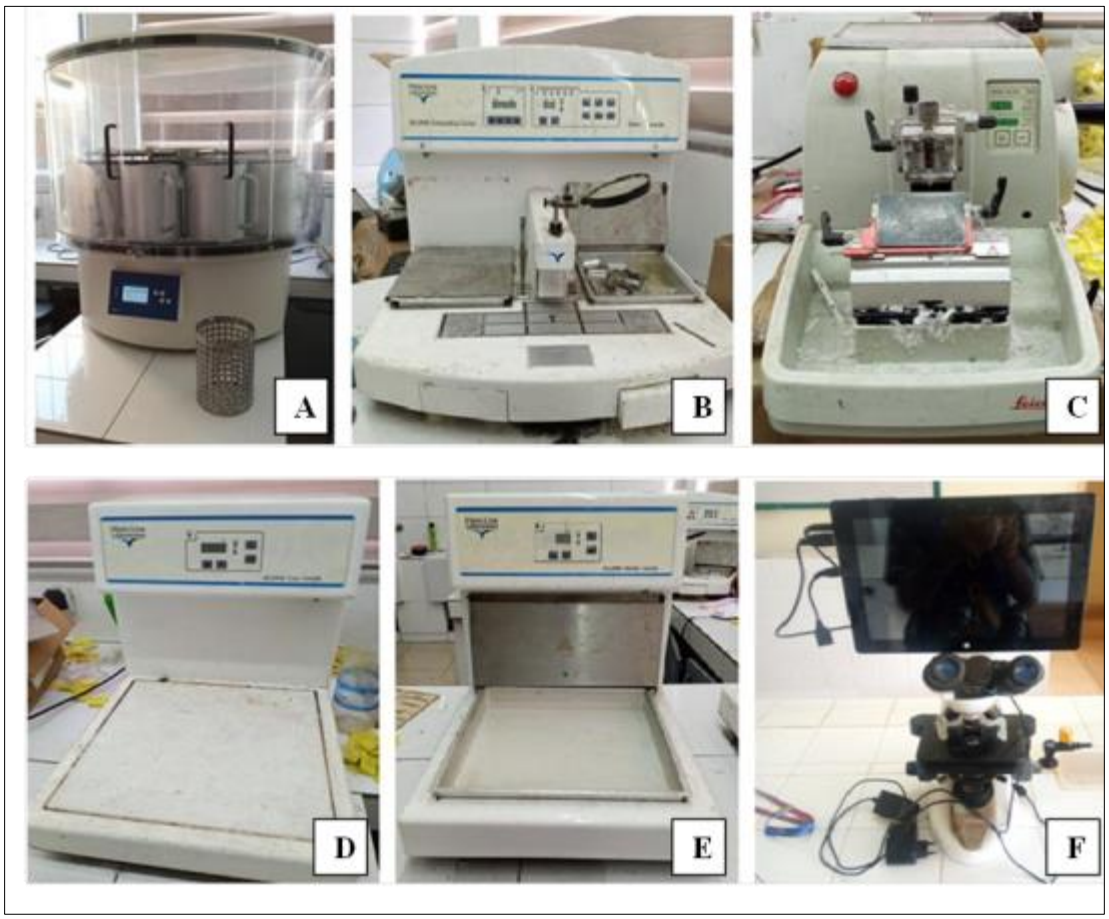
Gluing the slides

The mounting process consists of applying drops of Eukite (a special glue) to the slide and covering the cross-section of the slide with a few drops of toluene. The role of the Eukite is to lighten the slide, evacuate the air and bond the whole

assembly together so that the sample can be preserved and protected for years to come. The slide, coloured section and coverslip are heated in the oven at 55°C for 01 hour.

2.2.3. Photographing and describing the sections

In order to make the best use of each histological section, it was attached to the information (age, sex, height and weight) previously taken. The sections were then photographed at different magnifications in the laboratory of the University Jean Lorougnon Guede in Daloa and described using an optical microscope equipped with an android tablet (Figure 3F).



A: Automated dehydration machine; B: Embedding Center; C: Rotary Microtome; D: Cryo Console; E: Thermal Console; F: Optical microscope with tablet.

Figure 3 Laboratory equipment used for histological sections

2.3. Gonadal-somatic ratio

According to Yin (1993), the gonadal-somatic ratio (GSR) or 'gonadal index' reflects the growth of the gonads during the reproductive cycle. It is defined asratio of gonad weight to eviscerated weight expressed as a percentage. Its formula is as follows.

$$GSR\ (\%) = \frac{\text{Gonad weight}}{\text{eviscerated weight of the frog}} \times 100$$

A very high ratio indicates advanced maturation of the gonads. On the other hand, alow ratio indicates sexual rest.

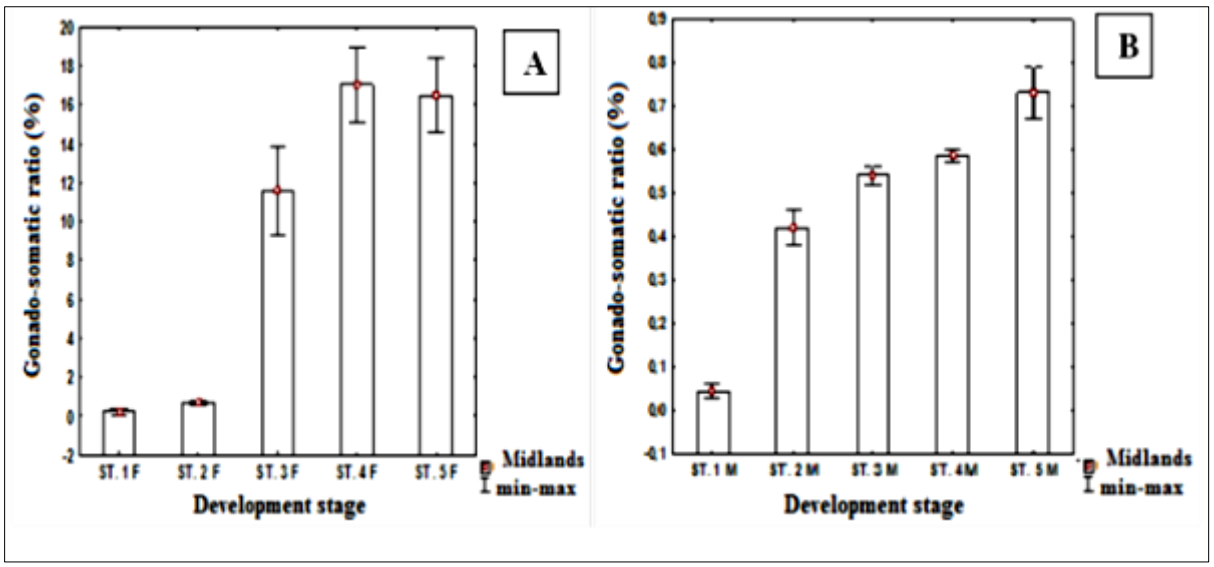
2.4. Statistical tests

Parametric tests, in particular the Anova test and Student's t-test were used for means or standard deviations [10] , as well as for the differentiation of gonado-somatic ratios (GSR) of males and females of *Hoplobatrachus occipitalis*

3. Results

3.1. Gonado-somatic ratio

Gonado-somatic ratios increased overall with age in both males and females. In males, there was a significant difference between the GSR of 06-month-old males and that of other stages. In fact, the average RGS of males aged 06 months was 0.045 ± 0.015%, whereas at 07.5 to 12 months the RGS ranged from 0.42 ± 0.04% to 0.73 ± 0.06%. For females, the SGRs at 06 and 07.5 months were well below those of the upper stages (Figure 4). The mean RGS values differed between males and females (Anova, p>0.05).



A: female gonadal-somatic ratio; B: male gonadal-somatic ratio; ST.1F: females at 06 months; ST.1F: females at 07.5 months; ST.1F: females at 09 months; ST.1 F: females at 10.5 months; ST.1F: females at 12 months; ST.1M: males at 06 months; ST.1M: males at 07.5 months; ST.1M: males at 09 months; ST.1M: males at 10.5 months; ST.1M: males at 12 months;

Figure 4 Gonado-somatic ratio as a function of five developmental stages

3.2. Histological state of the gonads according to the physiological stages of the frogs

In males, the vocal sacs that are the distinctive signs of this sex appear from 7.5 months of age, as can be seen in Figure 5. Figure 6 shows photographs of male and female gonads at different stages of development. Histological sections in Figures 7 and 8 show different stages of oocytes with a progressive increase in diameter from 50 µm to 1800 µm. At 06 months, pre-vitellogenic stage I and II oocytes were observed, giving the ovary a yellow colour with granulations. At 07.5 months (69.75 ± 02.25 mm), vitellogenic stage III and IV oocytes were observed, marking the beginning of the ovarian maturation process. Stage V vitellogenic oocytes with thicker zona pellucida and large yolk platelets appeared 01.5 months later. From 10.5 months (92.5 ± 0.5 mm) to 12 months (103 ± 02.1 mm), the ovaries of female *Hoplobatrachus occipitalis* frogs possess vitellogenic stage VI oocytes in addition to the other stages. These are the largest cells (1800 µm) with large plates of vitellus.

Table 1 Average lengths and weights of male and female individuals at different stages of development

Development stages	Age (months)	Females		Males	
		Weight (g)	Sizes (mm)	Weight (g)	Sizes (mm)
Stage 1	06	19.95 ± 2.72	61.5 ± 4.25	21.61 ± 2.23	60.5 ± 2.5
Stage 2	07.5	38.09 ± 7.75	69.75 ± 2.25	33.42 ± 0.12	71.75 ± 1.75
Stage 3	09	56.16 ± 4.86	83 ± 1.05	46.38 ± 0.95	77.25 ± 0.25
Stage 4	10.5	74.23 ± 0.15	92.5 ± 0.5	58.87 ± 4.66	85.0 ± 4.05
Stage 5	12	98.34 ± 4.08	103 ± 2.1	74.32 ± 2.88	93.0 ± 1.01

Male frogs at 06 months (60.5 ± 02.5 mm), do not have vocal sacs enabling them to be identified and have very small testicles (01 mm x 02.5 mm). At this stage, early germ cells such as spermatogonia and spermatocytes were observed (Figure 9 and 10). Vocal sacs appeared 07.5.

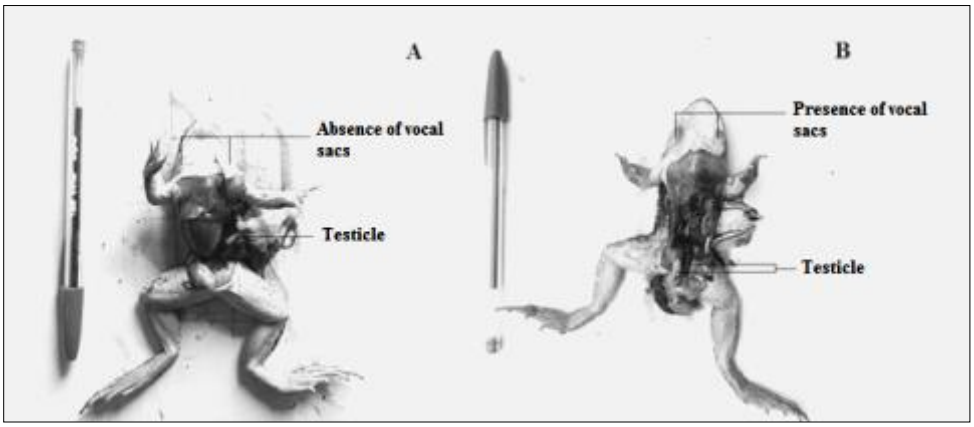
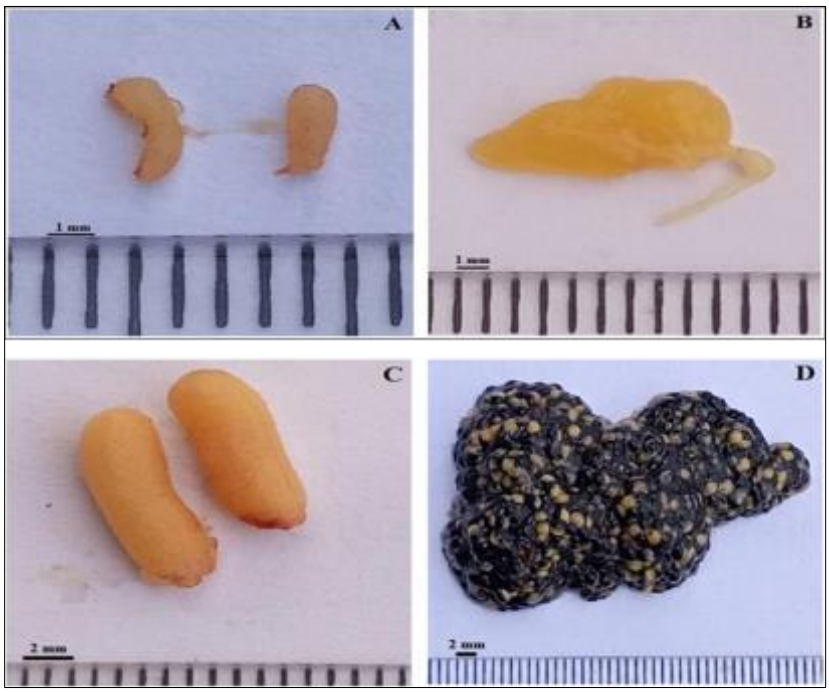
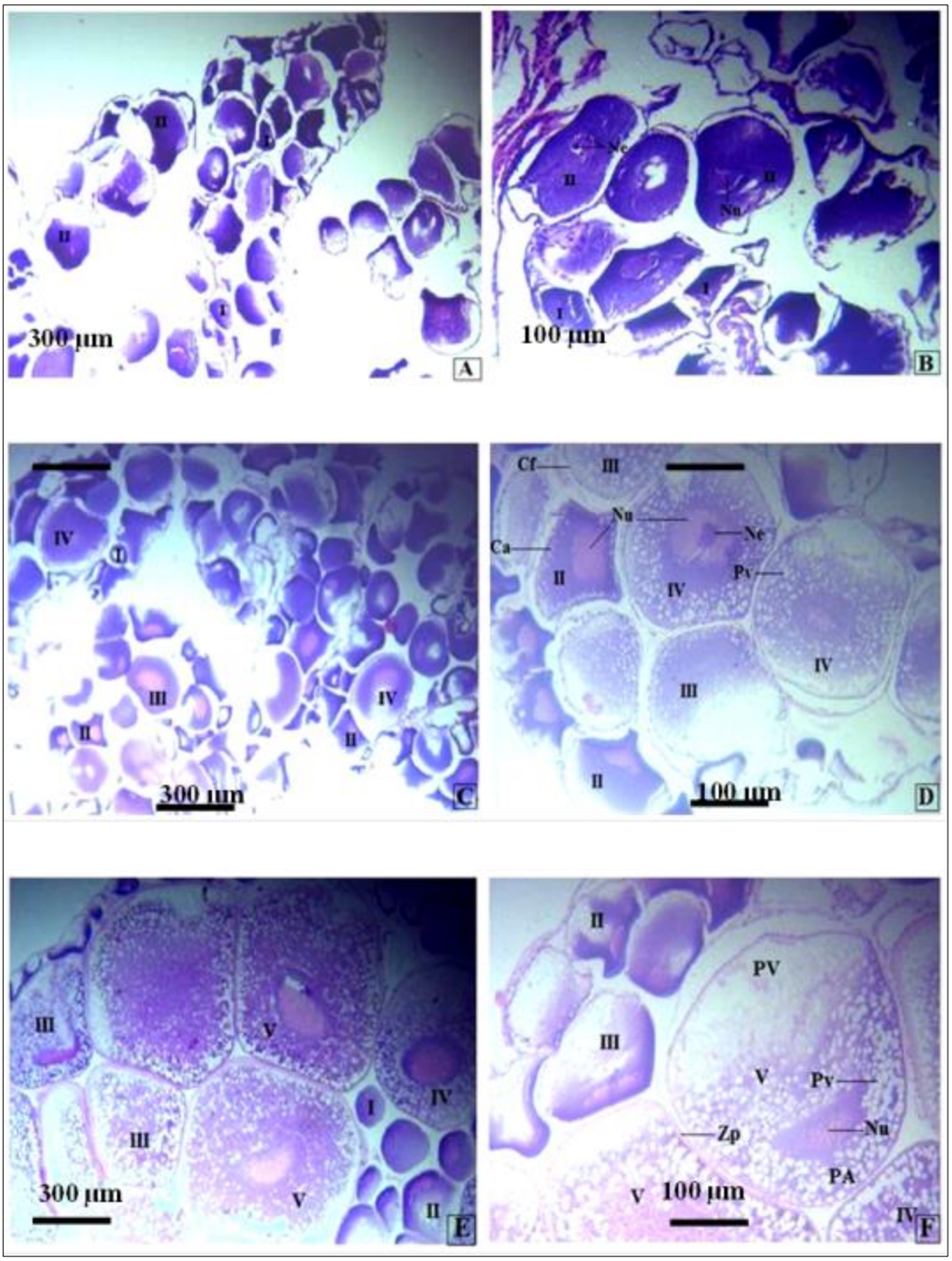


Figure 5 Dissection photographs of two male specimens of *Hoplobatrachus occipitalis* at 06 months (A) and 07.5 months (B)



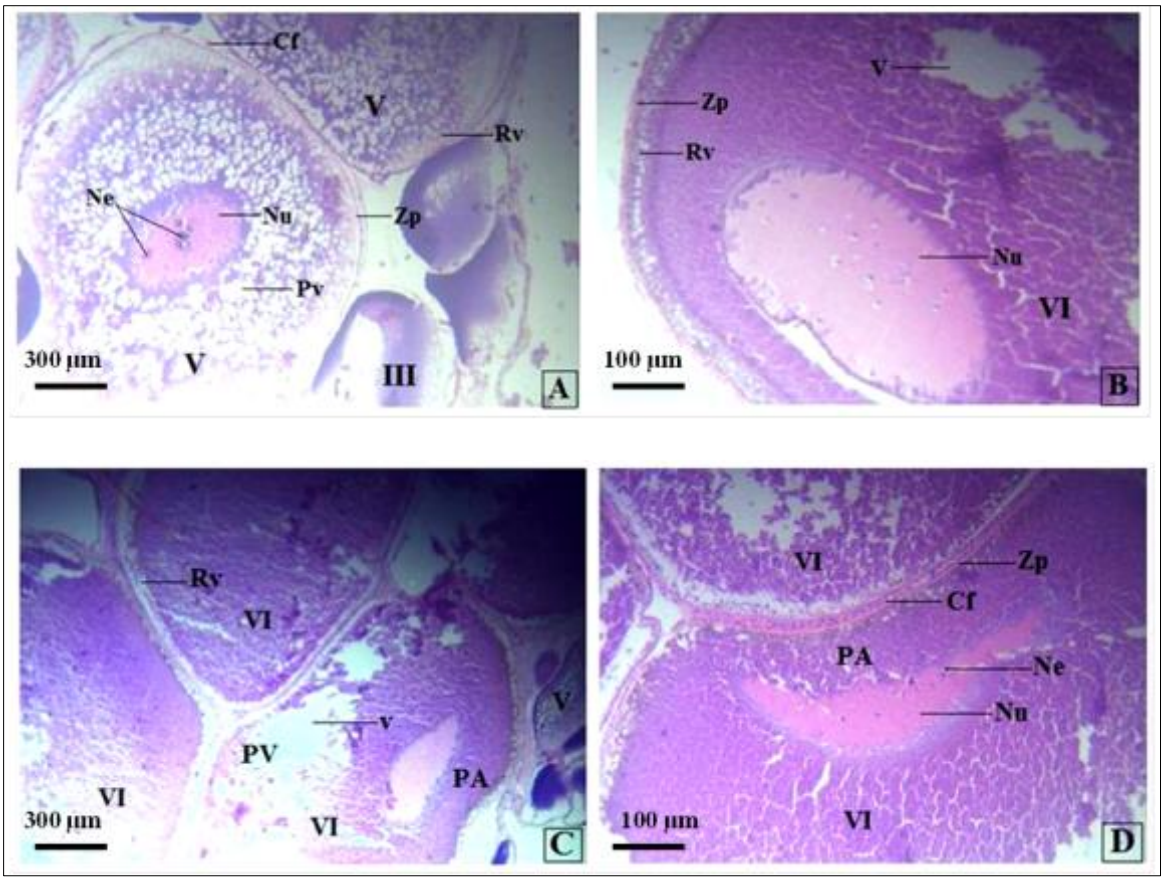
A: Testis at 06 months; B: Ovary at 06 months; C: Testis at 12 months; D: Ovary at 12 months

Figure 6 Male and female gonads of *Hoplobatrachus occipitalis* aged 06 and 12 months



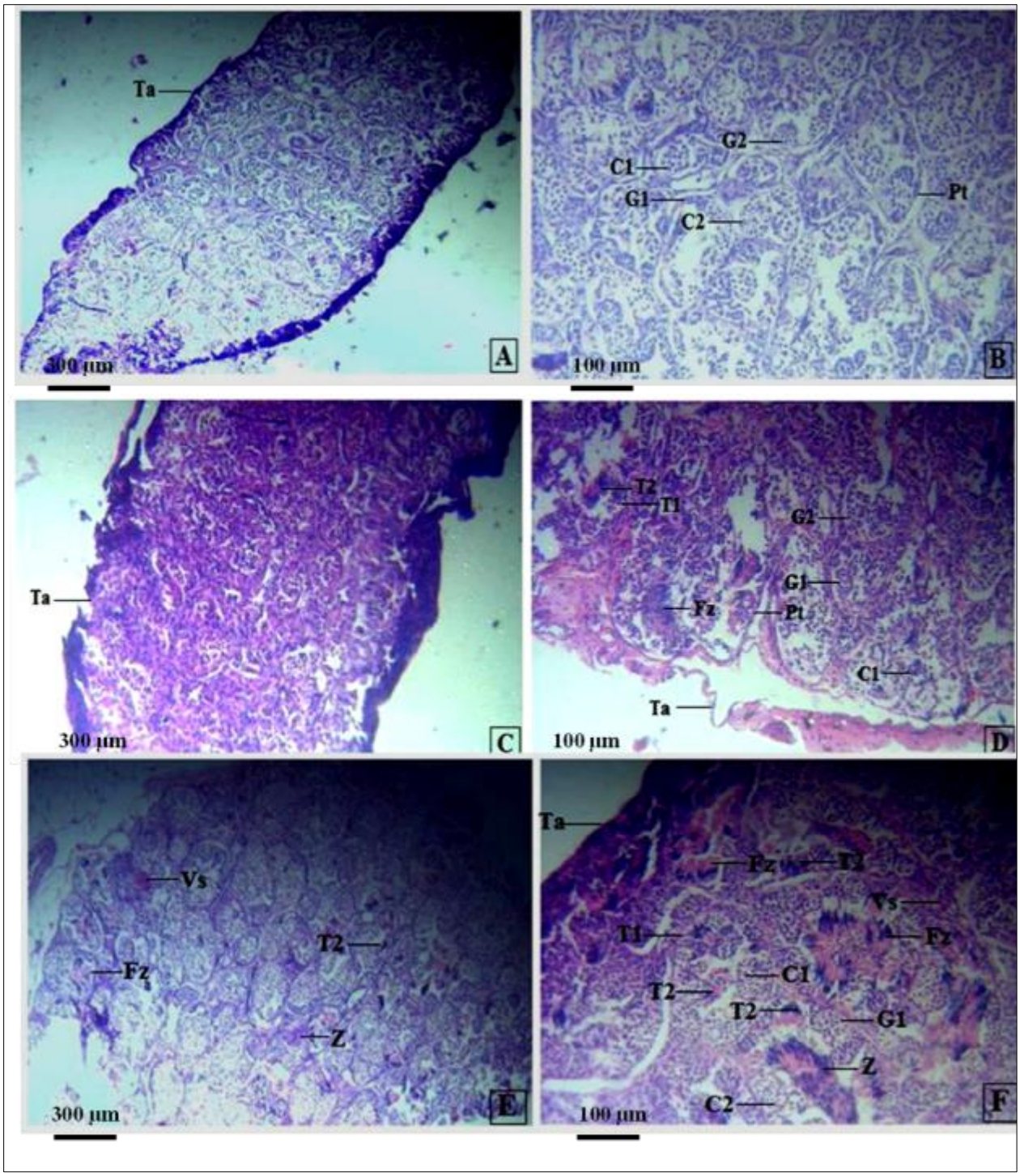
A & B: ovary at 06 months; C & D: ovary at 07.5 months; E & F: ovary at 09 months; I: stage 1 oocyte; II: stage 2 oocyte; III: stage 3 oocyte; IV: stage 4 oocyte; V: stage 5 oocyte; Nu: nucleus; Ne: nucleolus; Cf: follicular cell; Ca: cortical alveolus; Pv: vitelline platelet; Zp: zona pellucida; PA: animal pole; PV: vegetable pole.

Figure 7 Histological sections of ovaries of *Hoplobatrachus occipitalis* aged 06 to 09 months



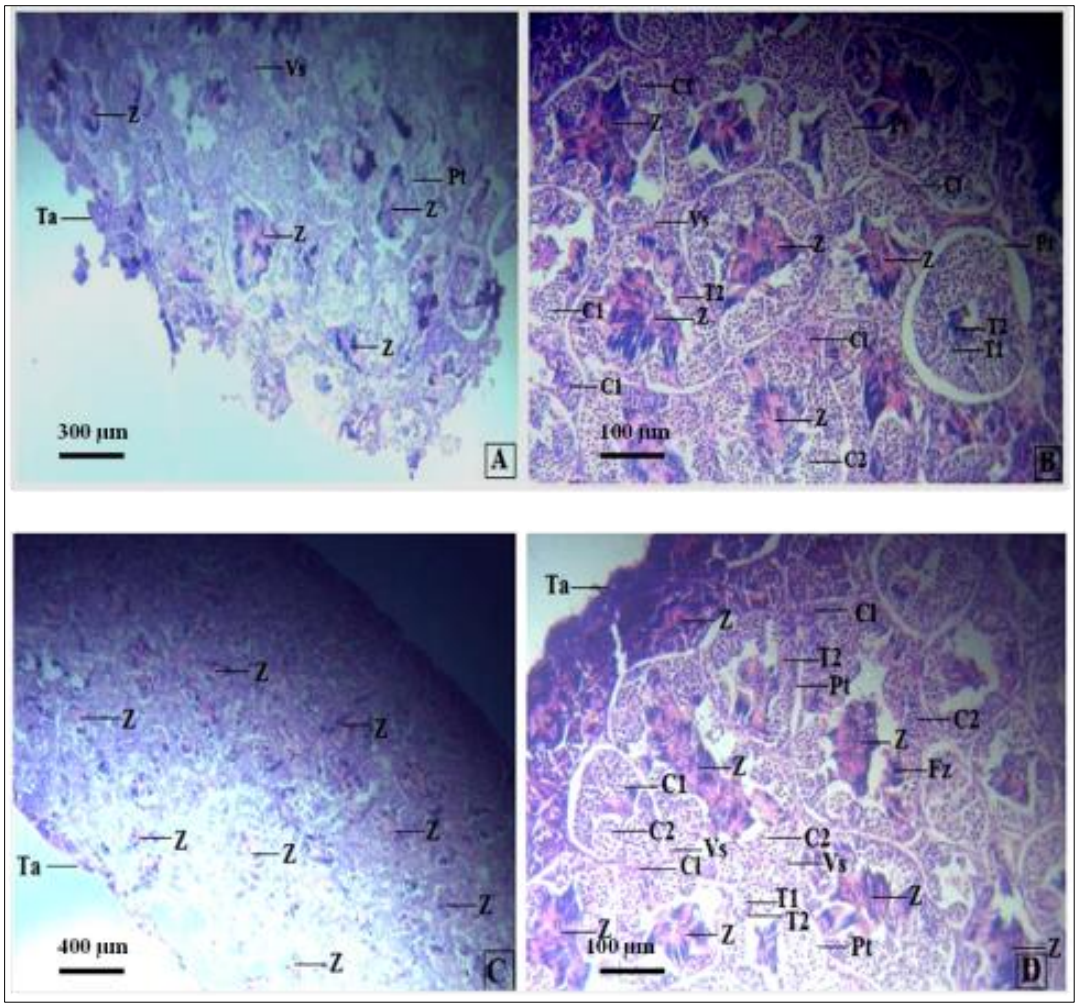
A & B: ovary at 10.5 months; C & D: ovary at 12 months; III: stage 3 oocyte; V: stage 5 oocyte; VI: stage 6 oocyte; Nu: nucleus; Ne: nucleolus; Cf: follicular cell; Pv: platelet vitrellins; Zp: zona pellucida; v: vitellus; Rv: vacuole row; PA: animal pole; PV: vegetable pole

Figure 8 Histological sections of *Hoplobatrachus occipitalis* ovaries aged 10.5 to 12 months



A & B: testis at 06 months; C & D: testis at 07.5 months; E & F: testis at 09 months; G1: primary spermatogonia; G2: secondary spermatogonia; C1: primary spermatocyte; C2: secondary spermatocyte; T1: early spermatid; T2: late spermatid; Fz: sperm bundle; Z: sperm in lumen; Pt: tubular wall; Ta: tunica albuginea; Vs: blood vessel.

Figure 9 Histological sections of testicles from *Hoplobatrachus occipitalis* aged 06 to 09 months



A & B: testis at 10.5 months; C & D: testis at 12 months; C1: primary spermatocyte; C2: secondary spermatocyte; T1: early spermatid; T2: late spermatid; Fz: sperm bundle; Z: sperm in the lumen; Pt: tubular wall; Ta: tunica albuginea; Vs: blood vessel; Cl: leydig cell.

Figure 10 Histological sections of testes from *Hoplobatrachus occipitalis* aged between 10.5 and 12 months

4. Discussion

To determine the sexual maturity of *Hoplobatrachus occipitalis* under rearing conditions, the gonado-somatic ratio (GSR) and the histological state of the gonads as a function of the age of the frogs were analysed. The results showed that the GSR of males (0.045 to 0.73%) was lower than that of females (0.22 to 17.03%). This superiority of the RGS of females could be due to the high weight of ovaries containing a high number of oocytes. In the case of females, the GSR at 06 months to 07.5 months of age was lower (0.22 to 0.67%) than in older females (11.58 to 17.03%). This could be explained by the fact that from 09 months of age (RGS = 11.58%), the development of *Hoplobatrachus occipitalis* ovaries is more important. Histological sections of ovaries from 09 month old females confirm this increase with the presence of oocytes from all stages except stage VI. The ovaries of females aged 06 to 07.5 months were less diverse in terms of the number of oocyte stages. These results are corroborated by [11], according to whom there are only stage I oocytes in the ovaries of frogs aged 01 to 04 months and stage II, III and IV oocytes become evident during the fifth month. The high RGS values of females aged 10.5 to 12 months (16.99 to 17.03%) are in agreement with the histological sections which show the presence of all stages of oocytes (I to VI) but with a lower RGS.

In males, gonado-somatic ratios evolve differently from females. Indeed, the GSRs of 06-month-old males *of Hoplobatrachus occipitalis* are lower (0.045%) than those of older males (0.42 to 0.73%). These results are thought to be due to the very small size of the testes at this stage (01 mm x 02.5 mm) and the presence of only early germ cells, including spermatogonia and spermatocytes, in the histological sections of the gonads. These results were observed by [7] in *Rana catesbeiana*. According to these authors, the presence of spermatogonia can be distinguished at[4] months and spermatocytes appear in frogs aged 05 months. Furthermore, the morphocytological characteristics of the primary spermatogonia analysed in this work are similar to those reported in the literature for various groups of anurans [3]

[15]. However, these RGS values for males of *H. occipitalis* are lower than those of [9], which ranged from 01.33 to 01.74%. The significant increase in RGS from 07.5 months of age shows high testicular activity confirmed by histological sections. At 07.5 months of age, these sections showed evolved germ cells including spermatids (early and late) and above all bundles of spermatozoa, even if the latter were limited in number. From 09 months of age, the presence of*H. occipitalis* is similar to that of females. At 09 months of age, males in a semi-controlled environment can fertilise eggs because they have free spermatozoa in the lumen of certain seminiferous tubules. However, it is at 10.5 months of age that he is fully mature with a great capacity for sperm production. In fact, the predominance of spermatids (early and late) and spermatozoa (in fascicles and free) confirms the sexual maturity and reproductive activity of the organisms [6].

5. Conclusion

This study shows that sexual maturity is reached at 10.5 months in both males and females of *Hoplobatrachus occipitalis*. The corresponding mean sizes are 92.5 ± 0.5 mm for females and 85.0 ± 4.05 mm for males. At this stage the average RGS for females was 11.58 ± 2.32% and for males 0.54 ± 0.02%. These results are basic data that will enable us to optimise the reproduction of this species of frog in a breeding environment.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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