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RESEARCH ARTICLE

Artificial Reproduction and Embryogeny of the Tiger Frog *Hoplobatrachus occipitalis* (Günther 1858)

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Abstract The objective of this study was to investigate the embryonic development of the frog *Hoplobatrachus occipitalis* after their artificial reproduction with the Ovaprim®, a combination of gonadotropin releasing hormone analogue and dopamine antagonist domperidone. Male and female *H. occipitalis* were collected from the wild and were intramuscularly injected with Ovaprim® at doses of 6 µL/g and 8 µL/g body weight, respectively. The eggs were laid 6 h after hormone injection. The numbers of eggs laid by each female frog were 1726 ± 5.4 (SD) with 94.4% and 88.2% mean fecundity and hatching rate, respectively. The diameter of the eggs at laying was 2.73 ± 0.33 mm while it was 5.04 ± 0.05 mm just before hatching. The stages of embryonic development starting from fertilization to tadpoles' formation took 27 h to complete at 26.4 ± 0.3 °C. The average height of the tadpoles after hatching was 5.1 mm. Circular tadpole movement in the chorion with an irregular respiratory movement was observed at 21 h after egg-laying. In summary, the study shows that *H. occipitalis* could reproduce in controlled environment by hormonal induction; the reproductive structures have no significant effect on egg-laying; and the embryonic development time of *H. occipitalis* differs from that other Anuran species.

Keywords *Hoplobatrachus occipitalis* · Reproduction · Ovaprim® · Fecundity · Egg-laying

Introduction

For centuries frogs are being consumed as food by humans. The international frog market is currently supplied by animals captured in the wild (Negroni and Farina 1993). Also, frog's value is well known for economical purposes and teaching. Frog consumption is essentially focused on legs especially the thigh. However, in many countries, the frogs are consumed entirely except the viscera (Negroni and Farina 1993). Due to the continued exploitation of these wild animals many frog species are now endangered. For instance, out of the 6285 species of amphibians listed in International Union for Conservation of Natre (IUCN) redlist, 1895 species (30%) are endangered or threatened with extinction (Reinier et al. 2010). This exploitation of frog led to a decrease in their population thus provoking natural imbalances. Among other causes of frog population decrease, is uncontrolled use of pesticides and chemical products (Negroni and Farina 1993).

Frog farming is on the increase due to its high demand for human consumption (Negroni and Farina 1993). According to Woynarovich and Horvàth (1981), the artificial reproduction has several advantages including year-round production, genetic improvement of stocks, and hybrids and, offsetting attacks by parasites. It is noteworthy that captive breeding (Holt et al. 2003) is important to conserve the viability of the approximately 32% of the world's amphibian species that are threatened (Browne et al. 2006b). However, the efficient use of captive breeding requires the reliable production of oocytes, usually through hormonal induction (Holt et al. 2003). The use

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of either pituitary hormones or their analogues to induce ovulation of amphibians is well established in some species of laboratory anurans including the northern leopard frogs (*Rana pipiens*), and the wood frog (*Rana sylvatica*) (Light and Pickoff 1976; Light et al. 1987). Also, one of the components of assisted reproductive technologies is the artificial manipulation of reproductive events using exogenous hormones. Specifically, males and females are administered hormones to stimulate the production and release of gametes (spermatozoa and oocytes), which are then used to generate embryos via in vitro fertilization, also referred to as artificial fertilization (Kouba et al. 2009). Several reproductive hormones have been proven to induce ovulation and spermiation in anuran species, acting either at the level of the pituitary or directly upon the gonads. Gonadotropin releasing hormones (GnRH), also called luteinizing hormone releasing hormone (LHRH), is the hypothalamic hormone controlling vertebrate reproductive physiology (Reinier et al. 2010). Intraperitoneal or intralymphatic administration of LHRH has been successfully used to induce ovulation and spermiation in some anurans (Browne et al. 2006a, b; Michael et al. 2004).

In Africa, *H. occipitalis* is the biggest and fleshiest of all edible frogs. For this reason, it is widely exploited for human consumption (Mazyambo 1981). *H. occipitalis* is a commercially exploited frog in many West African countries such as Burkina Faso, Benin and Nigeria where it is sold either dried or fried (Mohneke et al. 2011). Therefore, a perfect reproduction of H. occipitalis is necessary to facilitate the preservation of this threatened vertebrate (Chanson et al. 2008). In this context, it seems critically important to record the basic characteristics such as age at first reproduction, age at puberty (e.g., first appearance of nuptial pads in males), seasonality, environmental conditions that trigger reproductive events, fecundity, internal or external fertilization, egg size, oviparity or ovoviviparity, parental care, etc. (Poole and Grow 2012). This information will have significant implications for captive husbandry and breeding especially when establishing an amphibian captive assurance colony for the first time. However, there is a lack of information on the artificial reproduction and ontogeny of *H. occipitalis*. Major research works on *H. occipitalis* focused on its biological diversity and feeding habits (Rödel and Spieller 2000; Rödel and Branch 2002; Rödel 2003; Rödel and Ernest 2004; Assemian et al. 2006; Hirschfeld and Rödel 2011). The purpose of this study was to reproduce *H. occipitalis* in captivity by the spawning agent Ovaprim® and to document the different stages of its embryonic development.

Materials and Methods

Sampling and Spawners' Selection

Artificial reproduction trial was carried out at the Research Laboratory on Wetlands (LRZH) of the University of Abomey-Calavi, between July and August 2018. The adopted reproduction technique was those of Poole and Grow (2012) and Browne et al. (2006a). The spawners (8 adult frogs, male and female) were captured in ponds in the research station (Longitude E: 2° 20′ 18.7″; Latitude N 6° 24′ 53.4″). These frogs were acclimatized for 10 days in a circular cement pond (diameter 1.5 m and height 1 m) until the selection of the spawners. During this period, they were fed with live maggots and commercial feed (Coppens). Sun shine during experiment was low (about 4 h per day), day temperature ranged between 25 and 27 °C, while night temperature averaged to 24 °C.

Female spawners were selected based on weight (123–190 g) and sexual characters such as gravid females with well-developed abdomen and distended legs (Byrne and Roberts 2004, Fig. 1). Males were selected according to weight and their height at maturity (Tohé et al. 2016). In all, four females and four males were selected, weighed and measured individually.

Hormone Induction and Spawning

Ovaprim® (Syndel, Canada) that contains an analogue of salmon gonadotropin releasing hormone (sGnRHa) and a dopamine antagonist domperidone, was used to induce

Fig. 1 Photograph of a mature female *H. occipitalis*

gonad maturation in frogs. Following the recommendations of Poole and Grow (2012), and Browne et al. (2006a), females and males received a dose of 8 µL/g and 6 µL/g respectively (Table 1). Ovaprim® was injected intramuscularly in the thigh muscle at 7 am as recommended by Poole and Grow (2012). Following injection, the frogs were randomly distributed in pairs (a male and a female) and placed in four circular ponds containing 40 L of water each and covered with grid to avoid direct sun light exposure and to accelerate gamete maturation. The injected spawners were monitored every 20 min for gamete maturation and to determine the exact egg laying time.

During these operations, water temperature was 26.4 ± 0.3 °C, dissolved oxygen 6.55 ± 0.35 mg/L and pH was 7.8 ± 0.7 in the four basins.

Study of the Embryonic Development

The observations of the eggs began immediately after egg-laying and continued till hatching. A total of fifteen eggs were collected at random and observed every 30 min and then photographed by using a camera coupled with the binocular magnifier (Jeulin 2 × 10) during the experiment.

Measurement of Egg Characteristics

To estimate the total number of eggs laid, 12 samples of different egg masses of 0.57 g, 0.85 g, 1.36 g, 2.28 g, 2.68 g; 3.30 g, 3.83 g, 4.44 g, 5.28 g, 6.12 g and 7.06 g were taken and counted just after the egg laying. The eggs were weighed using electronic balance of 0.01 g precision. The unfertilized eggs were detected as those with no morphological or physiological changes and counted.

The size of the eggs was measured using Image J software. The linear axes measurements of 50 eggs were used to determine the mean diameter at each developmental stage. Three samples of 0.85 g of fertilized eggs were counted and weighed every 30 min to determine the mean mass of egg in relation to time. The volume of the eggs in cubic millimetres was calculated from axes' measurements.

Calculations

The absolute fecundity (AF) is the total spawned eggs by the female, relative fecundity (RF), spawning rate (SR), fecundity rate (FR), hatching rate (HR) and egg's volume were calculated according to the following formula:

AF: total eggs released in water;

$$\mathrm{RF}\left(\frac{\text{eggs}}{\text{g}}\right) = \frac{\text{Absolute Fecundity}}{\text{female weight (g)}}$$

$$\mathrm{SR}\ (\%) = \frac{\text{stripped eggs} + \text{eggs released in water}}{\text{Absolute Fecundity}} \times 100$$

$$\mathrm{FR}\ (\%) = \frac{\text{Number of fertilized eggs}}{\text{Number of incubated eggs}} \times 100$$

$$\mathrm{HR}\ (\%) = \frac{\text{Number of hatched eggs}}{\text{Number of fertilized eggs}} \times 100$$

$$\mathrm{V}\left(\mathrm{mm}^3\right) = \frac{\pi}{6} \times (\mathrm{Lp})^2 \times \mathrm{Lg}$$

With Lg, Length of the big axe and Lp, Length of the small axe of the egg.

Reproduction parameters and link between spawners weight, sizes and spawning times were determined from the data collected. The mean and standard deviation were computed for each parameter.

One way analysis of variance (ANOVA) was used to compare the parameters of the different treatments. Student–Newman–Keuls test (SNK test) using pair comparisons of means was performed to determine the link between the weight of spawners, their size and the spawning time. All statistical analyses were done using R software (version 3.5.0) and a threshold of 5% was considered for decision making.

Results

Spawning and Difference Between Non Fertilized and Fertilized Eggs in *H. occipitalis*

Spawning occurred 6 h after hormone injection (Table 1) at 26.4 ± 0.3 °C. Ten minutes before egg-laying, the male climbed on the female for mating. The male water the eggs

Table 1 Spawning time after Ovaprim® administration

Femeles	Weight of spawners (g)	Sizes (mm)	Spawning times (PM)
1	128.4	11.5	1.45
2	190.1	12.5	12.30
3	123.3	10.9	2.30
4	145.1	12.3	1

Values are not significantly different ($p > 0.05$)

laid by the female with it laitance. The eggs appeared opaque and spherical at the time of laying. They were black–whitish in colour at the two poles, covered by adhesive and transparent substance (Fig. 2). The egg clusters were maintained due to the adhesive and transparent substance. After laying, the eggs showed no morphological differentiation. A robust positive correlation ($R^2 = 0.998$) was found between the egg mass and the number of eggs (Fig. 3).

Table 2 summarizes the diameter, mass and volume of the eggs. The diameter of the unfertilized eggs was 2.73 ± 0.03 mm. In contrast, the fertilized eggs were 85% larger having a diameter of 5.04 ± 0.05 mm ($p < 0.05$).

Reproduction Parameters

Mean values of reproduction parameters are summarized in Table 3. The average number of eggs laid by females of *H. occipitalis* was 1726 ± 5.37 (SD) and the mean fertility rate was 93.07 ± 3.23%. The average weight of eggs laid by females was 75.71 ± 2.35 g, the mean of absolute fertility was 1467 ± 217.5 eggs and with a hatching rate of 88.2%.

Development of Fertilized Eggs in Relation to Time

The major stages of embryonic development in this study were: zygote stage, mitosis stage (cell divisions), gastrula stage and tadpole formation stage (Fig. 4).

Zygote Stage

At 0 h: egg presents at spawining a transparent external membrane. It was spherical with a big central nucleus. At this stage there is a peri-vitelline space filled with transparent liquid, which separates the chorion (nuclear mass) from the external membrane of egg. There was no proof for a nuclear division. Macroscopically, the first half of the

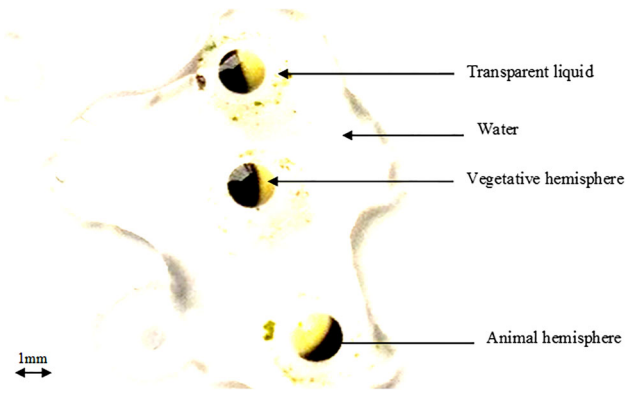


Fig. 2 Macroscopic view of the eggs of *H. occipitalis* after spawning

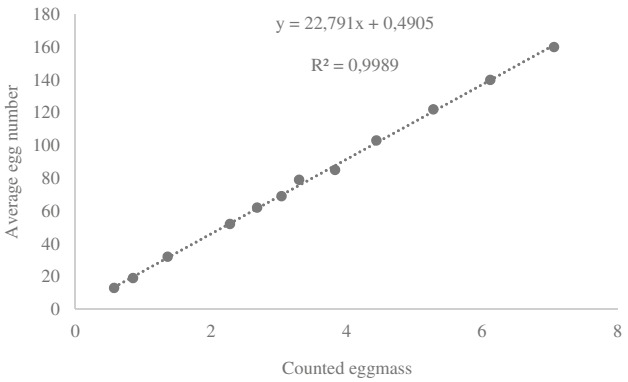


Fig. 3 Relationship between the number of eggs laid and their mass in *H.occipitalis*

nuclear mass was black though the second one was white. At this stage, there was no difference between non-fertilized and fertilized-eggs. As the eggs were opaque, it was not possible to see detailed modifications in the nucleus.

Mitosis Stage

At 2 h and half, the nuclear mass of fertilized eggs underwent a slight decrease in profit of the perivitellin space. At this stage, distinction between fertilized and non fertilized eggs was visible because the nuclear mass of non fertilized eggs didn't undergo any decrease.

At 7 h: An important regression of the nuclear mass was observed. The perivitelline space became larger, transparent, limpid and bright.

At 9 h: the regression started was even more pronounced. We also noticed a beginning of unsticking of the chorion from the nuclear mass: marking the beginning of embryo formation.

Gastrula Stage

At 13 h: chorion unsticking was more accentuated giving a form to the embryo. Both the nuclear mass and chorion volume are increased. Chorion unstuck progressively by showing a clear and easily identifiable region.

At 16 h and half: a progression in the decrease in the black mass contained in the chorion for the benefit of the clear region was noticed. It became a transparent liquid in which the developing embryo dwells. At this stage, the external membrane disappeared for the benefit of chorion.

At 17 h and half: the embryo's form became sharper. The formation of the head and body of the embryos were observed.

19 h and half: the formation of the head was complete. The structuration and the formation of the tail was noticed. At this stage, active rotation movement of the embryo in its capsule following by body lengthening was noticed.

Table 2 Variation in color, form, mass and measures of fertilized eggs during the embryonic development

Periods	Big axe (mm)	Small axe (mm)	Mass (g)	Volume (mm^3)	Eggs' form	Eggs' color
Fertilized egg	2.73 ± 0.03	2.52 ± 0.08	0.04 ± 0.02	9.09 ± 0.03	Spherical	Black–whitish
Mitosis	2.84 ± 0.05*	2.47 ± 0.08b*	0.09 ± 0.10*	9.09 ± 0.01*	Spherical	Black–whitish
Mitosis	2.93 ± 0.02*	2.46 ± 0.05*	0.12 ± 0.01*	9.36 ± 0.02*	Oval	Black–whitish
Gastrula	3.16 ± 0.03*	2.38 ± 0.02*	0.15 ± 0.01*	9.43 ± 0.01*	Oval	Black–whitish
End gastrula	3.87 ± 0.01*	2.21 ± 0.27*	0.17 ± 0.03*	9.89 ± 0.01*	Oval	Colorless-black
Tadpole formation	4.86 ± 0.09*	1.97 ± 0.03*	0.18 ± 0.07*	9.91 ± 0.01*	Oval	Colorless-black
Tadpole ready to hatch	5.04 ± 0.05*	1.98 ± 0.31*	0.18 ± 0.07*	10.36 ± 0.01*	Oval	Colorless-black

*$p < 0.05$ compared with that of fertilized egg

Table 3 Reproductive parameters of *Hoplobatrachus occipitalis* after hormone injection

Parameters	Absolute fecundity	Relative fecundity (eggs/g)	Spawning rate (%)	Incubated eggs' number	Fertilized eggs' number	Fecundity rate (%)	Hatching rate (%)
F_1	1360	10.59	100	1360	1238	91.03	88.93
F_2	1726	9079	100	1726	1629	94.38	92.14
F_3	1230	9.97	100	1230	1193	96.99	84.74
F_4	1552	10.69	100	1552	1395	89.88	86.88
Mean ± SD	1467 ± 217.5	10.08 ± 0.74	100	1467 ± 217.5	1363.75 ± 196.89	93.07 ± 3.23	88.17 ± 3.14

Formation Stage of the Tadpoles

At 21 h: and half: Head, tail and vitellin development were noticeable with a roundish tail. The rotative movement continued and the embryo's development pursued.

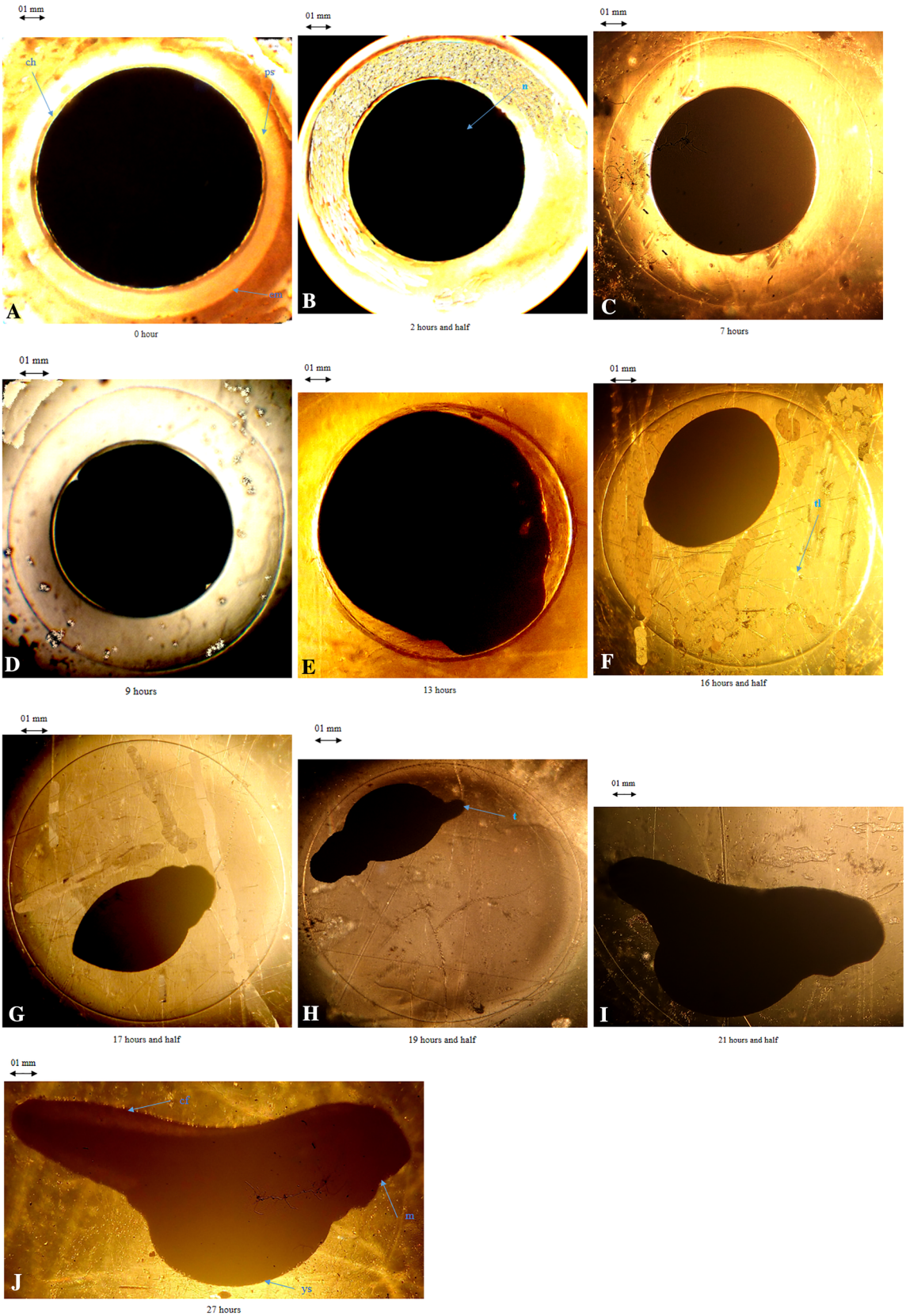
27 h: the tail lengthened progressively. A small transparent fins surrounding the caudal part of the embryo was observed. Vitellin became big and visible. Hatching began 27 h after fertilization. The average length of tadpole was 5.65 ± 0.77 mm.

During embryonic development, the color of fertilized eggs varied from black–white to black (Table 2). Eggs increased in size and became supple and transparent.

Discussion

The induced reproduction in *H. occipitalis* in the current study provided insights into the potential use of Ovaprim® for captive reproduction of the species. The synchronization of ovulation the application of a stimulator is important for induced reproduction in frogs. After 6 h, frogs injected with Ovaprim® produced milt and eggs. This observation could be attributed to the chemical composition of Ovaprim® and the reproduction environment. In a similar study, reproduction was induced in *Pseudophryne corroboree* by using the hormone LHRHa (Byrne and Silla 2010). In another study, Browne et al. (2006b) induced the reproduction in *Bufo baxteri* through the combined action of LHRHa and progesterone.

During our preliminary experiments, all attempts to induce reproduction in wild-caught adult *Hoplobatrachus occipitalis* with human chorionic gonadotropin (hCG) have failed. In the present study, the combined use of hCG and Ovaprim®, was ineffective in inducing reproduction. These results may be linked to challenges caused by the specific action of hormones, the lack of environmental stimuli regarding reproduction and the stress due to the spawners capture like the case of *Lithobates (Rana) sevosus* (Poole and Grow 2012). The 8 µL/g of Ovaprim® favored egg laying in *H. occipitalis* female. This dose used was optimal for the final maturation and release of the eggs. This result is similar to those obtained by Browne et al. (2006a) who combined hCG, LHRHa and progesterone in the species *Bufo fowleri*. From these prior studies, only progesterone favored egg laying in *Bufo fowleri*. So, the use of Ovaprim® in the artificial reproduction of *H. occipitalis* is different from progesterone used in hormonal induction in *Bufo fowleri*. We noticed the different hormones have specific actions on the reproduction of frog species. It is important to mention that hormonal treatment induces efficiently egg laying and final eggs' maturation (Mansour



◀**Fig. 4** Photographs showing different embryonic developmental stages of *H. occipitalis*. **a** egg's fertilization; **b** mitosis; **c** mitosis; **d** begining gastrula; **e** gastrula; **f** gastrula (nuclear mass decrease); **g** gastrula; **h** end gastrula; **i** tadpoles are making up; **j** hatching. The pictures are magnified 2×. *em* external membrane, *ch* chorion, *ps* perivitellin space, *n* nucleus, *h* head, *t* tail, *tl* transparent liquid, *m* mouth, *ys* yolk sac, *cf* caudal fin

et al. 2011). This shows that Ovaprim® could be specific for the induction of spermiation and releasing of eggs of *H. occipitalis* because the relative efficacy of the hormones is very specific to the species (Kouba et al. 2009).

The temperature can influence both egg maturation in the female and milt production in the male (Jorgensen 1992). For example, temperature ranging between 9 and 12 °C was found conducive for reproduction of the frog *Lithobates pipiens* (Morin 2008). In contrast, temperatures ranging between 4 and 15 °C and between 18 and 23 °C were found favourable for the reproduction of *Rana temporaria* (Vignes 2010) and *Colostethus machalilla* (Del Pino et al. 2004), respectively. In the present study, the temperature during egg laying was relatively higher at 26.4 ± 0.3 °C. The discrepancies between the present study and that of previous studies with regard to optimum temperature for reproduction could be explained by species-specific thermal preferences for reproduction among the amphibians.

Freshly laid eggs of *H. occipitalis* were ovate, opaque, white-blackish in color and covered with a limpid membrane. The color and egg diameter were similar to those of certain catfishes (Santos et al.2013). The mean diameter of *H. occipitalis* eggs observed in the present study (2.73 ± 0.03 mm) was similar to those reported by Channing (2001), Rödel (2000), and Tohé et al. (2016) in *H. occipitalis* from Central African Republic, West Africa and South Africa, respectively. However, the egg diameter observed in our study is higher than that of 1.6–1.8 mm in *H. occipitalis* from Sierra-Leone (Zug 1987). This observation suggests that the size of *H. occipitalis* eggs could be influenced by the geographical area, environmental cues and physiology of the species. The egg diameter increased to 5.04 ± 0.05 mm just before hatching. This increase can be linked to the embryonic development and swelling of the external membrane due to the absorption of liquid contained in the incubator. It is important to note that the perivitelline space of *H. occipitalis* eggs is highly vitellin-rich facilitating the growth of the embryo (Sayim and Kaya 2008). The mean fecundity observed in the current study (1467) was significantly lower than the 3225 oocytes reported in the same species earlier (Tohé et al. 2016). This difference could be explained by variation in height of the female frogs because the authors observed that spawners spawned more eggs than their smaller counterparts did. Alternatively, the lower number of eggs could be attributed to insufficient diet since the majority of the spawners were caught from the natural environment with no artificial feeds provided. Nevertheless, the fecundity recorded in *H. occipitalis* during this study was within the range of 469 and 3752 previously reported (Channing 2001). The average fecundity rate recorded in this study was 93.07 ± 3.23%, which was higher than the 73 ± 5% recorded by Browne et al. (2006a) in *Bufo baxteri*. Moreover, in this study, eggs' hatching occurred 27 h after fertilization at 26.5 ± 0.3 °C. In, *Hyla arborea*, hatching of eggs occurred 52 h and half after fertilization at 20 °C (Sayim and Kaya 2008). In *Colostethus machalilla* eggs' hatching occurred 197 h after fertilization when temperature ranged between 285 18–23 °C (Del Pino et al. 2004). Similarly, in *Engystomops* genus egg hatching occurred 29 h after fertilization (Romero-Carvajal et al. 2009). The discrepancies in these results could be explained by differences in species and temperature medium.

In the present study, the different stages of nuclear divisions occurred at 7 h intervals after fertilization at 26.5 ± 0.3 °C. This observation was comparable to that reported in *H. arborea* (Gosner 1960) but contradicted the temperature of 20 °C which was reported by Del Pino et al. (2004). Nevertheless, these observations are different from those of who reported the occurrence of nuclear division after fertilization in *C. machalilla* to be 06 h at a temperature of between 18 and 23 °C. We observed early gastrula 9 h after fertilization which is at variance with the observations of Romero-Carvajal et al. (2009) in genus *Engystomops* and Del Pino et al. (2004) in *C. machalilla*. Similarly, different gastrula stages in this study were observed between 13 and 17 h interval after fertilization which differ markedly from those of *H. arborea* with 25 h duration (Sayim and Kaya 2008). The results of the present study showed that embryonic development stages vary according to species. *H. occipitalis* had short incubation time compared to many frog species such as *H. arborea* (211 h), *C. machalilla* (20 days), *Xenopus laevis* (20 days), and frogs belonging to *Engystomops* genus (76 h) (Light and Pickoff 1976; Del Pino et al. 2004; Sayim and Kaya 2008; Romero-Carvajal et al. 2009). Indeed, there was close similarity among the frog species regarding embryonic characters Also, eggs and tadpoles are aquatic in nature, thus their embryonic development are expected to be similar though they belong to different families with some inter-specific differences. In this study, the time of the different stages of embryonomic development were much lower than those of previous workers (Del Pino et al. 2004; Romero-Carvajal et al. 2009). This observation could be linked to the relatively high temperature that occurred in our study area.

The results of artificial reproduction and embryogenesis of *H. occipitalis* provided deep insights into the

reproduction, and survival conditions of the species, as well as their embryonic development cycle. The optimal dose of the hormone Ovaprim® for effective egg-laying in females was 8 µL/g and for milt production in males was 6 µL/g.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Availability of Data and Material All relevant data are within the paper and its supporting information files.

Consent to Participate The animals used in this work have not been ill-treated or killed.

Consent for Publication Not applicable' for that section.

Ethical Approval All procedures performed in studies involving the use of animals were in accordance with the ethical standards of the Research Committee of the "Laboratoire de Recherche sur les Zones Humides"– in the Republic of Benin.

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