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Gametogenesis in Pubescent *Heterobranchus longifilis* (Teleostei: Clariidae) (Val. 1840) Attaining Puberty Under Artificial Conditions; and Under Rainy and Dry Season Conditions, in Tropical Earthen Ponds

C.O. OFOR

College of Natural Resources and Environmental Management
Michael Okpara University of Agriculture
Umudike, P.M.B. 7267, Unuahia, Abia State
Nigeria

Abstract

Three groups of *Heterobranchus longifilis* attained puberty under three environmental conditions. In experiment 1, 23 fish (10 males, 13 females) were raised in the rainy season, (group 1). In experiment 2, 90 fish (39 males, 51 females) were raised in a recirculation system (group 2). In experiment 3, 160 fish (73 males, 87 females) were raised in the dry season (group 3). Groups 1 and 3 were raised in outdoor earthen ponds. Gametogenesis in the groups was studied using histology and gonadosomatic index. Oocytes maturation commenced at 9½ months in all groups and lasted 67 days in group 1, and about 130 days in groups 2 and 3. Sexual maturity occurred at 352, 427, and 425 days respectively in groups 1, 2 and 3. It seems that the key point in ovarian maturation in pubescent *Heterobranchus longifilis* is 9½ months, with further oogenesis being influenced by rainfall. The time spent from here to sexual maturity onset may be shortened if it falls within the rainy season. The period may run its full course if it falls within the dry season, or the rearing environment is indoor. The application of these observations in the aquaculture of the species is discussed.

Introduction

The influence of environmental factors on reproduction of *Clarias gariepinus* has been documented by Clay (1979), Van den Hurk et al.

(1984), and for *Heterobranchus longifilis* by Legendre (1986), Richter et al. (1989) Freunde et al. (1995), and Nunez Rodriguez et al. (1995). Some of the environmental factors implicated are water temperature, pH, salinity, flow-volume, flow rate, turbidity, photoperiodicity, etc. Seasonal changes in these factors act as cues for gonadal recrudescence, and the more dominant factor depends on locality. The influence of rainfall on reproduction is exerted indirectly via rainfall-associated changes in some of these physico-chemical parameters.

In ponds, environmental influences on fish reproduction exist, especially those caused by rainfall. Freunde et al. (1995) found that adult pond-adapted and adult pond-reared female *H. longifilis* have a breeding season coinciding with the rainy season. Plasma levels of testosterone increased with the onset of the rainy season in both groups of females. Plasma levels of estradiol in pond-adapted females was the highest at the beginning of the breeding season in February/March. However, for pond-reared females, estradiol levels were high from January-August.

A review of methods of assessing ovarian development in fishes has been done by West (1990). Moiseyeva and Kukharev (1993) studied gonadal maturity stages of *Psenopsis cyanea* using histological methods. They found that in the ovaries of adult individuals, only oogonia and oocytes in early meiotic prophase and the first three stages of protoplasmic growth were observed all year, and were thus the reserve pool of sex cells. Recruitment to this pool was due to an increase in the number of oogonia and early meiocytes in the immediate post-spawning season (fall-winter season). From the concluding phases of protoplasmic growth, growth and development of sex cells were due to the transition of the same oocytes from one stage of oogenesis to the other, without any recruitment to the reserve pool.

It is known that in some animal species, the onset of sexual maturity is associated with a reduction in growth rate, especially in females, due to the diversion of resources to egg development. Henken et al. (1987) found sex differences in production in *C. gariepinus,* which was most pronounced in the higher weight ranges. This was attributed to the attainment of sexual maturity by the fishes, after which the females utilized nutrients for the growth of their gonad. Also in *C. gariepinus* , the ovaries can account for more than 20% of the body weight, leading to a reduction in product after gutting. In production of *H. longifilis,* it is therefore important to delimit the period before sexual maturity onset.

As a basis for *H. longifilis* culture, gonad maturation was studied under tropical pond conditions (Ofor 2001a), and under controlled conditions of a water re-circulation system. Attainment of puberty under these two conditions was compared (Ofor 2001b), and it was suggested that the influence of rainfall on the gonad was not restricted to ovarian recrudescence, but was also demonstrated in ovarian maturation of pubescent conspecifics. It was further suggested that this influence was responsible for the difference in age at onset of sexual maturity between pond and recirculation system raised *H. longifilis.* Literature on gametogenesis and gonad maturation in pubescent *H. longifilis* is lacking. Most of the literature is on adult individuals. Because of this and to facilitate grow-out of the species in Nigeria, a study was conducted comparing gametogenesis, gonad maturation, and age at sexual maturity onset, in *H. longifilis* attaining puberty in (1) outdoor earthen ponds in the rainy season (2) re-circulation system (3) outdoor earthen ponds in the dry season. This is expected to provide further information on the influence of rainfall on the processes.

Materials and Methods

Source of fish

Experiment 1

Adult *H. longifilis* sourced from rivers in south east Nigeria, and held in the ponds in the fish farm of the Institute of Oceanography University of Calabar, Nigeria for more than 1 year, were bred in June 1990, by induction with carp pituitary suspension. Twenty three fingerlings (10 males, 13 females) from this breeding were stocked into replicate earthen ponds and were reared to sexual maturity. They are designated as group 1.

Experiment 2

Adult *H. longifilis* similarly sourced and husbanded (in the same ponds) as in experiment I, were bred by induction with cPS. The fingerlings were transferred to the in-door recirculation system of the Forschungs und Studienzentrum fur Veredelungswirtschaft, of the University of Gottingen, Germany, where they were reared to sexual maturity. They were then bred by induction with cPS in 1992, to produce the fish used. Ninety of these (39 males, 51 females) were stocked into replicate tanks. They were reared to sexual maturity in the recirculation system and are designated as group 2.

Experiment 3

Adult *H. longifilis* sourced from the rivers of south east Nigeria and held in ponds in the Michael Okpara University of Agriculture, Umudike for more than I year, were bred with cPS in April 2003. One-hundred and sixty of the fingerlings (73 males, 87 females) were stocked into replicate earthen ponds, also in the Michael Okpara University of Agriculture, Umudike fish farm, and were reared to sexual maturity. They are designated as group 3.

Husbandry conditions

Larval feeding for all groups was with *Artemia* nauplii. At I month of age, fish of groups 1 and 3 were transferred to their respective earthen ponds.

Experiment 1

The fish were held in outdoor earthen ponds of dimensions 9x4x1.5m. They were fed with minced trash fish only. The feeding rate realized at the end of the experiment was 1.5% fresh body weight per day. Physico-chemical parameters were as follows: Mean daily oxygen level was 87% saturation (range 76%-126%), mean daily temperature was 27°C (range 25.8°C-30°C), pH had a range of 6.54-7. Sex ratio was 1:1.3 male to female.

Experiment 2

The fish were held in glass fibre flow through tanks of 1 m^3 volume, in a recirculation system. Temperature, pH, and oxygen levels were respectively maintained at 26°C, 7.04, and 80% saturation. Feeding was with Trouvit pellets of 55% protein content from fry to fingerling stage (10 g), and 45% protein content from fingerling stage until the end of the experiment. Feeding rate realized at the end of the experiment was 3% fresh body weight per day. Sex ratio was 1:1.3 male to female.

Experiment 3

The fish were held in out-door earthen ponds of dimensions 9x4x1.5m. They were fed with locally available commercial pellets of 36% protein content, at the rate of 3% fresh body weight per day. Mean daily temperature and oxygen levels were respectively 26.8°C (range 25.2°C to 31°C) and 85% saturation (66%-120%). pH had a range of 6.1-7.2. Sex ratio was 1:1.2 male to female.

In the earthen ponds the lower pH values were measured immediately after a rain. The earthen ponds were fertilized with chicken droppings and periodically limed. In the areas where they were located, the most important season-defining environmental influence was rainfall. The rainfall and temperature data for the areas of experiments 1 and 3, were obtained respectively from the department of Geography and Regional Planning, University of Calabar; and the National Root Crops Research Institute, Umudike, both located in south east Nigeria.

Sampling

The experiments were conducted in sequence. Experiment 1 was used to establish critical periods (appearance of sexual dimorphism, commencement of vitellogenesis). These served as guides in the development of a sampling scheme for experiment 2, under controlled conditions. But due to the paucity of fish, fish in experiment 1 were sampled only twice. Six fish (3 males, 3 females) were taken on the 285th day. Eight (4 males, 4 females) were taken on the 352nd day. Sampling (frequency) in the re-circulation system experiment was determined by the critical periods established in the pond experiment, and by the rate of advancement of the gonad through gametogenesis. Fishes of experiment 2 were sampled 9 times at 176, 238, 266, 297, 308, 336,360, 399, 427 days, for histological examination. Experiment 3 was sampled at 240, 290, 336, 352, 390 and 425 days. The sampling scheme for the experiments is shown in table 1.

Selection of males and females was done by careful examination of the genital papilla. The fish were stunned, weighed, dissected and the testes, seminal vesicles and ovaries taken and weighed. The influence of extraneous factors feed quality, feed quantity, stocking density. on the processes under investigation is expected to be mediated through size (condition) differences. To control this influence, only fish of comparable weight from all groups and at all ages were used. (Table 1). The similarity in sex ratio among the groups also enhanced this control.

Table1. Mean weight of sacrificed fish

Age (Days)			Male mean weight (g)			Female mean weight (g)		
Group			Group			Group		
1	2	3	1	2	3	1	2	3
	176			402 ; N=4			391; N=4	
	238	240		804; N=4	615; N=4		1067; N=4	680;N=4
	266			986;N=4			1418;N=4	
285	297	290	1200;N=3	1152;N=4	862;N=4	770;N=3	1249;N=4	965;N=4
	308			618;N=4	714;N=4		754;N=4	
	336	336					619;N=6	690;N=4
352	360	352	910;N=4			1016;N=4	960;N=4	852;N=4
	399	390					1293;N=6	1024;N=6
	427	425					1251;N=11	1194;N=12

Immediately after weighing, midsections of testes and seminal vesicles were fixed in Bouin's fluid. Midsections of ovaries were fixed in Gendre's fluid. Fixation was for 24 hours. Histological sections were cut at 5 micra, and prepared for staining following standard methods. The following staining procedures (Baker and Silverton 1978) were used: Weigert's Iron haematoxylin countered with Van Gieson stain in demonstrating blood cells in the interstitium, and to determine extent of invasion of the testes by blood vessels; Heidenhain's Iron haematoxylin countered with eosin, to demonstrate various cell types in spermatogenesis and oogenesis; Periodic Acid Schiff to demonstrate yolk incorporation. The sections were then examined with a Carl Zeiss binocular microscope. The number of the various cells types in each ovary was expressed per field of view at x 100 magnification. The result for all the ovaries examined at each age for each group was pooled and used to create a frequency distribution of cell types. From this, the relative abundance of the various cell types was determined, and hence the predominating cell type. The relative abundance of cell types was matched against rainfall and temperature patterns in experiments I and 3. The cell types presented in table 2 were worked out for the ovaries examined, and are modified after those observed in *Ictalurus punctatus* by Tucker (1984); the cell types presented in table 3 were worked out for the testes.

Table 2. Characterization of oocytes

Cell Type	Designation	Histological Characteristics
1	Oogonia	Located closest to the ovarian lamellae. Have large nucleus to cytoplasm ratio.
2	Chromatin nucleoli type	Larger nucleus than type 1. Cytoplasm stains more intensely than oogonia.
3	Early perinucleolar type	Characterised by a cytoplasm staining an intense colour. Nucleus has many nucleoli located near the nuclear envelope.
4	Late perinucleolar type.	Differentiated from type 3 by the presence of a granulosa and a thin layer of follicular cells.
5	Endogenous vitellogenic	Characterized by dense, vacuole-filled cytoplasm. The vacuoles did not stain with haematoxylin and eosin, and are progressively peripherally displaced. Presence of follicular cells.
6	Early exogenous vitellogenic type	Vacuoles exclusively peripheral. Granulosa layer well developed. Often more than one layer of follicular cells. Yolk uncoalesced, making the cytoplasm very dense and opaque.
7	Late exogenous vitellogenic type.	Characterized by the presence of only coalesced yolk.

Sexual maturity

Males

To determine the onset of sexual maturity in males, the milt from males sampled from all groups of fishes at different ages (8 months, 9½, and 11 months) was used to fertilize the eggs of a sexually mature female, using the following procedure: After mid-portions had been taken for histological examination, 1ml of milt from the testis was made up to 2 ml with physiological saline. If no fluid or milt was observed, the testis was crushed and washed with 2 ml physiological saline. The fertilization fluid so produced in both situations was then mixed with 1 g of eggs of an adult (previously sexually mature) fish. Water in which the eggs were to be incubated was added until the eggs were just covered. The entire mixture was then agitated for I minute and transferred to hatching trays. Hatching rate was calculated as the percentage hatching larvae per 1 g egg mass (700 eggs). Conditions of incubation were approximately 26°C temperature, 7.08 pH, 88% oxygen saturation. An individual was considered sexually mature when it caused a hatching rate of 50% and above. A group was considered sexually mature when all of its members sampled gave this response.

Females

To determine the onset of sexual maturity in females, starting from 240 days age, a batch of female fish from each group was repeatedly induced with cPS at the rate of 6mg/kg body weight (at intervals averaging 3½ weeks). An individual was considered sexually mature when it

Table 3. Characterization of Spermatocytes

Cell Type	Designation	Histological Characteristics
1	Spermatogonia	Occur in cysts located in close proximity to the walls of the seminiferous tubules. Stain lightly with haematoxylin.
2	Spermatocytes	Liberated from spermatogenic cysts. Stain darker with haematoxylin than type 1. Located peripherally in the lumen of the seminiferous tubules.
3	Maturing and mature sperm cells	Stain darker than type 2. Characterized by central location in the lumen of the seminiferous tubules.

The Gonadosomatic index (GSI) was calculated as:

$$\text{GSI} = \frac{\text{Weight of Gonad(g)}}{\text{Fresh body weight of fish (g)}} \times 100$$ (Van den Hurk et al. 1984)

Seminal vesicle somatic index (SVSI) was calculated as:

$$\text{SVSI} = \frac{\text{Weight of seminal vesicle(g)}}{\text{Fresh body weight of fish (g)}} \times 100$$

yielded mature eggs, irrespective of number or weight of the eggs. A group was considered sexually mature when all of its members in the sample gave this response.

Results

Table 4 shows the rainfall and temperature data for the areas of experiments 1 and 3, during the study. Onset of sexual maturity set in for males of all groups at 9½ months. At 352 days (15/6/91) sexual maturity had set in for females of group 1, which occurred at the peak of the rains. Sexual maturity set in for females of groups 2 and 3 at about 14½ months.

The results of sexual maturity tests, as well as the sequence of events in the gametogenesis of *H. longifilis* in the three experiments, are presented in table 5. The appearance of histological sections of ovaries and testes at different ages are presented in figures 1 to 9. Figure 10 shows the pattern of abundance of various cell types in comparison to rainfall and temperature in the area of experiments 1 and 3.

Table 4. Monthly total rainfall (mm) and mean monthly temperature (°C) in the the study areas of experiments 1 and 3

Year		Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
90	Rainfall (mm)	5.6	4.6	2.9	65.4	292.0	475.6	660.5	253.5	288.0	266.6	126.3	54.5
	Temp. (°C)	27.2	28.2	30.0	29.2	27.5	26.5	24.6	24.9	25.5	26.4	27.3°	27.1
91	Rainfall (mm)	1.5	14.8	47.6	275.8	205.0	468.6	477.7	485.6	136.5	193.2	75.4	5.2
	Temp. (°C)	27.0	28.5	28.5	27.5	28.0	27.3	25.6	26.0	26.1	25.8	27.0	26.6
2002	Rainfall (mm)	3.1	107.1	68.5	259.0	436.3	240.1	359.8	333.7	238.5	248.5	57.8	0.0
	Temp. (°C)	26.5	28.0	28.5	28.0	28.0	26.5	26.5	25.5	25.5	26.0	27.5	27.0
2003	Rainfall (mm)	0.0	37.9	119.5	159.8	231.4	282.4	447.5	372.6	340.8	180.2	69.2	0.0
	Temp. (°C)	27.5	28.5	29.0	29.0	27.5	26.5	26.5	26.0	26.0	27.0	27.5	26.0

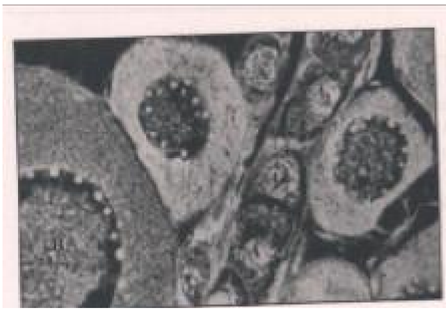

Fig. 1. T/S of ovary of pubescent *H. longifilis* at the proliferation phase, showing previtellogenic oocytes: A - Oogonia (Cell type 1); B - Early perinucleolar oocytes (Cell type 3); Gendre's fluid; Heidenhain's iron haematoxylin, eosin (X100)

Fig. 2. T/S of ovary of pubescent *H. longifilis* at the end of proliferation phase, showing previtellogenic oocytes: A - Chromatin nucleolar oocytes (Cell type 2); B - Early perinucleolar type (Cell type 3); Gendre's fluid; Heidenhain's iron haematoxylin, eosin (X100)

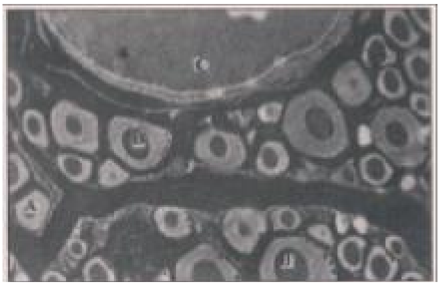

Fig. 3. T/S of ovary of pubescent *H. longifilis* at the beginning of maturation phase, previtellogenic oocytes:
A - Early perinucleolar oocytes (Cell type 3)
Oocytes undergoing endogenous vitellogenesis: B - Late perinucleolar type (Cell type 4); C - Endogenous vitellogenic oocytes (Cell type 5); Gendre's fluid; Heidenhain's iron haematoxylin, eosin (X100)

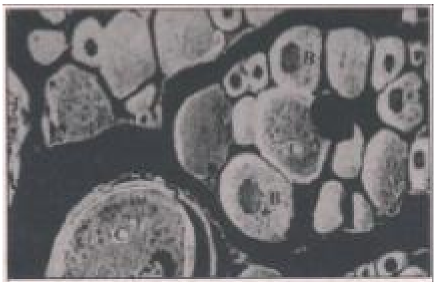

Fig. 4. T/S of ovary of pubescent *H. longifilis* during maturation, A - Early perinucleolar oocyte (Cell type 3); B - Late perinucleolar oocyte (Cell type 4); C - Early exogenous oocyte (Cell type 5); Gendre's fluid; Heidenhain's iron haematoxylin, eosin (X100)

Fig. 5. T/S of ovary of pubescent *H. longifilis* during maturation; Oocytes undergoing endogenous vitellogenesis: A - Late perinucleolar oocyte (Cell type 4); B - Endogenous vitellogenic oocytes (Cell type 5); C - Early exogenous oocyte (Cell type 6); Gendre's fluid; Heidenhain's iron haematoxylin, eosin (X100)

Fig. 6. T/S of ovary of pubertal *H. longifilis* A - Early exogenous oocyte (Cell type 6); B - Late exogenous oocyte (Cell type 7); Gendre's fluid; Heidenhain's iron haematoxylin, eosin (X100)

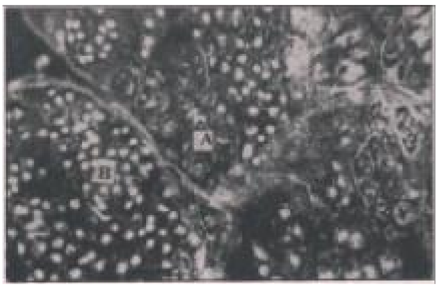

Fig. 7. T/S of testis of pubescent *H. longifilis* A - Spermatogonia; B - Spermatocyes; Bouin's fluid; Heidenhain's iron haematoxylin, eosin (X100)

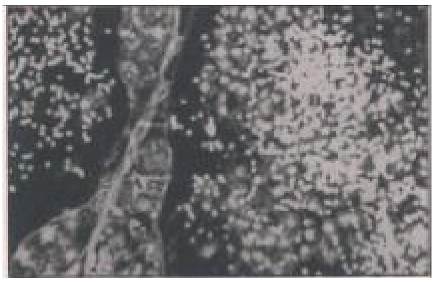

Fig. 8. T/S of testis of pubescent *H. longifilis* A - Spermatogonia; B - Spermatocyes; Bouin's fluid; Heidenhain's iron haematoxylin, eosin (X100)

Fig. 9. T/S of testis of pubertal *H. longifilis*, showing seminiferous tubule filled with mature and maturing sperm (A); Bouin's fluid; Heidenhain's iron haematoxylin, eosin (X100)

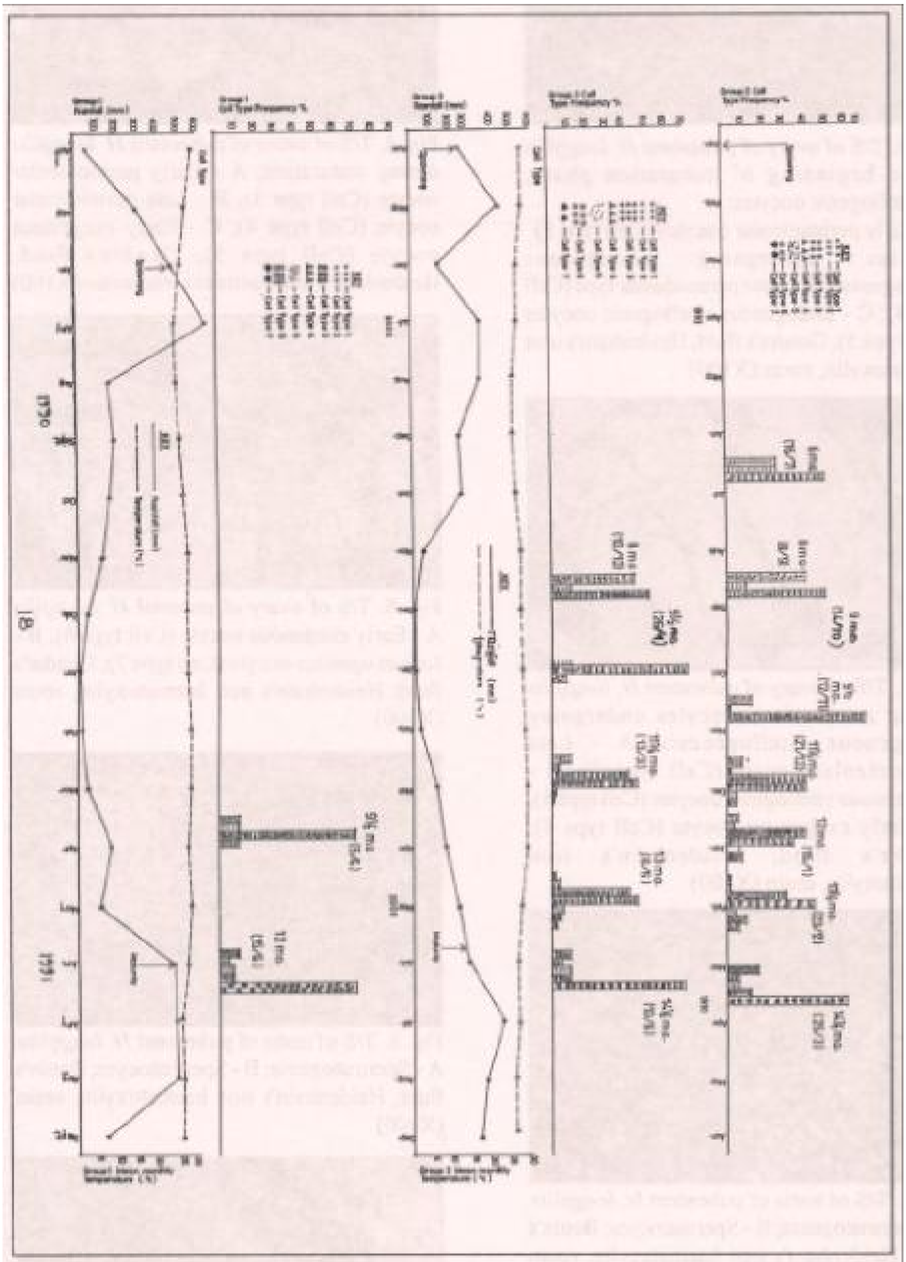

Fig. 10. Sexual maturity onset in female *H. longifilis* commencing and attaining puberty in the rainy season (group 1) in the dry season and dry season-rainy season transition (group 3) and in a recirculating system (group 2)

Table 5. The sequence of events during pubescent gonad development in *H. longifilis* in earthen ponds in the rainy season (Group1), in a recirculation system (Group 2) and earthen ponds in the dry season (Group 3)

Characterization of Ovaries	GS1(range)			Age (Days)			Characterization of testes	GSI /SVSI		
	Group 1	Group2	Group 3	Group 1	Group 2	Group 3		Group 1	Group 2	Group 3
Ovaries of reddish-brown coloration. Filled with cell types 3,2,1, in increasing other of abundance. Eggs can be seen with a hand lens. Types 4,5,6,7 absent (fig 1)		0.04± 0.007			176		Seminal vesicles not yet present. Testes thin, ribbon-like and filled with cell type 1. Seminiferous tubules compact. Sexual dimorphism not yet obvious. Genital papilla has not yet assumed characteristic shape for males.		0.017± 0.009	
Cell type 3 predominates. Eggs can be seen only with a hand lens. Types 4,5,6,7 absent. Negative response to cPS. (fig 2).		0.05± 0.02	0.04± 0.04		238	240	Seminal vesicles present. Sexual dimorphism obvious (Gential papilla assumes characteristic shape for males). Seminiferous tubules compact. (Fig. 7).		0.05± 02	0.03± 0.02
Ovaries of reddish-brown coloration. Cell type 3 predominates.Cell types 4,5,6,7 absent. Negative response to cPS. (fig. 2)		0.1± 0.03			266		Seminal vesicles well developed. Cell type 2 predominates. Interstitum developing. Vascularization obvious. (Fig 8).		0.03± 0.02/ 0.13± 0.005	
Ovaries of reddish-brown coloration. Cell type 3 predominates. Cell type 4, can be seen. Cell type 5,6,7 absent	0.38± 0.04	0.15± 0.02	0.07± 0.05	285	296	290	Vascularization prominent. Milk-white patches seen at the free edge of testes. Cell type 3 predominates. Seminiferous tubules expanding. Spermatogenic cysts discontinuous with the wall of seminiferous tubules. Hatching rate 50% or more (Fig. 9).	0.26+ 0.02	0.12± 0.03/ 0.25± 0.06	0.16± 0.05/ 0.31± 0.07
Ovaries dark brown. Individual eggs can be seen without a hand lens. Cell types 3 and 4 co-predominate. Cell types 5 and 6 can be seen in some ovaries (fig 3).		0.19± 0.02			308		Vascularization prominent. Majority of Seminiferous tubules fully expanded. Testicular cell type 3 predominant. Cysts discontinuous with the wall of seminiferous tubules. Milk- white patches extensive (Fig. 9).		0.14± 0.12/ 0..35± 0.06	
Ovaries a darker shade of brown. Cell types 3 and 4 predominate. More of 5 and 6 seen. Negative response to cPS (fig 4).		0.29± 0.02	0.27± 0.07		336	336	Fully expanded seminiferous tubules, with lumen filled with cell type 3. Whole milk-white in colour. Hatching rate more than 50%.	0.28+ 0.09	0.17± 0.01/ 0.3± 0.3	
Cell types 3 and 4 predominate. Cell types 5 and 6 increasing in number. Ovaries light green in colour. Negative response to cPS (Fig 5)		0.40± 0.01	0.52± 0.03		360	352				
Cell type 4 predominate. Cell type 7 appears. Ovaries light green in colour. Negative response to cPS.		0.40± 0.01	0.6± 0.05		399	390				
Cell type 7 predominate. Ovaries greenish in colour. Positive response to cPS (Fig 6.)	2.13± 0.03	1.4± 0.05	1.6± 0.1	352	425	427				

Testes

Histological examination of the seminal vesicles revealed that at maturity they contained no sperm cells. A comparison of the external appearance with histological sections of testes showed that the distribution and density of type 3 cells followed the distribution of white patches on the testes, and its degree of whiteness. The anterior portion of the testes was always at a more advanced stage of spermatogenesis than the other parts. At the beginning of the experiment, the testes contained compact seminiferous tubules. With age, the seminiferous tubules expanded. Spermatogonia occurred in cysts.

With further spermatogenesis cysts progressively moved away from the wall of the seminiferous tubules and seemed to eventually void their contents into the lumen of the tubule. There were no differences in the progression and completion of spermatogenesis between the testes of males of the groups.

Oogenesis

Cell types 1-3 were the previtellogenic oocytes. Cell types 4 and 5 were endogenous vitellogenic oocytes. Cell types 6 and 7 were the exogenous vitellogenic oocytes. Increases in size of the ovaries were seen to occur the same time as the increase in number of advanced oocytes (Table 4). Oogonia were the only cell types found undergoing division. Other cell types were involved in growth and maturation only. Three stages in the oogenic activity in the ovaries of pubescent *H. longifilis* were seen. These were proliferation, growth, and maturation. Proliferation stage, characterized by the active multiplication of cells, involved only cell type 1. Growth stage, characterized by the increase in size of proliferated cells, involved only cell types 2 and 3. Maturation stage involved cell types 4 to 7 and was characterized by the development of follicular cells and granulosa layer in the oocytes, as well as yolk deposition. Maturation depressed proliferation.

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