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Histochemical and biochemical study of lipids during the reproductive cycle of the toadfish, *Halobatrachus didactylus* (Schneider, 1801)*

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SUMMARY: Histochemical and biochemical aspects of the lipids of the female toadfish, *Halobatrachus didactylus*, during its annual reproductive cycle were studied. Oil droplets containing neutral lipids, and protein-yolk granules containing phospholipids and glycolipids were observed in the vitellogenic oocytes. The cytoplasm of the hepatocytes also contained neutral lipids (oil droplets). The highest lipid concentrations were observed in the liver and the lowest in the serum. In the liver, triglycerides predominated in the fractional composition of lipids. The ovarian and hepatic total lipids and phospholipids, and the hepatic triglyceride levels were higher during the reproductive period (from January to May). A positive relationship between the gonadosomatic index (GSI), hepatosomatic index (HSI) and ovarian total lipids, cholesterol, and phospholipids was found. The HSI was also positively correlated with the hepatic and serum lipids, whereas the GSI showed a negative relationship with the serum lipids. Results obtained show that the GSI and HSI variations observed, during the annual reproductive cycle of *H. didactylus* females, are associated with histological-histochemical changes and to modifications in the ovarian, hepatic and serum lipid concentrations.

Key words: toadfish, reproductive cycle, lipids, ovary, liver, serum, histochemistry, biochemistry.

RESUMEN: ESTUDIO HISTOQUÍMICO Y BIOQUÍMICO DE LÍPIDOS DURANTE EL CICLO REPRODUCTIVO DEL PEZ SAPO, *HALOBATRACHUS DIDACTYLUS* (SCHNEIDER, 1801). – Se estudian aspectos histoquímicos y bioquímicos de lípidos durante el ciclo reproductivo anual de hembras del pez sapo, *Halobatrachus didactylus*. En el citoplasma de los ovocitos vitelogénicos se han observado glóbulos lipídicos conteniendo lípidos neutros y gránulos proteicos constituidos por fosfolípidos y glicolípidos. Los lípidos neutros (gotas de grasa) también están presentes en el citoplasma de los hepatocitos. Las mayores concentraciones de lípidos se han observado en el hígado y las menores en el suero. Los triglicéridos fueron los lípidos predominantes en el hígado. La concentración de lípidos totales y fosfolípidos hepáticos y gonadales, así como los niveles de triglicéridos hepáticos han sido superiores durante el periodo reproductivo (de Enero a Mayo). Se han establecido coeficientes de correlación positivos entre el índice gonosómico (GSI) y hepatosómico (HSI) y la concentración de lípidos totales, colesterol y fosfolípidos gonadales. Mientras el HSI mostró una correlación positiva con los lípidos hepáticos y séricos, el GSI presentó una coeficiente de correlación negativo con los lípidos séricos. Estos resultados muestran que los cambios que experimentan el GSI y el HSI, a lo largo del ciclo reproductivo de hembras de *H. didactylus*, están asociados con modificaciones histológico-histoquímicas y con variaciones en la concentración de lípidos ováricos, hepáticos y séricos.

Palabras clave: pez sapo, ciclo reproductivo, lípidos, ovario, hígado, suero, histoquímica, bioquímica.

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INTRODUCTION

The reproductive activity of teleosts implies important morphological and histochemical changes, as well as variation in the concentration of carbohydrates, proteins, enzymes and hormones. Such changes occur in certain organs and tissues, especially in the ovary and liver, and lead to annual variation in the gonadosomatic and hepatosomatic indices (Love, 1980; Van Bohemen *et al.*, 1981; Matsuyama *et al.*, 1991; Grau, 1992; Rosety *et al.*, 1992; Garcia-Garcia, 1995). Considering these changes, and particularily the ovarian macroscopic and microscopic characteristics, Blanco (1991) divided the annual cycle of the toadfish, *Halobatrachus didactylus* into two periods: (1) reproductive period from January to May (final or exogenous vitellogenesis and maturation-spawning) and (2) non-reproductive period from June to December (regression and/or repose and initial vitellogenesis stages). At the end of the reproductive period (April-May), 65% of the oocytes are in final vitellogenesis and maturation stages. At the beginning of the non-reproductive period (regression and repose stages), 70-90% of the oocytes are basophil previtellogenic, the others being atretic oocytes and oogonia. The histological characteristics of the ovary (postovulatory follicles and atretic oocytes) and the presence of young toadfish (19-25 mm) in the Bay of Cádiz (Arias and Drake, 1990; Blanco, 1991), indicate that *H. didactylus* spawns during spring (April-May).

The chemical composition of the yolk and the distribution of carbohydrates, proteins, and lipids have been studied during the reproductive cycle of different teleosts (Khoo, 1979; Gutierrez *et al.*, 1985; Mayer *et al.*, 1988; Gonzalez De Canales *et al.*, 1992; Grau, 1992; Sarasquete *et al.*, 1993b; Garcia-Garcia, 1995). Data published by these authors suggest the existence of intra and interspecific differences, such as: absence of lipid globules in the oocytes, presence or absence of phospholipids and neutral lipids in the yolk granules and variations in the composition of the cortical alveoli.

During the reproductive cycle of teleost fish, a transfer of carbohydrates, proteins, and lipids between different organs and tissues, via plasma, take place. The changes in energy reserves such as glycogen and lipid in the liver and ovary occur according to the metabolic needs of the organs at different seasons (Van Bohemen *et al.*, 1981; Dasmahapatra and Medda, 1989; Singh and Singh, 1979, 1990).

As in higher vertebrates, lipids play a very important role in metabolism and reproduction of fishes. During gonadal development, phospholipids and sterols perform important functions as essential constituents of biological cell membranes, affecting their structural and physiological properties (Tocher *et al.*, 1985). Neutral lipids, particularily triacylglycerides, are a major energy source and the predominant form of energy storage. Cholesterol, in addition to its role as a membrane constituent, is a precursor of gonadal steroid synthesis (Dannevig and Norum, 1982; Singh and Singh, 1990). Some lipids are also the major transport form of macromolecules as vitellogenin and are used in yolk synthesis (Love, 1980; Lal and Sing, 1987). The increasing levels of gonadal and hepatic lipids during vitellogenesis and maturation of teleosts are well established (Love, 1980; Dasmahamatra and Medda, 1989; Singh and Singh, 1990). However, contradictory patterns are found in the annual changes of serum lipid levels (Tandon and Chandra, 1976; Singh and Singh, 1979; White, *et al.* 1986; Lal and Singh, 1987; Garcia-Garrido *et al.*, 1990).

H. didactylus is a sedentary teleost that belongs to the family Batrachoididae and is very common along the South Atlantic coasts of Spain and Portugal (Blanco, 1991). Its easy catch and maintenance in laboratory conditions make this species an excellent model for physiological studies. Although some aspects of its biology have been widely studied, only a few physiological and biochemical studies concerning its reproduction have been conducted. In order to extend such information, this study focuses on the distribution and quantity of lipids (total lipids, cholesterol, phospholipids and triglycerides) in the ovary, liver and serum of *Halobatrachus didactylus* females during the annual cycle.

MATERIALS AND METHODS

Female toadfish, *Halobatrachus didactylus* (n=70), were caught by dragging in the Bay of Cadiz (SW, Spain) from March 1992 to February 1993, and grouped according to Blanco (1991) into two periods (reproductive and non-reproductive). Gonadosomatic (weight of ovary x 100/total body weight) and hepatosomatic (weight of liver x 100/total body weight) indices were estimated. Blood was drawn under anaesthesia (inmersion in ethyl-4-aminobenzoate, 0.1 g/l marine water) by heart puncture and allowed to clot. The serum was then obtained by

low-speed centrifugation and samples were stored at -20 ºC until lipid analysis.

For the histomorphological study, samples of liver and ovary were fixed in formaldehyde-phosphate buffer 0.1M (pH 7.2) and embedded in paraffin or Historesin (Hydroxyethylmethacrylate, Reichert-Jung, Heidelberg FRG). Paraffin sections (6-7 µm) were stained with Haematoxylin-eosin and Haematoxylin-V.O.F. and plastic sections (0.5-1 µm) were stained with Toluidin blue and Cytopanchrome according to Sarasquete *et al.* (1993a).

Histochemical reaction for lipids (Sudan Black B for lipids in general, Red oil O for neutral lipids, Nile Blue and Luxol Blue for phospholipids, and Chloramine T-performic acid-dinitrophenylhydrazine-PAS for glycolipids) were carried out according to techniques described by Culling *et al.* (1985) and Pearse (1985). These methods were applied to sections directly processed in a cryomicrotome and to sections from paraffin and Historesin embedding procedures. Cold acetone was used to remove neutral lipids, chloroform/methanol (1/1) to remove all lipids, and phospholipids were extracted with pyridin (Pearse, 1985).

For biochemical study, ovarian and hepatic lipids were extracted with a mixture of methanol:chloroform (1:2 v/v). Aliquots of these extracts and serum samples (stored at -20 ºC) were used for determination of different lipids according to standardized methods of Biochemica Boehringer (Mannheim). Sulphophosphovainilline Test-Combination method was used to determine total lipid concentration. This method shows a pink coloration of lipids after the reaction with sulphuric acid, phosphoric acid and vainilline. Cholesterol was analyzed by using the cholesterol oxidase/peroxidase/Trinder method (CHOD/POD/Trinder). This method is based on the conversion of cholesterol esters in cholesterol and fatty acids by the action of the cholesterol esterase enzyme. Then, cholesterol is oxidated by cholesterol oxidase in 4-cholestenona and hydrogen peroxide, that is detected by Trinder reaction. Triglycerides were analyzed by using the glycerol phosphate oxidase/ peroxidase/Trinder method (GPO-POD-Trinder). In this method, triglycerids are hydrolized in glycerol and fatty acids by the action of a lipase; glycerol is transformed by a glycerol-kinase in alfa-glycerol phosphate that is converted by the action of a glycerol phosphate oxidase in dihydroxyacetone and hydrogen peroxide and revealed by the Trinder reaction. Phospholipids were shown by the Trinder-PAP enzymatic reaction. In this method, choline obtained after the hydrolisis of phospholipids by the action of phospholipase D is oxidated by the choline oxidase; then the reaction of peroxidase with hydrogen peroxide in the presence of a cromogen is shown by a pink coloration. All the analytical procedures were performed using commercial kits supplied by Boehringer GmbH Diagnostics (Mannheim). Lipid concentrations are indicated as mg of lipid/100 ml of tissue extract or serum sample.

Lipid concentrations (total lipids, cholesterol, triglycerides and phospholipids) in gonad, liver and serum, during reproductive and non reproductive periods, were compared by two way and three way analysis of variance, followed by the Newman-Keuls multiple comparison test. A simple linear regression analysis between GSI, HSI and the different lipids was performed.

RESULTS

Histochemical study of lipids

The lipid content (oil droplets) of the vitellogenic oocytes (Fig. 1) and hepatocytes (Fig. 2) were dissolved during the paraffin and Historesin embedding procedures (Fig. 1a and 2b). These lipids react with Red oil O (Fig. 1b) and Black Sudan B (Fig. 2a). They were stained in pink (neutral lipids) with Nile Blue and extracted with cold acetone.

The proteic yolk granules, observed in the vitellogenic oocytes of the *Halobatrachus didactylus*, did not contain neutral lipids (Red Oil O). They showed a weak sudanophilia in sections incubated with pyridine and stained with Sudan Black B. These results, as well as its tinctorial affinity to Nile Blue, Luxol Blue and Chloramine T-performic acid-dinitrophenylhydrazine-PAS techniques, indicate the absence of neutral lipids and the presence of phospholipids and glycolipids in the yolk granules. Neutral lipids and phospholipids were present in the follicular envelope of the vitellogenic oocytes (Fig. 1b). However, the lipid reactions performed were negative in the cytoplasm of the previtellogenic oocytes, cortical alveoli (glucidic vesicles), as well as in the zona radiata of the oocytes (Fig. 1b). On the other hand, the intrahepatic pancreas samples were negative to the lipid reactions studied (Fig. 2a); at the beginning of the vitellogenesis, an increase in the number of oil droplets was observed in the cytoplasm of the hepatocytes.

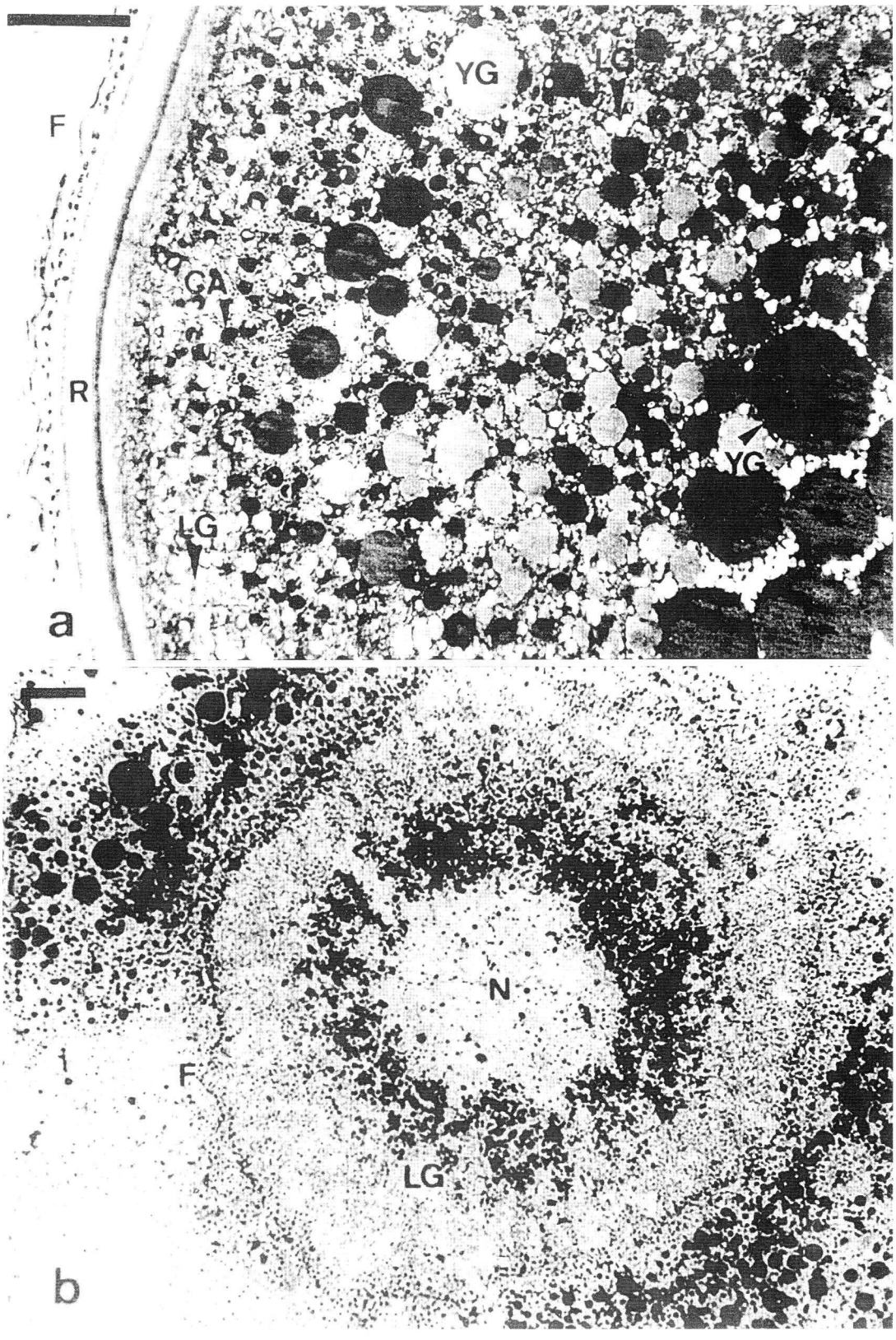

FIG.1. – Histological sections of the ovary of the toadfish, *Halobatrachus didactylus*. **a.** Vitellogenic oocyte with different cellular structures. The content of the lipid globules is dissolved during Historesin embedding procedure. Cytopanchrome staining. Bar = 50 µm. **b.** Initial vitellogenic oocyte showing positivity, in the follicular envelope and globules (oil droplets), to Red Oil 0 reaction. Cryomicrotome processed section. Bar = 30 µm. F: follicular envelope; R: zona radiata; CA: cortical alveoli; LG: lipid globules; YG: yolk granules. N = nucleus.

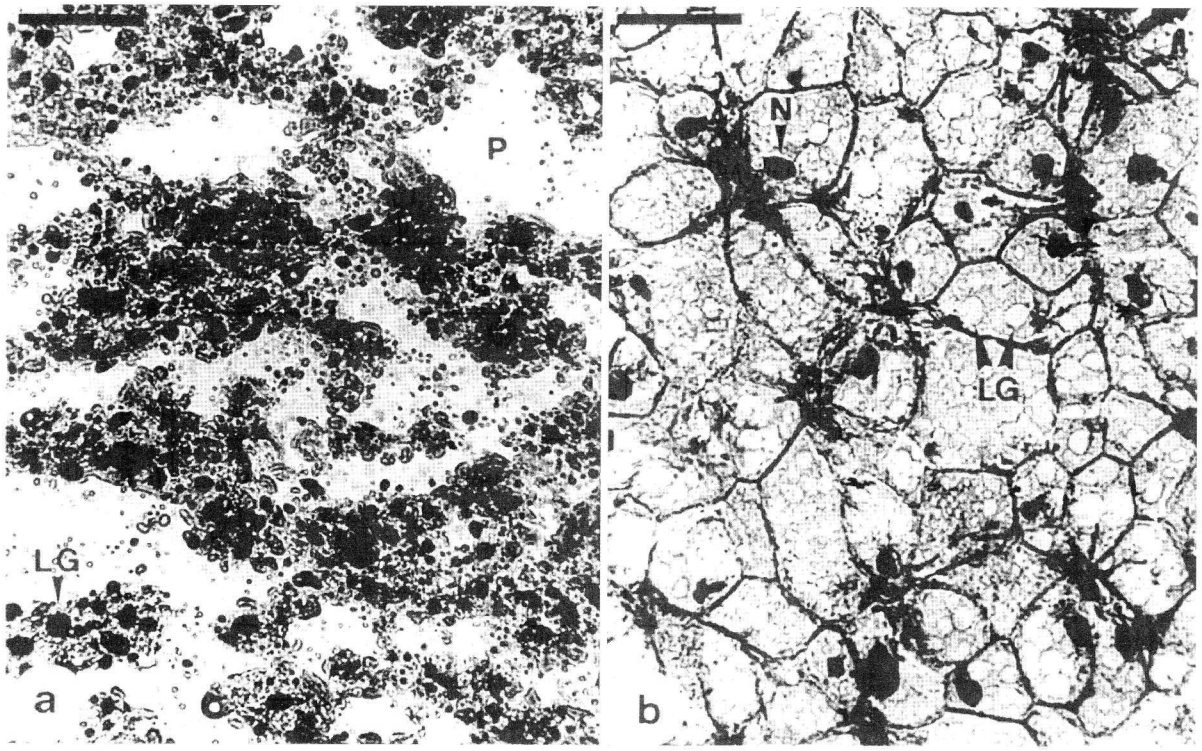

FIG.2. – Histological sections of the liver of the toadfish, *Halobatrachus didactylus*. **a.** Histological section of liver processed in the cryomicrotome. Neutral lipids are evident within the globules of the hepatocytes whereas the intrahepatic pancreas is negative to Sudan Black B reaction. **b.** Neutral lipid present in the cytoplasm of the hepatocytes are dissolved during paraffin embedding procedure. Haematoxylin-V.O.F. staining. LG: lipid globules; P: intrahepatic pancreas; N: nucleus. Bar = 30 μm.

Biochemical study of lipids

The two way ANOVA carried out for the total lipid concentrations revealed significant differences by period ($F=14.943$, $P<0.0002$) and tissue ($F=46.396$, $P<0.00001$). Interaction was also significant ($F=7.516$, $P<0.0007$), suggesting that there is a synergistic effect between both variables. Total lipid mean annual values were higher in liver than in gonad ($P<0.05$) and serum ($P<0.01$), showing the ovary higher lipid concentrations than the serum ($P<0.01$) (Table 1). These results were also found during the non reproductive period (June to December). However, during the reproductive period (January to May), lipid concentrations in gonad and liver were significantly higher than serum lipid concentrations ($P<0.01$).

Total lipid concentrations were higher during the reproductive period than in the non reproductive period ($P<0.01$); these differences were due to higher lipid levels in the ovary ($P<0.01$) and liver ($P<0.05$) (Table 1). Cholesterol, phospholipids and triglycerides were determined in ovary, liver and serum (Table 1). The three way ANOVA revealed the existence of significant differences by period ($F=24.607$, $P<0.00001$), lipid type ($F=96.771$, $P<0.00001$), and tissue ($F=74.19$, $P<0.00001$). Differences in lipid levels observed between the periods were exclusively due to the existence of higher concentrations of ovarian phospholipids ($P<0.01$) and hepatic phospholipids and triglycerides ($P<0.05$) during the reproductive period.

In the ovary, phospholipid concentrations were significantly higher in relation to cholesterol and triglycerides ($P<0.01$). In the liver, triglyceride concentrations were higher than the concentration of phospholipids and cholesterol ($P<0.01$). No differences were observed in the concentrations of serum phospholipids and cholesterol, both being higher than triglyceride concentrations ($P<0.05$).

Cholesterol concentrations showed no significant differences in serum, ovary and liver. Phospholipids were more abundant in gonad than in liver and serum ($P<0.01$). Phospholipid concentrations in liver were also higher than in serum ($P<0.05$). Finally, triglyceride concentrations were higher in liver than in ovary and serum ($P<0.01$).

During oocyte development, coinciding with a GSI increase, variations in some lipid levels were observed (Table 1). Simple regression analyses performed between gonadosomatic index (GSI), hepa-

TABLE 1. – Gonadosomatic index (GSI), hepatosomatic index (HSI) and ovarian, hepatic and serum lipid concentrations in females of the toadfish, *Halobatrachus didactylus*.

	REPRODUCTIVE PERIOD (January to May)	NON REPRODUCTIVE PERIOD (June to December	MEAN VALUES (January to December)
GSI	13.37±1.49	4.69±0.60	9.03±1.05
HSI	5.67±0.78	3.47±0.60	4.57±0.45
TOTAL LIPIDS			
Ovary	2522±212	1468±135	1920±119
Liver	2645±235	2022±161	2305±138
Serum	783±110	955±57	884±56
CHOLESTEROL			
Ovary	399±39	233±26	304±25
Liver	225±20	195±15	209±12
Serum	269±46	326±23	302±23
PHOSPHOLIPIDS			
Ovary	1857±167	1035±113	1387±109
Liver	836±88	563±32	687±46
Serum	371±40	397±24	386±22
TRIGLYCERIDES			
Ovary	186±14	147±17	164±12
Liver	1472±151	1168±116	1306±95
Serum	95±20	155±12	130±12

Data represent mean values (mg/100 ml) ± standard error of the mean. Reproductive period (n = 30), non reproductive period (n = 40).

TABLE 2. – Correlation coefficients (r) between gonadosomatic index (GSI), hepatosomatic index (HSI) and different gonadal, hepatic and serum lipids in *Halobatrachus didactylus* females.

TISSUE	LIPID	GSI	HSI
OVARY	Cholesterol	0.74**	0.27*
	Phospholipids	0.77**	0.28*
	Triglycerides	0.01	0.20
	Total lipids	0.76**	0.30*
LIVER	Cholesterol	–0.14	0.25*
	Phospholipids	0.20	0.29*
	Triglycerides	0.15	0.49**
	Total lipids	0.14	0.45**
SERUM	Cholesterol	–0.34**	0.42**
	Phospholipids	–0.27*	0.47**
	Triglycerides	–0.34**	0.34**
	Total lipids	–0.35**	0.45**

Asterisk represent significant correlations: *: $P < 0.05$ ($r = 0.23$); **: $P < 0.01$ ($r = 0.30$).

tosomatic index (HSI) and different lipids are shown in Table 2. There was a positive correlation between GSI and gonadal cholesterol, phospholipid and total lipid levels, a negative correlation between GSI and the serum lipid levels, and a lack of any significant correlation between GSI and the hepatic lipids. The HSI showed a positive correlation with almost all gonadal, hepatic and serum lipids (Table 2).

DISCUSSION

In females of the toadfish, *Halobatrachus didactylus*, lipid globules (oil droplets) present in the cytoplasm of the vitellogenic oocytes and hepatocytes only contain neutral lipids, which become dissolved during paraffin and Historesin embedding procedures. This fact indicates that oil droplets could represent free unsaturated lipids (triacylglycerides, wax esters and/or cholesterol) as it has been found in other species (Mayer *et al.*, 1988; Grau, 1992; Garcia-Garcia, 1995). Lipid globules were not present in some species, and phospholipids and/or neutral lipids could (or not) be observed in the yolk granules of various teleosts (Khoo, 1979; Mayer *et al.*, 1988; Sarasquete *et al.*, 1993b).

On the othe hand, the presence of glycoproteins (Gonzalez De Canales *et al.*, 1992) and phospholipids/glycolipids in yolk granules of the vitellogenic oocytes of the toadfish as in other species (Gutierrez et al., 1985; Grau, 1992; Sarasquete et al., 1993b; Garcia-Garcia, 1995), could be related to the vitellogenin. Wallace and Selman (1985) pointed out that vitellogenin, synthesized in the liver of females under gonadotropin control, is released into the blood and transported to the ovary. There, it is enzymatically degraded to lipo-

vitellin and phosvitin, which are the precursors of the yolk granules in teleosts.

During the annual cycle of *H. didactylus*, the ovary shows higher total lipid and phospholipid concentrations from January to May (reproductive period) and significant relationships between the GSI and HSI and these lipids. Both indices are higher during the reproductive period, as previously observed by Rosety *et al.* (1992) in this species. In other teleosts (Htun-Han, 1978; Obando and Leon, 1989), the correlation between the liver weight and the gonadal activity was associated with the energetic requirements of the reproductive process. The gradual enhancement of lipid content in the ovary during the breeding season is likely to be due to its increased formation and deposition in ooplasm at the time of development of oocytes, and is related (Dasmahapatra and Medda, 1982; 1989) to high gonadotropin levels inducing oocyte maturation.

In *H. didactylus*, the hepatic total lipids, triglycerides and phospholipids were higher during the reproductive period. The increase in lipid content of liver is possibly caused by a higher level of endogenous estrogen during the breeding season. This fact could indicate that no marked mobilization of this compound has taken place, or that estrogen-induced formation of lipids has markedly surpassed its mobilization (Dasmahapatra and Medda, 1982). On the other hand, the reduction in the hepatic lipid levels after spawning may be (Idler and Bitners, 1959; Dasmahapatra and Medda, 1989) the result of increased breakdown, mobilization and/or decreased synthesis, and associated with a decrease in the levels of endogenous oestrogens and gonadotropins.

The presence of higher hepatic triglyceride levels during the reproductive period of the toadfish was correlated with the histochemical results. The beginning of vitellogenesis of the toadfish, as in *Fundulus heteroclitus* (Garcia-Garcia, 1995), a higher number of oil droplets (neutral lipids) was observed in the cytoplasm of hepatocytes. Dasmahapatra and Medda (1989) and Singh and Singh (1990), in different species, also pointed out an increase in liver triglycerides (energy source) and diglycerides (vitellogenin precursor). In toadfish, Rosety *et al.* (1992) observed an increase in serum calcium and gonadal and hepatic proteins, as well as a decrease in hepatic glucose, in relation to the hepatic synthesis of the yolk-precursor. Haux and Norberg (1985) also stated the importance of phospholipids and triglycerides during vitellogenin synthesis, since they are functional components of this molecule. On the other hand, Van Bohemen *et al.* (1981) related the absence of lipids and hepatic glycogen in *Salmo gairdneri*, at the end of vitellogenesis, with a great metabolic demand of hepatocytes during a period of active vitellogenin synthesis.

In teleosts, the annual changes in serum lipid levels present interspecific differences. So, in some species (Love, 1980), blood lipid levels become higher at the time of ovarian growth due to the mobilization of body lipids including hepatic ones. However, in others species, a decrease or an increase in different serum lipids has been reported with increasing gonadal activity (Tandon and Chandra, 1976; Singh and Singh, 1979; White *et al.*, 1986). In toadfish, a negative relationship between the GSI and the serum lipids, as well as a positive correlation with the HSI was observed. According to Korsgaard and Petersen (1979) the decrease in blood lipids, during some stages of the reproductive cycle, could be related to a fast incorporation to the liver, where a parallel rise of lipids is observed.

Although further work should be directed to elucidate the role and the metabolism of lipids in the toadfish, these findings could establish the basis for future studies of processes related to the reproductive activity in teleost fish such as the control of photoperiod, captive conditions, hormonal regulation and feeding-starvation.

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