ANNUAL REPRODUCTIVE ACTIVITY OF A SPECIES OF *CREAGRUTUS* GÜNTHER (TELEOSTEI: CHARACIFORMES) IN A NEOTROPICAL MOUNTAIN RIVER

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Annual reproductive activity of a species of *Creagrutus* Günther (Teleostei: Characiformes) in a neotropical mountain river

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Running head: Reproductive activity of *Creagrutus*

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Figure 6. Monthly percentages of microscopic maturity stages of a) mature females and b) all males. In a) ░, Regeneration; ▒, Total Spawning; ▓, Partial Spawning; █, Late Vitellogenesis. In b) ░, Immaturity and Early Spermatogenesis; ▒, Regeneration; ▓, Late Spermatogenesis; █, Mid-Spermatogenesis. In both figures, the darker colours are more associated with reproductive activity.

ABSTRACT

TITLE: ANNUAL REPRODUCTIVE ACTIVITY OF A SPECIES OF *CREAGRUTUS* GÜNTHER (TELEOSTEI: CHARACIFORMES) IN A NEOTROPICAL MOUNTAIN RIVER[*]

AUTHOR: RAFAEL MAURICIO TORRES MEJÍA [**]

KEY WORDS: Gametogenesis, reproductive pattern, seasonality, tropics, maturity scale, histology.

DESCRIPTION:

The annual reproductive cycle of a species of *Creagrutus* (Characidae) was studied in a mountain river in Colombia. Micro- and macroscopical maturity scales were developed for this species. Oocytes exhibited a funnel with furrows that lead radially to the micropyle, a characteristic previously reported for other characids, which could have a phylogenetic utility. Ovaries indicated group-synchronous, total spawning. Sperm was of the aquasperm type and fertilization was most likely external. Macroscopic observations were less precise than the microscopical ones, demonstrating the necessity of histology for this sort of study. Gonadal mass did not vary significantly among sampling date, but the percentages of mature stages did, revealing a reproductive activity concentrated mainly during periods of low rainfall, what is relatively uncommon in tropical freshwater fishes. There were observed months of high rainfall with females filled with vitellogenic oocytes and fat but not spawning, and low rainfall months with few reproductive mature females, respectively before and after the main reproductive season. It is emphasized that it is not possible to generalize a reproductive pattern for all fishes of tropical rivers, and that the understanding of the factors that determines the reproductive timing of fishes in tropical mountain streams is far from known and still deserves further attention.

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RESUMEN

TÍTULO: ACTIVIDAD REPRODUCTIVA ANUAL DE UNA ESPECIE DE *CREAGRUTUS* GÜNTHER (TELEOSTEI: CHARACIDAE) EN UN RÍO NEOTROPICAL DE MONTAÑA [*]

AUTOR: RAFAEL MAURICIO TORRES MEJÍA [**]

PALABRAS CLAVE: **G**ametogénesis, Patrón reproductivo, Estacionalidad, Trópicos, Escala de madurez, Histología**.**

DESCRIPCIÓN

La actividad reproductiva anual de una especie de *Creagrutus* (Characidae) se estudió en un río neotropical de montaña. Se desarrollaron escalas de madurez micro y macroscópicas para la especie. Los ovocitos exhibieron una concavidad con surcos dirigidos radialmente hacia el micrópilo, una característica previamente reportada para otros carácidos y que podría tener cierta utilidad filogenética. Los ovarios indicaron madurez sincrónica de grupos y desova total. Los espermatozoides fueron del tipo acuaesperma y la fertilización al parecer externa. Las observaciones macroscópicas fueron menos precisas que las microscópicas, demostrando la necesidad de la histología para ésta clase de estudios. La masa gonadal no varió entre muestras mensuales, al contrario de los porcentajes de los estados de madurez, que revelaron una actividad reproductiva concentrada principalmente durante los meses de menos lluvias, lo cual es relativamente raro para peces tropicales de agua dulce. Se observaron meses lluviosos con hembras cargadas de ovocitos vitelogénicos y grasa pero que no estaban desovando, y meses secos con muy pocas hembras maduras reproductivas, respectivamente antes y después de la principal temporada reproductiva. Se enfatiza que no es posible generalizar un patrón reproductivo para los peces de ríos tropicales, y que aun están lejos de comprenderse los factores que determinan la estacionalidad reproductiva de los peces en éstos ríos, por lo cual se requiere mayor investigación en ésta área.

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INTRODUCTION

While reproduction of organisms is determined mainly by temperature and day length in temperate zones, it has been recognized the importance of rainfall as the principal environmental factor influencing the reproductive pattern of tropical fishes (Lowe-McConnell, 1987). In the case of tropical freshwater habitats, reproductive seasonality of fishes has been reported for floodplains, where most of species spawn during the high water period (Welcomme, 1979; Lowe McConnell, 1987; Galvis *et al.*, 1989, Araujo-Lima & Oliveira, 1998), and in intermittent streams, where reproduction is generally occurring during the rainy season (Alkins-Koo, 2000). In more stable habitats such as small lowland tropical rivers, which do not present the flood-pulse phenomena, fish reproductive patterns differ among species and may be continuous or seasonal, with peak a different seasons even within the same river system (Payne, 1975; Kramer, 1978; de Silva *et al.*, 1985).

In mountain or piedmont tropical rivers, another apparently stable habitat, reproductive activity of fishes has been scarcely investigated. Some of the few examples are the studies of Winemiller (1989), Winemiller & Taphorn (1989), and Taphorn (1992), who analyzed aspects of the natural history of a community of fishes inhabiting a river basin in Venezuela, finding that all lowland, piedmont and mountain river species spawned during rainy months, except by one mountain stream species, *Bryconamericus deuterodonoides* Eigenmann 1914, which spawned during the dry season. Flecker *et al.* (1991) confirmed *B. deuteronoides* reproductive pattern through the analysis of larval drift. Similarly, Dudgeon (2000) generalizes that in Asian rivers fishes spawn during rainy months, with few exceptions of reproduction occurring partially or totally during the rainy season (de Silva *et al.*, 1985;

Wang *et al.*, 1995). Moreover, in the tropical northern Australia three rainbowfish species reproduced during the dry season in piedmont rivers, even though one of them was known to spawn during the wet season in lowlands (Pusey *et al.*, 2001, 2002). The occurrence of such contradictory patterns justifies more studies on the reproductive biology of tropical fishes in mountain rivers. To contribute to the knowledge on this area, the main objective of this study was the description of the annual reproductive activity of a species of the genus *Creagrutus* in a mountain river, and its relation to selected environmental variables.

Creagrutus are restricted to freshwater neotropical and subtropical, seem to prefer fast currents, and are omnivores that feed mainly on benthonic macro-invertebrates, algae, and allochthonous insects and seeds (Taphorn, 1992; Román-Valencia, 1998; Ortaz, 1992, 2000; Vari & Harold, 2001). Maximum standard length (L_S) of most species is less than 100 mm (Vari & Harold, 2001). The reproductive activity pattern of five species of *Creagrutus* has been investigated [*C. bolivari* Schult 1944, (Taphorn, 1992; Ortaz, 1997), *C. brevipinnis* Eigenmann 1913 (Román-Valencia, 1998), *C. gyrospilus* Vari & Harold 2001, *C. taphorni* Vari & Harold 2001 (Taphorn, 1992), and C. melasma Vari & Harold 2001, (Winemiller, 1989; Taphorn, 1992)]. Nevertheless, among these investigations, the conclusions of Ortaz (1997) have been questioned because two new species were discovered in Ortaz' (1997) collecting sites, making it impossible to establish the exact taxonomic identity of the specimens analyzed in that study (Vari & Harold, 2001:81). Similarly, the specimens considered by Taphorn (1992) as a single species, *C.* cf. *beni*, were subsequently described as *C. gyrospilus* Vari & Harold 2001 and *C. taphorni* Vari & Harold 2001. Therefore, the research on the annual reproductive activity of species of *Creagrutus* still deserves attention.

In order to determine the reproductive cycle, it was first necessary to determine reproductive stages. Previous studies have used the gonadal mass, macroscopic stages of gonadal maturity, and, less frequently, microscopic stages of gonadal maturity. In this study all three methods were compared. Additionally, the presence of sperm within the female reproductive tract, the type of oocyte maturation [synchronous, group-synchronous, or asynchronous, Nagahama (1983)], the type of spawning (total or partial), and the size at first maturity were examined.

2. MATERIALS AND METHODS

2.1 STUDY SITE

Fish were collected in the Río Fonce and 500 m upstream from the mouth of the one of its tributaries, the Río Mogoticos (6˚25'-31' N, and 73˚7'-9' W, between 1100 and 1350 m of altitude), the type locality of this species. The Río Fonce drains a region of the west side of the Eastern Cordillera of Colombia, and it is part of the Río Magdalena basin system. The Río Fonce transports a relatively high sediment load (historic average 1490 KTn d^{-1}). The rainfall historic average show a bimodal pattern (Fig. 1a), with two low-rainfall seasons (December to March and June to August) and two high precipitation seasons (April to May, and September to November). Historic rainfall and river flow patterns fit very well to each other except by a slight phase lag of the flow regarding to rains (Fig. 1a). Río Fonce's watershed is primarily forested and agricultural, and its soil is constituted mainly of sedimentary rocks.

2.2 FIELD SAMPLING

Twenty to 30 *Creagrutus* sp. longer than 2 cm L_S were collected monthly from 11/2003 to 11/2004, in field campaigns of two days toward the middle of each month. Capture methods included cast nets, seines, dip nets, and hooks with worms as bait, depending on water conditions. The capture of small juveniles noted. Specimens were fixed by immersing them into neutralized formalin (10%), transferred to ethanol (70%) 15 to 30 days later, and were deposited in the collection of the ICN-MHN (Instituto de Ciencias Naturales, Museo de Historia Natural, Bogotá). Secchi disk visibility, water temperature, conductivity and pH, were measured *in situ* at each collecting site.

2.3 LABORATORY PROCEDURES

Preserved specimens were dissected under a stereo microscope. Prior to dissection the L_S (±0.02 mm) and the total mass (M_T) (±0.001g) of each specimen were measured. During the dissection the gonads were photographed and observed to record their gender, colour, volume relative to abdominal cavity and the presence of oocytes. The degree of transparency and flaccidity of gonads was assessed subjectively and quantified from 0 to 1. After these observations maturity of gonads was categorized using the macroscopic stages of Vazzoler (1996), Alkins-Koo (2000) and Azevedo *et al.* (2000). Masses of the eviscerated specimen (M_E), its liver (M_L), fat within the abdominal cavity (M_F), and gonads (M_G) were measured (±0.001g).

2.4 MICROSCOPIC ANALYSIS

To obtain histological preparations, one of the gonads of each specimen was embedded in paraplast, following a modification of the Luna (1968)'s method. Gonads were cut longitudinally at 7-10 μm thickness with a manual microtome. All slides were stained with Meyer's haematoxylin and eosin, duplicates of a sub-sample of ovaries were stained with Berg's method (Luna, 1968) to look for spermatozoa (to determine whether males introduce sperm into females), and with PAS to detect various carbohydrates (Luna, 1968; Sheehan & Hrapchak, 1973).

To determine the characteristics of oocytes, the continuity of oogenesis and the type of spawning transects were made through the gonads of five females chosen randomly from each reproductive stage. Along transects, the mean diameter of more than 50 oocytes per gonad were recorded. The oocytes were staged microscopically using an adaptation of the maturity scale of Matkovic & Pisanó (1989) (Table I). After the observation of the oocyte proportions a microscopic maturity scale was designed to classify the maturity stages of the ovaries. The classification was based on the observation of the most advanced oocytes, the atretic oocytes, and the post-ovulatory follicles, along a complete gonadal section. Testes were classified microscopically using an adaptation of the scale of Blazer (2002). The proportion of cyst with each germ cell type, and the abundance of spermatozoa in the lobular lumens were assessed subjectively.

2.5 MATURITY SIZE CALCULATION

The minimum size at maturity was designated as the size of the smallest specimen already with spermatozoa, in the case of males, and late vitellogenic oocytes or post-ovulatory follicles, in the case of females. The mean maturity size (L_{50}), the length at which 50 % of specimens were mature, was deduced by two methods, based on pooling of specimens according to L_S, in 5 mm groups. The first method, used for both sexes, consisted in the observation of the percentage of mature specimen in relation to L_S (Vazzoler, 1996), and was used for both sexes. The second method was the elaboration of a logistic curve that fitted the proportion of mature specimens (p), and the L_S. Data were transformed to obtain a simple linear regression with the formula $\ln[-\ln(1-p)] = \ln \alpha + \beta \ln L_S$ (Castro, 1999). The coefficients α and β were calculated by the least squares method, and used to obtain the logistic curve $p = 1\text{-}e \wedge (-\alpha\ L_S \wedge \beta)$ (Castro, 1999). The second method could not be applied to males due to their abrupt transition to maturity, which made it impossible to plot more than one point to calculate the logistic curve.

2.6 MACROSCOPIC ANALYSIS

Gonadal stages determined by microscopic observations were compared with macroscopic photographs and data of the same gonads, in order to delimit macroscopic maturity stages. To deduce whether variation in gonadal macroscopic characteristics followed the microscopic maturity stages, the Euclidean distances among microscopic maturity stages were calculated using a Cluster Analysis with the averages of macroscopic characteristics as variables. The macroscopic variables used were gonado-somatic index (I_G, $100\mathrm{M_G}\ M_E^{-1}$), volume, transparency, flaccidity and the presence of oocytes (the latter for females); their values were standardized to percentages. The groupings of the obtained dendogram, plus the comparisons of the macroscopic characteristics of stages, were used to collapse maturity stages with similar and overlapping characteristics, until macroscopic maturity stages were discrete enough to avoid most of ambiguity among them.

2.7 ANNUAL REPRODUCTIVE ACTIVITY ANALYSIS

The environmental data were explored using a Principal Component Analysis (PCA) in order to determine the variables that explained most of the variation. Additionally, months of sampling were compared with the PCA to detect any segregation among them.

The monthly reproductive activity of adult specimens was determined using two approaches. The first one was the study of the M_G variations. A traditional approach for this kind of analysis would have been to use somatic indices, such as the I_G. However, the use of percentages to normalize physiological data has been strongly questioned [see Packard & Boardman (1999) for a review on the issue]. Therefore, it was used the analysis of covariance (ANCOVA), the most recommended methodology alternative to the somatic indices (García-Berthou & Moreno-Amich, 1993; Packard & Boardman, 1999). Specifically, the design consisted in a multivariate analysis of covariance (MANCOVA) of the log-transformed data for each sex, with M_L, M_F, M_G as dependent variables, M_E as the covariant to control variation due to body size, and the month of capture as the independent variable. Subsequently, univariate tests (ANCOVA) and Tukey HSD pairwise tests were used to check for group differences. Homogeneity of slopes of MANCOVA models, a requirement to apply that statistics, was confirmed in each case.

The second method employed to characterize the reproductive activity was the comparison of monthly proportions of 1) reproductive females to all mature females, and of 2) males with “intense maturity” (Mid- and Late-spermatogenetic) to all mature males. Proportions were compared using the Pazer and Swanson test, with *a posteriori* Tukey-type test conducted in the case of finding significant differences of the proportions among months (Zar, 1999). Additionally, observations or capture of juveniles during sampling (evidence of relatively recent spawning) were compared with the results of the reproductive activity analysis.

3. RESULTS

3.1 FIELD SITE AND SAMPLING

The rainfall of the sampling year fitted very well to the historic bimodal pattern. The conductivity, pH, water temperature, and Secchi disk visibility were lower when the rainfall was high (Fig. 1b-c). The PCA revealed that all environmental variables studied were highly correlated with the two first components explaining 83% of the total variance (Fig. 2). The first factor was mainly defined by the rainfall and conductivity of the sampling year, and the historical mean flow, with percentages of correlation to this factor of 95, 94 and 92% respectively. The visual inspection of Fig. 2 allows the recognition of three main groups of months. The first, at the left side, included the months with highest rains. The second was composed by February, March and June, the driest months, at the upper right side. And the third group was formed by the remaining months at the lower right side, which were catalogued as months of transition between the dry and the rainy months.

Specimens of *Creagrutus* sp. were found in small embayments along the river bank and wide portions of the river, where water flow was minimal or null. In total 77 males and 135 females were captured and analyzed, plus an uncounted number of juveniles not included in the analysis except to point out the months of recruitment. Other species of fishes captured in the basin during this study were *Hemibrycon colombianus* Eigenmann 1914, *Hemibrycon* cf. *tolimae* (Eigenmann 1913), *Dolichancistrus* sp. Isbrücker, *Lasiancistrus caucanus* Eigenmann 1912, *Trichomycterus* sp. Valencienes, *Poecilia reticulata* Peters 1859, *Chaetostoma thomsoni* Regan 1904, *Geophagus steindachneri* Eigenmann & Hildebrand 1910, and *Oreochromis niloticus* (Linnaeus, 1758).

3.2 MICROSCOPIC DESCRIPTION OF OVARIES

Specimens less than 20 mm could not be sexed because their gonads were indistinguishable. Gonads of some specimens were difficult to cut or stain due to incomplete fixation. Oocyte characteristics are listed in Table I. Oogonia (stage I) were most abundant in immature females, usually aggregated into the ovarian epithelium, but they could be observed in some ovaries of other maturity stages. Pre-vitellogenic oocytes (stages II and II) were common in all the ovaries, except the mature gonads, where they were probably hidden by the bigger vitellogenic oocytes. Their granulosa layer forms a simple flat epithelium, PAS+, and the theca cells were not easily distinguished. In the late pre-vitellogenic oocytes (stage III) a thin chorion could be observed.

The formation of cortical alveoli, vacuoles containing a substance H-E negative and PAS+, distinguishes stage IV. These alveoli remain in the oocyte in the subsequent maturity stages, but masked in different degrees by the yolk granules. During the Cortical Alveoli stage, it is first observed a concavity at one side of the oocytes, a funnel with radially arranged furrows leading to the micropyle (Fig. 3).

The onset of vitellogenesis was observed in the Early Vitellogenic oocytes (stage V), which can be distinguished by the presence of very small orange yolk granules embedded in their cytoplasm. Oocytes in the Cortical Alveoli stage were scarcer than Pre-vitellogenic oocytes (stages I to III), and Early vitellogenic oocytes were much scarcer than vitellogenic oocytes. The late vitellogenic oocytes (stage VI) were recognized by the collapsing of their small yolk granules to form bigger ones and a thicker chorion. The last stage of the vitellogenesis, the oocyte maturation (stage VII), was characterized by the collapsing of the nuclear membrane, the slight migration of the nucleus to the animal pole (which was relatively close to the oocyte centre due to the micropyle funnel), and the enlargment of the yolk granules. At the end of this stage ovulation occurred. The remaining granulosa cells constitute the post-ovulatory follicle (POF); they were still PAS+, and became degraded until it was impossible to distinguish them from a mass of connective tissue. The observation of ovulated oocytes in the ovary preparations was very rare, occurring in only three gonads.

Follicular atresia was presented in stages IV to VI, and showed a variety of characteristics. Usually, early atretic oocytes were distinguished by the undulations of the chorion in the entire follicular periphery (not on only one side as in the case of the micropylar funnel), the fragmentation of the chorion, and the growth of the granulosa cells, which became cuboidal. Intermediate and late atresia show a cytoplasmic disarrangement, in some cases with the invasion by macrophage cells, and the multistratification of the granulosa layer. The final atretic oocyte was usually round, with a compact appearance, and characteristic convoluted remains of chorion inside.

The histological descriptions of the ovaries at each reproductive stage are set out in Tables I-III. Figure 4 shows the frequency of each type of oocytes at each microscopic reproductive stage. Most of immature ovaries showed no evidence of having been previously reproductive, although the ones from larger females could have been reproductive before and in this stage they could be in a stage of “resting”. If any vitellogenic oocytes remain after spawning, they appear to become atretic (Fig. 4e-f).
The search for spermatozoa was unsuccessful, they were not observed in any ovary.

3.3 MICROSCOPIC DESCRIPTION OF TESTES

Testes were classified into five stages, adapted from Blazer’s gonadal maturity scale: 1) Pre-spermatogenic (only with spermatogonia), 2) Early spermatogenic (mainly with spermatogonia to spermatids, but already with spermatozoa), 3) Mid-spermatogenic (with nearly equal proportions of spermatocytes, spermatids, and spermatozoa, the latter with spherical nucleus) 4) Late spermatogenic (with a higher proportion of spermatogonia and spermatids in the cysts, and of spermatozoa in the lobular lumen and into the cysts), and 5) Regressed (spermatogonia and cells in resorption mostly or totally predominant in the cysts, but the lobular lumens could still contain spermatozoa, even abundantly). The cysts of male gametic cells were arranged in anastomosing tubules, according to the nomenclature of Parenti & Grier (2004).

3.4 MACROSCOPIC DESCRIPTIONS OF GONADS

Macroscopic observations were less precise than microscopic observations for determining the maturity stage of females (Table III). The maturity stages derived from the macroscopic scales of Vazzoler (1996), Alkins-Koo (2000) and Azevedo (2000) often confounded maturing and totally spent ovaries. In addition, some characteristics used by these scales were never observed, including haemorrhagic zones, gonadal volumes greater than 60% of coelomic cavity and elimination of viscous liquids upon application of pressure (the latter for testes). Other characteristics such as flaccidity (observed in both immature and totally spent ovaries), or the partial filling of large-size oocytes (maturing, totally spent and regenerating ovaries possess them) were not exclusive to the stage they were supposed to define. Thus, in the macroscopic maturity scale proposed for *Creagrutus* sp., from the seven microscopic stages, only three macroscopic maturity stages could be defined (with the related microscopic stages in parenthesis): Immaturity (Immaturity and Pre-maturity), Non-reproductive maturity (Early Vitellogenesis, Totally Spawning and Regeneration) and Reproductive-maturity (Late Vitellogenesis and Partial Spawning).

In the case of males the macroscopic classification was even less precise than for females. The smallest testes, small and threadlike-shaped (sometimes with a mass less than one mg), were classified microscopically in the Pre- or Early-spermatogenic stages, while the largest ones were Mid- or Late-spermatogenetic. Between these extremes all spermatogenetic stages and the Resting stage presented similar macroscopic characteristics, which made it impractical to propose a maturity scale that had all its stages overlapped considerably.

3.5 ANNUAL CYCLE OF REPRODUCTION

Males were significantly smaller than females (Mann-Whitney U $\alpha_{0.05,\ (2),\ n\ =\ 153,\ 50}$, $P < 0.001$), matured at a smaller size (L_{50} = 28 mm in males and 48 mm in females, Fig. 5), and matured more abruptly than females (the transition from zero to 100% maturity occurred over a 5 mm size range in males and a 20 mm size range in females).

Variations of M_F, M_G, and M_L did not show a seasonal pattern, except for female's M_F. MANCOVA revealed that M_E was a significant covariant for both sexes ($P = 0.016$ and < 0.001, for males and females respectively), for all variables but M_F of females (ANCOVA, $P = 0.525$) (Table IV). This justifies the use of MANCOVA for this data set. However, no significant variation was explained by the month of capture for any of the body mass variables except for female fat mass (ANCOVA, $P = 0.006$), which was generally lower during dry months than during rainy months (Jan = Feb < May = Jun < Aug < Apr , Tukey HSD test).

The microscopic observations of gonads provided evidence of seasonal variation.

The proportions of reproductive females was statistically different among months ($\chi^2_{(0.05, 11)}$, $P < 0.001$), which were ordered according to their significant differences by the Tukey test as Sep = Oct = Apr < Nov = Dec = Jan = Feb = Jun (Fig. 6a). The months with a low proportion of reproductive females are all rainy and, except for November, the months with a higher proportion of reproductive females were drier. On the other hand, mature males occurred all year round. In fact, only three Pre-spermatogenic males were found. Analysis of the different stages of mature males showed that the proportion of males with "intense maturity" differed slightly among months ($\chi^2_{(0.05, 8)}$, $P = 0.035$), although the Tukey test failed to detect those differences. The months with the extreme lower and upper proportions of "intense-maturity" males were October and April, and June and July, respectively (Fig. 6b).

Juveniles were captured principally after dry months. Although data of juvenile samplings from December to March were missing, the among the remaining months sampled, juveniles less than 20 mm L_S were captured mainly in April and July, and much less abundantly from August to November.

4. DISCUSSION

4.1 ENVIRONMENTAL CHARACTERISTICS OF THE STUDY SITE

A bimodal rainfall pattern observed in the Río Fonce basin has been observed in various northern South American areas, and it is a consequence of the Bolivian High and the oscillations of the intertropical convergence zone (Lewis *et al.* 1995). Other attribute of the basin, the soils formed by sedimentary deposits, is characteristic of de Andean region, and may explain the relatively high sediment load the river transports (historic average 1490 KTn d^{-1}), as it is generally accepted for Andean rivers (e.g. Lewis *et al.*, 1995). Because of the sediment load, the Secchi disk visibility and water colour, the Río Fonce could be catalogued as a white-water river (Sioli, 1984). Finally, the high correlation of environmental variables in the PCA may indicate that the rainfall determined the pattern of the other environmental variables of the river during the sampling year.

4.2 MICROSCOPIC CHARACTERISTICS OF THE GAMETIC CELLS AND GONADS

The poor preservation of some specimens fixed with the standard method of immersing them into 10% neutralized formalin (e. g. AFS *et al.*, 2004), leads to recommend to improve fixation of viscera by injecting the fixative into the abdominal cavity of all specimens larger than 3 cm L_S, or even smaller if possible.

The gametic cells and gonads of *Creagrutus* sp. exhibited the general basic morphological and developmental pattern of teleosts (Tyler and Sumpter, 1996; Coward *et al.* 2002 ; Patiño & Sullivan, 2002). Some specific features, including the absence of oil droplets, the earlier appearance of cortical alveoli than yolk platelets, the non-fusion of the yolk platelets, and the non-hydration of maturing eggs, are common characteristics of freshwater teleosts (Nagahama, 1983; Mellinger, 2002).

One specific attribute is the duration of each of oogenesis phases. It seems that oogenesis in *Creagrutus* sp. is a process with two long period of growth, pre-vitellogenesis and Late

Vitellogenesis, each of which end in relatively rapid transitional phases, the Early Vitellogenesis and Maturation phases, respectively. The low abundance of oocytes found in stages V, VII, and VIII could be interpreted as a rapid transition of the oocytes through these phases during oogenesis. Conversely, the relative abundance of II, III and VI oocytes could reflect the longer duration of oocytes in these stages. The longer duration may be related to the greater increment in size observed in II, III and VI oocytes (Table I). In general, eggs of freshwater oviparous teleosts increase in size mainly during vitellogenesis, which may last from days to months (Tylor & Sumpter, 1996).

The type of oocyte maturation and spawning could be determined. In the case of Mature ovaries (Fig. 4c), the size-gap between the group of most advanced oocytes and next conspicuous smaller group implies that this species has group-synchronous ovaries. Shown in Fig. 4d-f , most of large vitellogenic oocytes are absent from ovaries after spawning, which implies that spawning is total, meaning that once a group of oocytes mature they are all released in a single spawning event or in a series of spawning events over a short period.

Another attribute observed microscopically was the coloration of the granulosa cells by PAS. This staining could be due to glycoproteins in the extracellular matrix. The staining of granulosa by PAS was useful to distinguish POFs, which are sometimes hard to differentiate from stromal tissue or atretic oocytes, especially when degeneration is advanced. Similarly, PAS+ cellular aggregates were also used by McBride & Thurman (2003) to distinguish females with regressed ovaries from immature virgin or maturing virgin females.

One unexpected feature of *Creagrutus* sp. eggs was the presence of furrows in the region of the micropyle. I two dimensions under the light microscopy, this region looks like undulations of the chorion, which also occurs in atretic eggs (e.g. Miranda *et al*., 1999), or as artifacts due to desiccation (pers. obs.). Nevertheless, the natural occurrence of this structure on the mcropylar region was confirmed by a tri-dimensional reconstruction after serial-section observation, by the presence of a micropylar cell in the bottom of the deepest groove, by the proximity of the animal pole to the funnel, by the distribution of furrows in just one pole of the chorion at a time, and by the cellular integrity of the oocytes with furrows. A chorion featuring grooves and ridges radially directed toward the micropyle has been reported previously for some cyprinids (Nagahama, 1983:243; Amanze & Iyengar, 1990), an osteoglossiform (Britz, 2004), and some species of Characidae (Rizzo *et al*., 2002; Brandao *et al*., 2003). Within the order Characiformes, the presence of furrowns only in some genera of the Characidae suggests that the origin of this characteristic is independent from other orders, although the homology of this trait for the family also needs to be confirmed. Since the phylogeny of Characidae remains poorly resolved (Vari, 1998; Weitzman & Malabarba, 1998), the presence of a characteristic, a chorion with furrows, that seems to segregate some genera from the bulk of the family may be very important. To determine the phylogenetic utility of the egg surface of Characiformes would necessitate an extensive sampling within the order, using SEM or light microscopy (through tri-dimensional reconstruction of serial sections). The function of chorionic furrows converging to the micropyle was studied by Amanze & Iyengar (1990), who found that most of sperm travelled along the grooves to finally arrive to the micropylar pit, enhancing the chances of egg fertilization to 99.7 % once the sperm had reached the micropylar region.

The species of *Creagrutus* examined here showed no evidence of insemination, the introduction of sperm into the female reproductive system, because sperm were not found in any ovary. Conversely, the nucleus of spermatozoa was spherical, a typical characteristic of aquasperm, the type of spermatozoa of most fishes with external fertilization (Jamieson, 1991). These observations lead to conclude that fertilization in *Creagrutus* sp. is likely external. Insemination has been confirmed for some genera of Characidae and at least other 21 other families of teleosts (Burns & Weitzman, unpublished). Within the Characidae, *Creagrutus* is one of the genera in which insemination occurs (aquasperm found in ovaries of two of 12 species examined so far, J. Burns, pers. com.). Independently of the non-occurrence of insemination in the species of *Creagrutus* studied here, this work could be useful to compare the reproductive activity of species included in taxa of Characidae where insemination has evolved.

Although thread-like testes are usually considered immature (e.g. Vazzoler, 1996), some of them showed small numbers of spermatozoa in this study, an observation that had not been reported previously. Nevertheless males with such testes should not be considered as mature. The biological function of such spermatozoa is unclear because low sperm concentration reduces the fertilization rates, as concluded by Nakatsuru & Kramer (1982) in a study on a characid.

4.3 MACROSCOPIC CHARACTERISTICS OF GONADS

A macroscopic maturity scale, with lower precision than the microscopic scale, could be developed for females but not for males. Macroscopic maturity scales have been widely used to stage maturity of fishes, in order to avoid the more time consuming and expensive histology. Nevertheless, pitfalls of macroscopic classifications have led to suggestions to avoid their use (e.g. Smith & Walker, 2004). Ovaries of *Creagrutus* sp. exhibited macroscopic variation sufficiently correlated with microscopic stages to support a macroscopic scale with three stages: Immaturity, Non-reproductive Maturity, and Reproductive Maturity. This scale does have some problems, for example the impossibility of differentiating ovaries in Late Maturity and Partial Spawning stages. The latter could reduce precision in the estimation of the time of spawning because partially spent females and the presence of POFs indicate a recent spawning event, since the maximum degeneration time of POFs reported for fishes is about three days (Fowler et al., 1999). However, the maturity scale proposed for females would be useful to perform simpler assessments of maturity in further studies with *Creagrutus* sp. that need only to differentiate reproductive from non-reproductive females.

Macroscopic variation in the testes of *Creagrutus* sp. was not discrete enough to propose a scale. For example, a thread-like shape, traditionally characteristic of immature gonads, was observed in both Pre- and Early-spermatogenic testes. Therefore histological methods are recommended every time determination of the maturity stage in male *Creagrutus* sp. is required. The conclusion that use of macroscopic scales requires their previous validation through histology should also be extended to other teleosts. While some authors dismiss macroscopic classifications (West, 1990), this study suggest that macroscopic maturity scales can be validated by contrasting the macro- and microscopic observations as demonstrated by Tomkiewick *et al*. (2003).

4.4 SEASONALITY OF REPRODUCTION

Fat of males, and gonads and liver of both sexes increase in mass with size (and consequently with age), but female fat mass variation cannot be explained by their size or age, as could be deduced by the results of the MANCOVA. The fat mass of females could have varied according to other factor than age, perhaps due to reproductive investment which could require a translocation of energy reserves for production of eggs.

Reproductive seasonality of *Creagrutus* sp. females was not evident in variation in gonadal mass, but was evident with microscopic maturity data. This apparently incongruence could be due to the variation of gonadal mass unrelated to maturity, as it has been found previously (e. g. Jons & Miranda, 1997). Another plausible explanation could be the effect of the reduced sample size, what diminishes the power of the sample to reject the null hypothesis (Hair *et al.*,1998). Microscopical maturity stages did not present the first flaw and could be considered more precise to determine reproductive season. The sample size required for the statistical analysis of proportions is not that exigent as for MANCOVA and in this case was enough to reject null hypothesis. An analogous source of evidence, data of juveniles, indicated that spawning resulting in recruitment occurred mainly during dry months [March (and perhaps earlier), and June], and less intensely during intermediate rainfall months (July to October). Therefore it is concluded that the reproduction of *Creagrutus sp*. is seasonal, coinciding with the flow oscillation of the river, with spawning occurring principally during dry months (December to February and June), and a rainy month, November. Data of males is congruent with this pattern, because the less intensity of spermatogenesis occurred in the months of maximum rainfall (April, November), although small sample size reduce confidence of this pattern.

The reproductive activity of females during November is intriguing. During November, females in Late Vitellogenesis accounted for 60 to 80% of all mature females, what could lead to include it as part of the reproductive season. Nevertheless, in both November samples, zero female with POFs was captured, which could mean that although gonads were mature, eggs were retained, perhaps delaying reproduction until the proper environmental clues or conditions occurred. Therefore, November could be considered a “month of preparation” prior to the reproductive season. The significantly increase in abdominal fat mass of females captured in November supports the latter conclusion, implying that the energetic reserves of females on that month are at their maximum; considering the abrupt diminution of M_F during the subsequent months (January to March), those energetic reserves are spent during the main reproductive season. It is notable that a histological analysis was indispensable to detect the differences between a reproductive and a preparation month, whereas analysis with M_G or macroscopical observations could not detect this subtle distinction.

The case of March is opposite to November. March is the driest month, the end of the first dry season when females had been spawning for some time. But in March reproduction is reduced, and few reproductively active females were captured, most of them being totally spent. Additionally fat reserves were also low. This could be the case of a month when an energetic limitation occurred, in the sense that environmental conditions were appropriate for reproduction but after a long reproductive season (December to February), energy reserves were depleted. It might also be a time when young would not have enough time to grow enough to survive the coming high water period, so there is little advantage to spawning at this time, supposing that fishes follow an environmental clue that indicate to them the upcoming of rainfalls.

The reproductive pattern of *Creagrutus* sp., a spawning concentrated in the dry season, is unusual for tropical freshwater fishes, in which reproduction generally take place only during rainy months (e.g. Welcomme, 1979; Lowe-McConnel, 1987; Taphorn, 1992). Similarly, other studies on *Creagrutus* inhabiting mountain rivers reported reproduction during dry months. Ortaz (1997), investigating the reproductive activity of what were possibly several species of *Creagrutus*, found using the analysis of I_G and macroscopic maturity scales that the main reproductive season was the dry months with a minor short one in the rainy season. In a general description of the natural history of *C. brevipinnis*, Román-Valencia (1998) reported an increment of I_G during a continuous season, from May to January, including both dry and wet months. Unfortunately, the I_G values were not validated or supported by any morphological observation of gonads, nor was their statistical significance variation confirmed. In contrast, Taphorn (1992:174) commented that in *C. melasma*, a species restricted to piedmont and mountain rivers, reproduction occurred throughout the wet season.

Examples of neotropical fishes with reproduction during dry months in floodplain rivers include members of Loricariidae (Galvis *et al.*, 1989), Scianidae and Clupeiformes (Araújo-Lima & Oliveira, 1998), but no Characiformes. However, there are examples of Characiformes spawning during the dry season, never as the general rule but rather as exceptions, in small lowland (Kramer, 1978) and piedmont rivers (Flecker *et al.*,1991; Taphorn, 1992). This is the same case of the cyprinids in Asia, where most of species have a reproductive season synchronized with monsoon rains, but there are exceptions of fishes inhabiting mountain rivers that spawn during dry seasons (Harikumar *et al.*, 1994; Wang *et al.*, 1995; Dudgeon 2000). In tropical Australia the few studies on the issue have shown that all mountain-river species of Melanotaeniidae spawn during the dry season (Pusey *et al.*, 2001, 2002). Interestingly, the reproductive pattern of *Creagrutus* sp. coincided also with that of a species of Cichlidae, observed by Rangel-Serpa & Ramírez-Pinilla (in prep.) in a parallel study (its samplings were made in the same localities and dates of this work).

Several hypotheses have been proposed to explain the reproduction during dry season in tropical stream fishes. The most likely of the five hypothesis posed by Kramer (1978) are that reproductive seasonality of a particular species 1) depends of food availability, either for authors or juveniles, 2) varies according the interspecific competition for food or breeding sites, and 3) is related to early evolution on past environmental particular conditions. The hypothesis favoured by Wang *et al.*, (1995) and Pusey *et al.* (2001, 2002), is based on the difficult conditions that flash floods create for small offspring. This idea has been developed by Humphries *et al.* (1999) as the "low flow recruitment" hypothesis, which states that during dry seasons tropical rivers have a higher concentration of larval food (zooplankton and algae) and a more stable habitat (no flash floods), favouring survival of offspring.

In the specific case of the *Creagrutus* population studied here, the reproductive pattern observed could be related to the food habits reported for the species of the genus, in congruence with the first Kramer's (1978) hypothesis. Other species of *Creagrutus* feed mainly on benthonic macro-invertebrates, algae, and allochthonous insects and seeds (Taphorn, 1992; Ortaz, 1992, 2000; Román-Valencia, 1998; Vari & Harold, 2001); these food items were found in the stomachs of *Creagrutus* dissected during this study (pers. obs.). The benthonic macro-invertebrates and algae, are washed out by floods (e.g. Fisher *et al.*, 1982; Pringle & Hamazaki, 1997). Thus, they are likely to be more abundant during the dry season. Nevertheless females showed a significant increase in the fat weight during the wet months, indicating that during these months they are able to acquire energy for reproduction. These females may have eaten other food items than benthonic macro-invertebrates and algae. In a study of the diet of some teleosts species in a neotropical mountain stream, Ortaz (2000) noticed that some species of *Creagrutus*, and *B. deutenodonoides*, which spawned during the dry season, were the most intense feeders on allochthonous material during the wet season. To determine whether this is the case for the population of *Creagrutus* sp. studied, additional specific analysis of its diet would be necessary. Thereafter, the wide-encompassing Kramer's (1978) hypothesis of the relationship between reproductive seasonality and food availability still requires additional research, for example studying the diet and reproductive activity of a community of fishes in a tropical watershed, including both mountain and lowland streams, and all ontogenetic stages of each species.

In relation to the "low flow" hypothesis, the reproduction of the population of *Creagrutus* sp. analyzed could be affected by heavy rainfall, because of the abrupt modifications of the water (reduction of visibility, pH, temperature and conductivity) and the alteration of the availability of food resources (benthic macro-invertebrates, algae and allochthonous insects and plant material). Nevertheless the hypothesis that reproduction during dry season prevents juveniles from being washed out by heavy rains needs further investigation because *Creagrutus*, like other tropical fishes that inhabit mountain rivers, prefers backwaters and embayments (Taphorn, 1992; Vari & Harold, 2001; pers. obs.), where flow speed is null even during the strongest floods (pers. obs.), and it is unlikely that juveniles can be washed out at those conditions. Additional studies on the habitat use by *Creagrutus* sp. along its ontogenetic development would help to clarify this question.

In conclusion, the reproductive pattern observed in this study should not be assumed to be the same for all fishes inhabiting piedmont or mountain rivers, as previously has been suggested Kramer (1978) and Ortaz (1997). Factors than abiotic environmental variables affect reproductive strategies of organisms (Stearns, 1992) and explanatory hypothesis about the reproductive patterns of fishes inhabiting tropical streams still lack support. Nevertheless, the importance of the unusual reproductive pattern described here focuses attention on the complexity of reproduction of tropical freshwater fishes. This emphasizes that the pattern observed in other habitats (like the well-known studied floodplain rivers) should not be considered as the generality in tropical rivers. The understanding of the factors that determines the reproductive timing of fishes in tropical mountain streams is far from known and still deserves further attention.

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TABLE I. Oocyte maturity scale, modified from Matkovic & Pisanó (1989).

Histological characteristic	I. Oogonia	II. Early primary oocyte)	III. Late primary oocyte	IV. Cortical alveoli	V. Early vitellogenesis	VI. Late vitellogensis	VII. Maturation
Cellular mean diameter (µm)	11-21	30-191	97-250	143-353	276-484	444-810	454-726
Nuclear shape (1=spherical 2=ellipsoidal 3=aster)	1, 2	1, 2	1, 2	2	2	3	3, --
Nucleus position (1=central 2=peripheric)	1	1	1	1	1	1	1, 2, --
Cariotheca: (1=smooth 2=folded 3=deeply folded)	1	1, 2	1, 2	2	3	3	3, --
Ratio nucleus:oocyte diameter	0.51-0.80	0.36-0.84	0.35-0.62	0.09-0.60	0.07-0.34	0.07-0.42	Absent-0.26
Cytoplasm (1=eosinophilic 2=basophilic)	1	2	2	2	2	2	2
Balbiani body (1=present)	--	1	1	1	--	--	--
Cortical alveoli (1=Peripheral 2=Dispersed)	--	--	--	1	1, 2	1	1, --
Yolk platelets diameter (µm)	--	--	--	--	15	15	15-25
Chorion thickness (µm)	--	--	0.8-3.3	1.6-5.0	2.5-6.3	3.8-8.8	4.2-8.8
Micropyle (1=present)	--	--	--	1	1	1	1?

TABLE II. Relative frequency of oocyte stages in the ovary maturity stages.

		Ovary maturity stages*						
Oocyte stage		Immaturity	Pre-maturity	Early Vitellogenesis	Late Vitellogenesis	Partial spawning¶	Total spawning¶	Regeneration¶
Oogonia	I	++	●	●	●	●	●	+
Early Primary oocyte	II	+++	++	++	+	+	+++	+++
Late primary oocyte	III	●	++	++	+	+	+	+
Cortical alveoli	IV		++	++	+	+	+	+
Early vitellogenesis	V			+	+	+		
Late vitellogenesis	VI				+++	++	●	
Mature	VII				●			
Post ovulatory	POF					+	+	
Atretic	AT				●	●	+	+

☐ Stage indicator.

* Relative abundance of oocyte stages: +++, dominant; ++, frequent; +, uncommon; ●, may be present.

¶ Ovaries also characterized by a disordered aspect, with abundant empty spaces.

TABLE III. Macroscopic maturity scale of preserved ovaries and its correspondence with the microscopic maturity scale.

Microscopic characteristics		I_G	Macroscopic characteristics
Oocytes*	Ovaries		
II	Immaturity	< 2.6 (< 1 always Immaturity)	IMMATURITY: Oval, translucid and flaccid, increasing length, opacity and firmness with size. Whitish or yellowish. Oocytes of mature size never observed. Volume¶ 5-30%. Gonads joined at their posterior extremes, except for smaller specimens.
IV	Pre-maturity		
V	Early Vitellogenesis	1.1-5.1	NON-REPRODUCTIVE-MATURITY: Long, sometimes with lobes. Usually yellowish-translucent except by the white or yellow or brown oocytes visible in 50-75% of cases. Ovaries in Early Vitellogenesis usually semi-opaque. Most of times flaccid. Volume 10-30 %.
POF, AT	Total Spawning		
II, AT	Regeneration		
VI	Late Vitellogenesis	2.7-26.2 (>11 always Late Vitellogenesis)	REPRODUCTIVE-MATURITY: Long with lobes, variably extended around viscera. Mostly opaque, whitish with yellow points (vitellogenic oocytes) to completely yellow. Oocytes always visible. Firm, easily broken-down. Volume 20-60 %. Gonads joined 25 to 75% of their length.
VI, POF	Partial Spawning		

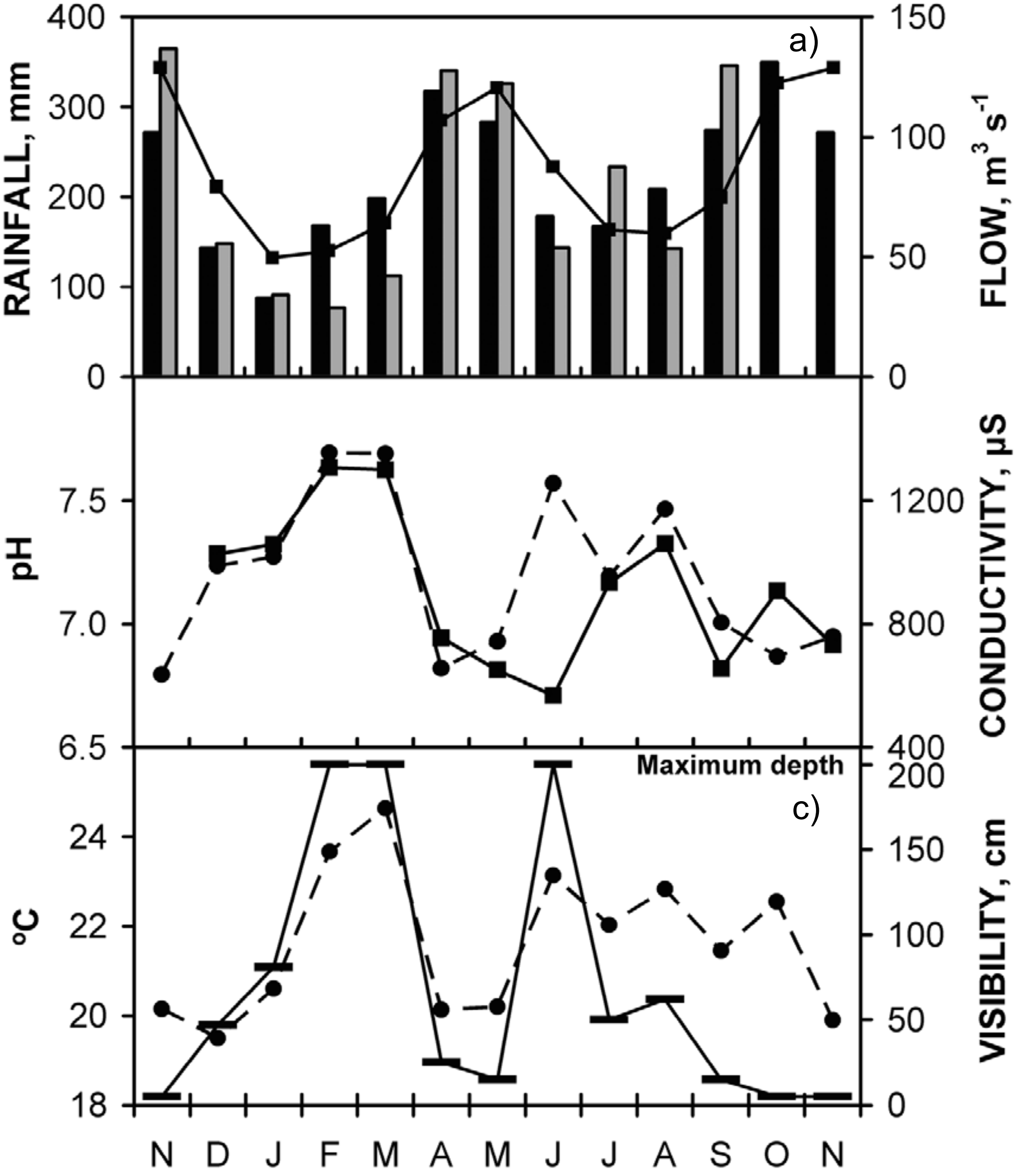
* Indicator stages, see Table II.

¶ Abdominal cavity volume.

TABLE IV. Multivariate analysis of covariance for the factor “Month”, with M_E as covariant, and M_F, M_G and M_L as dependent variables, for each sex.

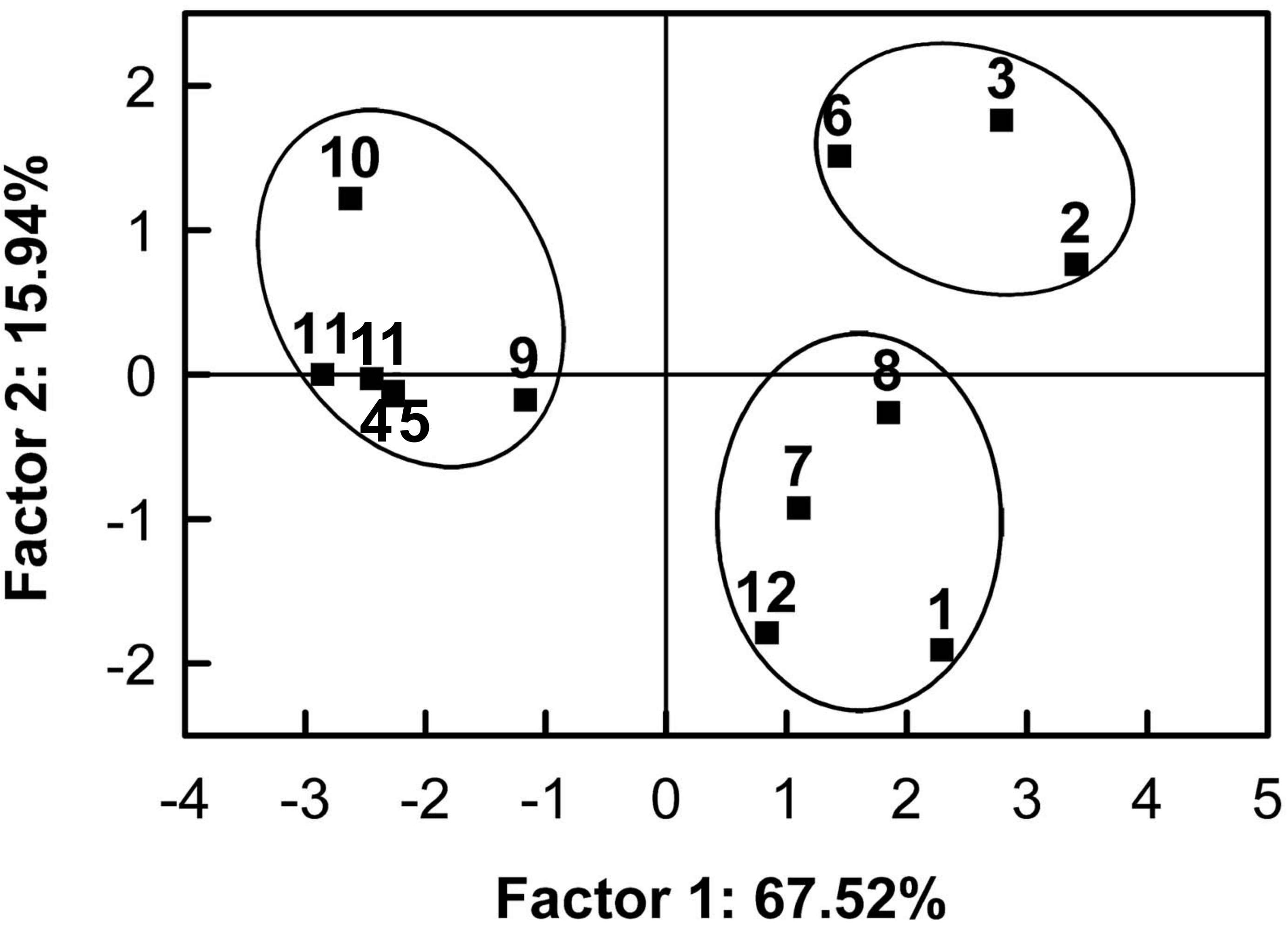
	MANCOVA		ANCOVA			
				M_F	M_G	M_L
Source	df	*P*	df	*P*	*P*	*P*
Females						
M_E	6, 234	0.016*	2, 119	0.525	0.036*	0.002*
Month	3, 117	0.046*	2, 119	0.006*	0.924	0.668
Males						
M_E	3, 70	<0.001*	1, 72	<0.001*	<0.001*	<0.001*
Month	3, 70	0.432	1, 72	0.364	0.266	0.707

* Significant, less than α = 0.05.



a)
RAINFALL, mm
400
300
200
100
0
FLOW, m^3 s^{-1}
150
100
50
0
pH
7.5
7.0
6.5
CONDUCTIVITY, µS
1200
800
400
Maximum depth
c)
°C
24
22
20
18
VISIBILITY, cm
200
150
100
50
0
N
D
J
F
M
A
M
J
J
A
S
O
N

Figure 1.



2
1
0
-1
-2
Factor 2: 15.94%
-4
-3
-2
-1
0
1
2
3
4
5
Factor 1: 67.52%
10
11
11
4
5
9
6
3
2
8
7
12
1

Figure 2.

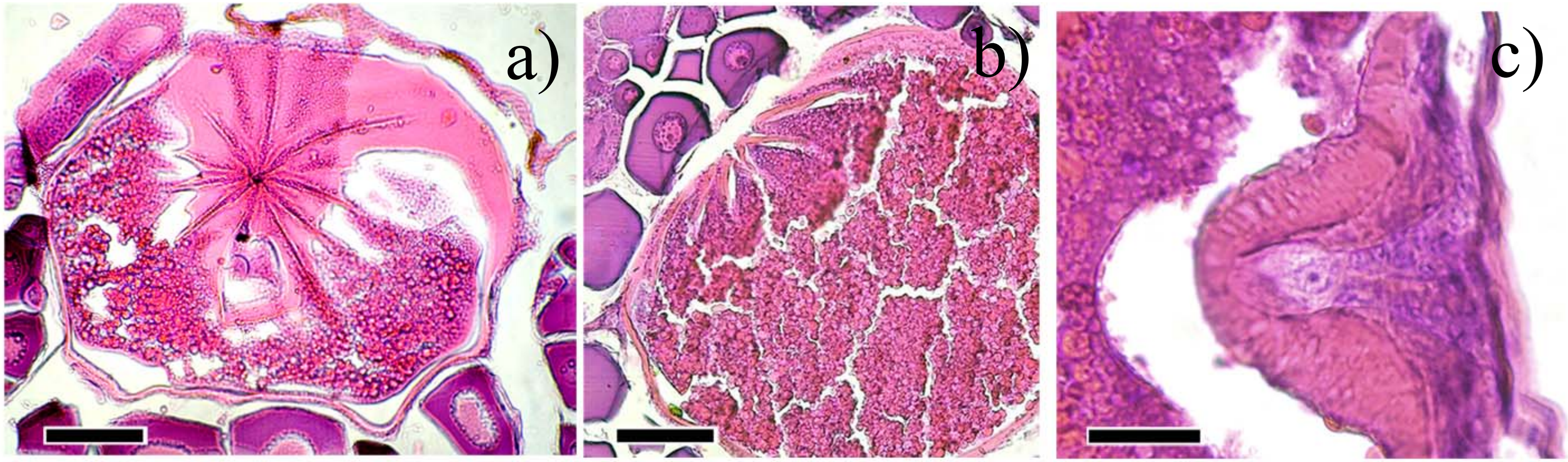


Figure 3.

[redacted]

Figure 4.

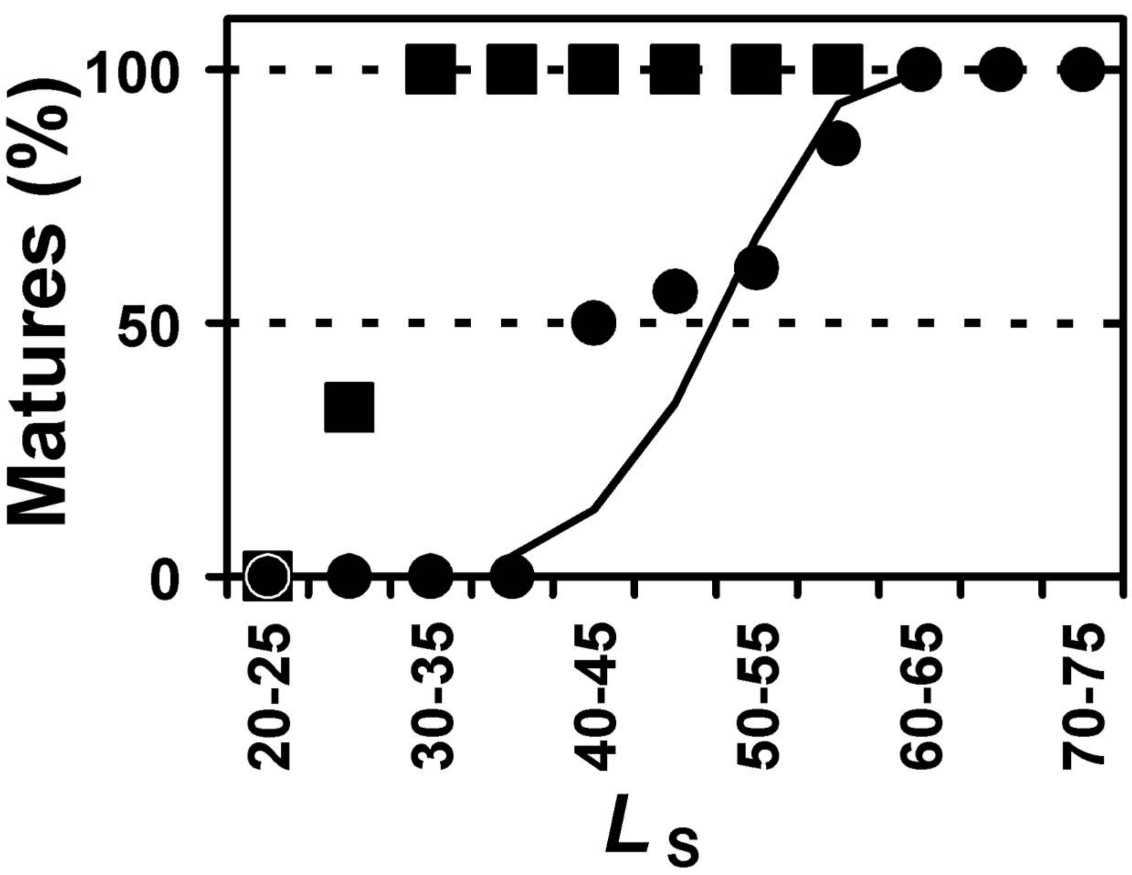


Figure 5.

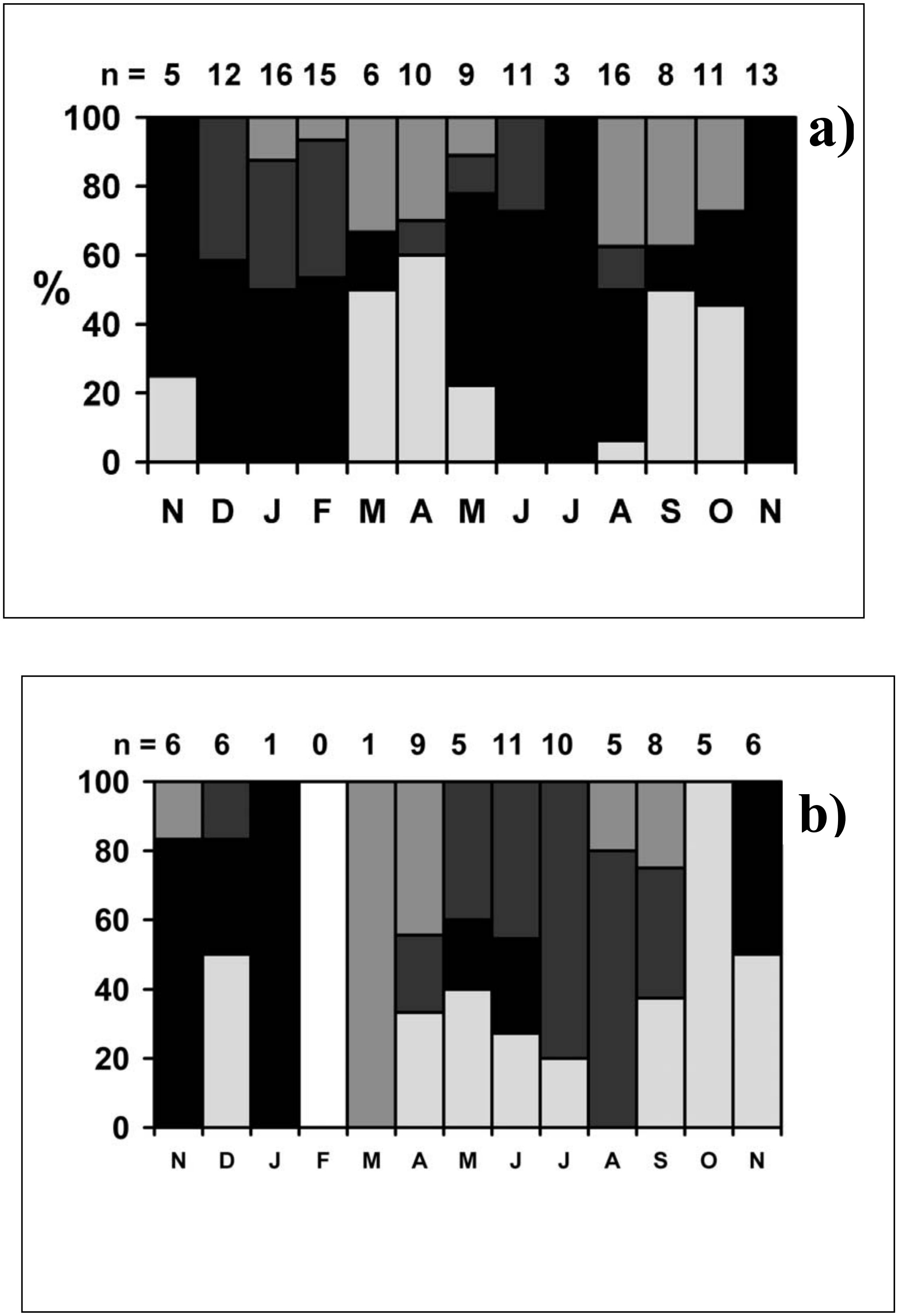

n = 5 12 16 15 6 10 9 11 3 16 8 11 13
100
80
60
%
40
20
0
N D J F M A M J J A S O N
a)
n = 6 6 1 0 1 9 5 11 10 5 8 5 6
100
80
60
40
20
0
N D J F M A M J J A S O N
b)

Figure 6.