



Zygoparity in Characidae – the first case of internal fertilization in the teleost cohort Otomorpha

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Most teleosts are externally fertilizing, with internal fertilization occurring as a relatively rare event. Until now, Euteleostomorpha is the only teleost cohort known to undergo internal fertilization. In the teleost cohort Otomorpha, it has been recorded the presence of sperm in the ovaries of some species of Characiformes and Siluriformes, but no fertilized eggs have been found so far in the female reproductive tract. It has been presumed that oocytes can be released into the water with associated spermatozoa and only there becomes fertilized, and the term insemination has been used to characterize the strategy adopted by these fish. Here, we present the discovery of the first case of internal fertilization in the teleost cohort Otomorpha, in *Compsura heterura* (Characiformes: Characidae). In the course of spawning, the eggs form the perivitelline space and the animal and vegetative poles within the ovaries, evidencing oocyte fertilization. The newly spawned eggs then continue to form the animal and vegetative poles and increase the perivitelline space. These eggs are in the zygotic stage. These data indicate that fertilized eggs are only retained for a short period, providing evidence that *C. heterura* is a zygoparous fish.

Keywords: Characiformes, Insemination, Ostariophysi, Reproduction, Spawning.

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A maioria dos teleósteos são espécies com fecundação externa, sendo a fecundação interna um evento relativamente raro. Até o momento, Euteleosteomorpha é a única coorte de teleósteos conhecida com espécies de fecundação interna. Na coorte de teleósteos Otomorpha, tem sido registrada a presença de esperma nos ovários de algumas espécies de Characiformes e Siluriformes, porém nenhum ovo fecundado foi encontrado até agora no trato reprodutor feminino. Presume-se que os oócitos possam ser liberados na água associados aos espermatozoides e que somente lá são fecundados, e o termo inseminação tem sido empregado para caracterizar a estratégia adotada por esses peixes. Apresentamos aqui a descoberta do primeiro caso de fecundação interna na coorte de teleósteos Otomorpha, em *Compsura heterura* (Characiformes: Characidae). Durante a desova, os ovos formam o espaço perivitelino e os polos animal e vegetal dentro dos ovários, evidenciando a fecundação interna. Os ovos recém-desovados continuam a formação dos polos animal e vegetal e aumentam o espaço perivitelino. Esses ovos estão na fase zigótica. Estes dados indicam que os ovos fertilizados são retidos por um curto período, fornecendo evidências de que *C. heterura* é um peixe zigóparo.

Palavras-chave: Characiformes, Desova, Inseminação, Ostariophysi, Reprodução.

INTRODUCTION

Fertilization (*sensu lato*) refers to several steps leading to and resulting in sperm-oocyte fusion. These steps include oocyte activation, expulsion of the second polar body, cortical reaction, formation of the perivitelline space, hardening of the envelope (chorion) and bipolar differentiation (cytoplasmic movement). Most teleost fish have centrolecithal unfertilized oocytes at oviposition and become telolecithal only after activation or even later in the process of fertilization (Kunz, 2004).

Fertilization may also occur internally in female's body at the gonoduct or ovary – lumen or ovarian follicle. Internal fertilization, however, is a relatively rare event in Teleostei, known only in 15 families of the Cohort Euteleosteomorpha (Fig. 1): in the Series Ophidiaria (viviparous species of Bythitidae and Dinematichthyidae), Series Ovalentaria (zygoparous, embryoparous or viviparous species of Adrianichthyidae, Anablepidae, Atherinopsidae, Clinidae, Embiotocidae, Goodeidae, Zenarchopteridae, Labrisomidae, Poeciliidae, and Rivulidae), and Series Eupercaria (zygoparous, embryoparous or viviparous species of Comephoridae, Sebastidae, and Zoarcidae) (Jakubowski *et al.*, 2003; Sequeira *et al.*, 2003; Kunz, 2004; Burns, Weitzman, 2005; Meisner, 2005; Evans, Meisner, 2009; Møller *et al.*, 2016).

Although no cases of internal fertilization have been shown thus far in the teleost cohort Otomorpha (Fig. 1), the presence of sperm has been verified in the female ovaries in some species of Characiformes (Kutaygil, 1959; Nelson, 1964; Burns *et al.*, 1995; Burns *et al.*, 1997; Malabarba, 1998; Burns *et al.*, 2000; Castro *et al.*, 2003; Weitzman *et al.*, 2005; Javonillo *et al.*, 2009; Quagio-Grassiotto *et al.*, 2012), and Siluriformes (von Ihering, 1937; Loir *et al.*, 1989; Burns *et al.*, 2000; Meisner *et al.*, 2000; Javonillo *et al.*,

2009; Spadella *et al.*, 2012). However, in all these cases, the exact moment of fertilization is unknown because fertilized eggs are not found within the ovaries (Burns *et al.*, 1995; Burns *et al.*, 1997; Azevedo *et al.*, 2000; Burns, Weitzman, 2005; Oliveira *et al.*, 2010). Furthermore, the presence of sperm in the ovaries does not prove internal fertilization because an oocyte can be released into the water with associated spermatozoa and only there becomes fertilized, which is known as internal gametic association (Munehara *et al.*, 1989). Given this occurrence, the term insemination has been used to characterize the strategy adopted by these fish (Burns *et al.*, 1997; Javonillo *et al.*, 2009), and the exact place that fertilization takes place remains unknown in Characiformes and Siluriformes of the teleost Cohort Otomorpha.

The objective of this study is to investigate and describe in an inseminating characid when fertilization occurs. For this purpose, the Cheirodontinae characid *Compsura heterura* Eigenmann, 1915 was used as a model. This species is inseminating, showing spermatozoa with slightly elongated nuclei in female ovaries (Burns *et al.*, 1997: Fig. 1e; Oliveira *et al.*, 2010: Fig. 1–2). The description of how fertilization takes place in such a member of the Cohort Otomorpha may serve as a starting point for comparison and discovery of the reproductive strategy of other inseminating Characiformes and Siluriformes.

MATERIAL AND METHODS

Fish Sampling. *Compsura heterura* specimens were collected with a 1 mm–plastic mesh hand net in 2001 from the Maxaranguape stream (05°30'45.00"S, 35°19'13.97"W), Maxaranguape city, Rio Grande do Norte state (RN), Brazil. Individuals were maintained at Ichthyology Laboratory, UFRGS, into twelve 60-litre aquariums, at a maximum of seven fish per aquarium. The aquariums contained dechlorinated tap water, were equipped with an undergravel biological filter, were lined with approximately 5 cm of gravel (5 mm grain size), and were planted with *Hygrophila difformis* and *Microsorium pteropus*. The lighting consisted of 20 W white fluorescent lamps with a timer set to a photoperiod of 12 h: 12 h light-dark. Aeration was constant by submerged pumps (280 l/h). The temperature was maintained at approximately 25°C with thermostat and heater, in an air-conditioned room. Fish were fed with commercial food flakes once a day *ad libitum*. The offspring of this wild population was analysed in this study.

Eight spawning females were euthanized using an overdose of Eugenol and dissected for ovaries removal. Twenty-two newly spawned oocytes were collected adhered to plant leaves (< 1 min after spawn).

Light Microscopy. Ovaries and oocytes were fixed by immersion in 2% glutaraldehyde and 4% paraformaldehyde in Sorensen's phosphate buffer (0.1 M, pH 7.2). The ovaries and oocytes were dehydrated in ethanol and embedded in historesin (Leica HistoResin). Serial sections (3 µm) were stained with periodic-acid-Schiff (PAS)/hematoxylin/metanil yellow (Quintero-Hunter *et al.*, 1991). Voucher specimens were deposited in the fish collection of the Departamento de Zoologia at the Universidade Federal do Rio Grande do Sul (UFRGS 22200, UFRGS 22201, UFRGS 22202).

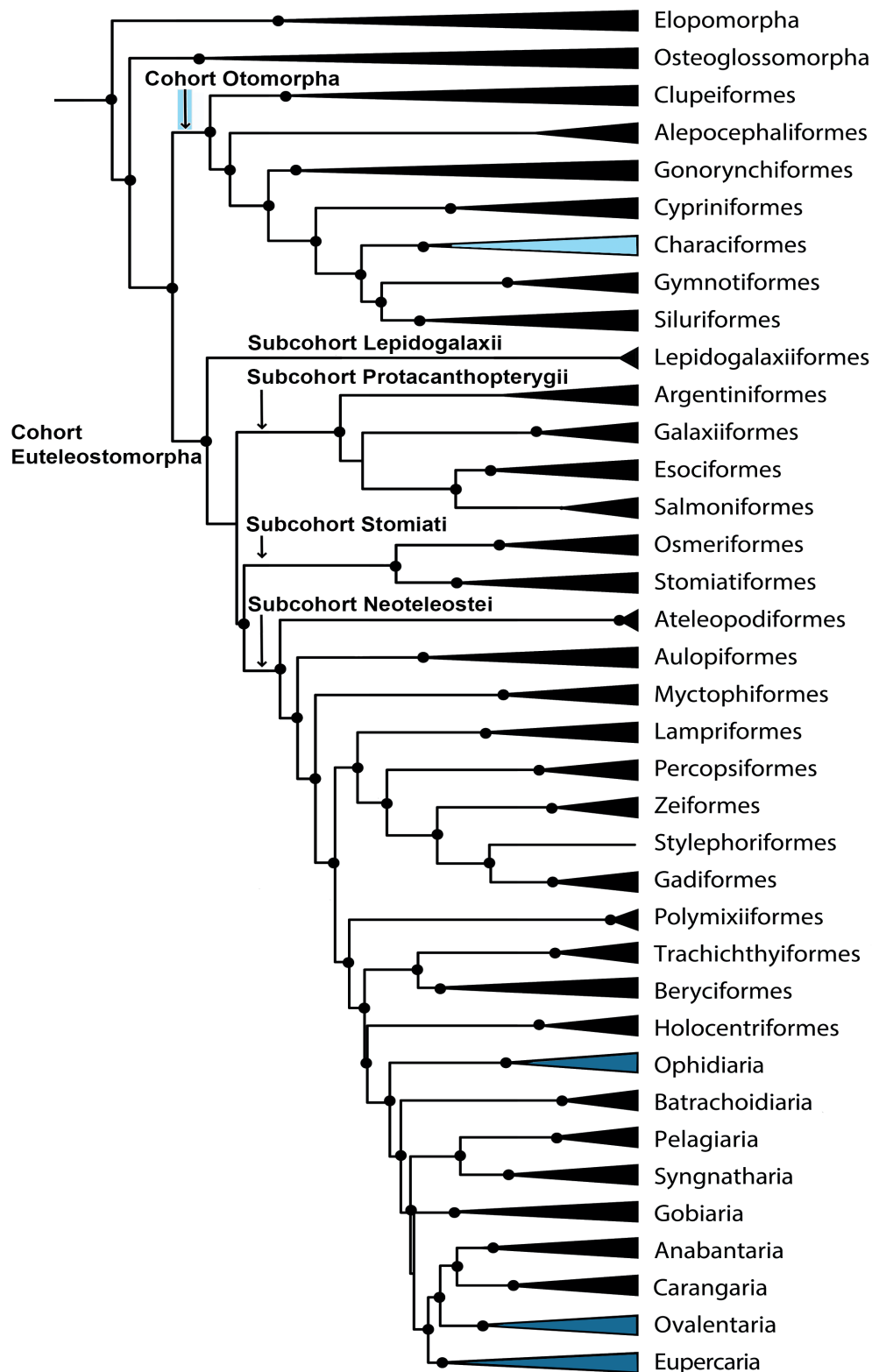


FIGURE 1 | Phylogeny of Teleostei (modified from Betancur-R *et al.*, 2017), showing clades previously known to include species with internal fertilization (dark blue) and the present record in the teleost cohort Otomorpha (light blue). Remaining clades (black) do not contain internally fertilized species.

Ethical note. This study conforms to the principles outlined in the Guide for the care and use of laboratory animals (Garber *et al.*, 2011) and was approved by the Ethics Committee on Animal Use of the Federal University of Rio Grande do Sul (Universidade Federal do Rio Grande do Sul) (Project n. 32291).

RESULTS

All spawning females had unfertilized oocytes in the follicle (follicular cavity) and fertilized eggs in the ovarian lumen (ovulated). All newly spawned eggs were fertilized. The observed oocytes (unfertilized) had a single micropyle and several cortical alveoli of different sizes at the peripheral region of the oocytes (cortical cytoplasm) (Fig. 2A). In the ovarian lumen, eggs were observed at the first cytoplasmic movements that define the animal and vegetative poles, containing few cortical alveoli and forming the perivitelline space (Fig. 2B). In the gonoduct, the eggs decreased the number of cortical alveoli (cortical reaction), and there was a clear distinction between the animal and vegetative poles (bipolar differentiation, Fig. 2C). The newly spawned eggs formed the animal and vegetative poles, and there was an increase in the perivitelline space. In the animal pole, there were no blastomeres (embryonic cells). These eggs were still in the zygotic stage (Fig. 2D).

DISCUSSION

Oviparity is defined as the laying of fertilized or unfertilized eggs with intact eggshells or jelly coats in the environment (Blackburn, 2015). At the time of laying, the egg may be unfertilized (*i.e.* oocyte; Oviparity), in the first stages of development (*e.g.* zygote; Zygoparity) or even in advanced embryonic stages (Embryoparity). Viviparity has been defined as a process in which females retain developing eggs inside their reproductive tracts and give birth to their young (*e.g.* larvae; Blackburn, 2015). Most teleosts (nearly 97.5%) are externally fertilizing, whose gametes are released and fertilization takes place in the water (Oviparity).

Teleost fish have been often referred to as “internally fertilizing” based on behavioral records, possible copulatory organs (*e.g.* male fin modifications), presence of sperm in the ovary or egg/embryo observations under stereomicroscopy, but showing no details on the stage of egg development (Meisner, 2005). Such is the case of the Glandulocaudinae characids (now part of the Stevardiinae), initially treated as bearing internal fertilization based on the observation of spermatozoa within the ovarian cavity (Burns *et al.*, 1995), but latter referred to inseminating based on the lack of fertilized eggs observed within the ovaries (Burns *et al.*, 1995; Burns *et al.*, 1997; Azevedo *et al.*, 2000; Burns, Weitzman, 2005; Oliveira *et al.*, 2010).

Here, in the ovaries of spawning females of *C. heterura*, the cytoplasmic movements, few cortical alveoli, absence of blastomeres, formation of perivitelline space and animal and vegetative poles in the eggs allowed us to infer that the spermatozoa fertilize the oocyte in the ovary and that this egg was in the zygotic stage (formation of the zygote or egg cell, before the first cleavage). This is the first case of internal fertilization in the

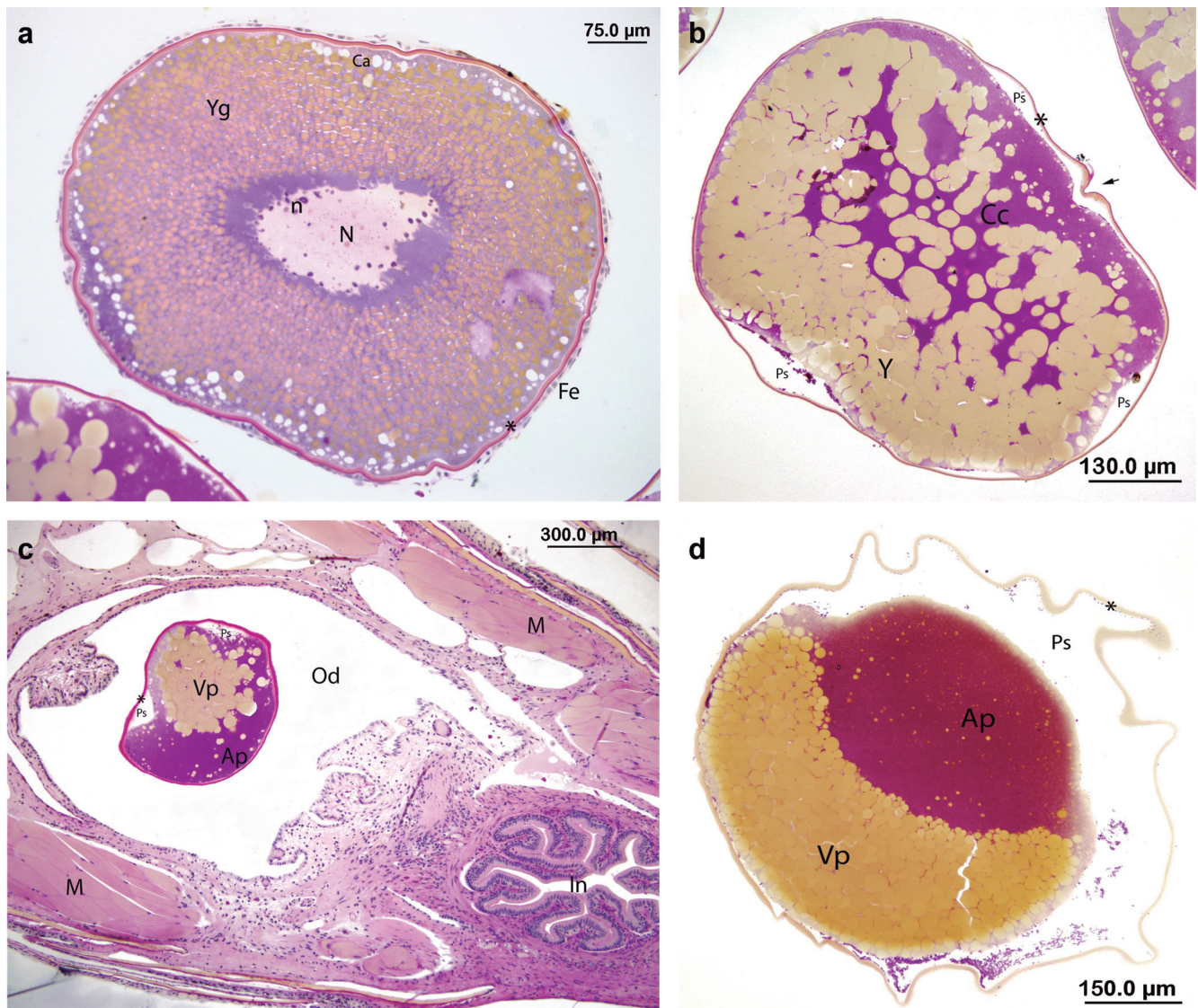


FIGURE 2 | Oocyte and fertilized eggs of *Compsura heterura*. **A.** Unfertilized oocyte in ovary (follicle); **B.** fertilized egg in ovary (ovarian lumen) and **C.** gonoduct (**Od**); **D.** fertilized spawned egg. **Yg**, yolk granules; **Ca**, cortical alveoli; asterisk, chorion; **Fe**, follicular epithelium; **N**, nucleus; **n**, nucleoli; **Y**, yolk; **Cc**, cortical cytoplasm; **Ps**, perivitelline space; arrow, micropyle; **Vp**, vegetative poles; **Ap**, animal poles; **In**, intestine; **M**, muscle tissue.

teleost cohort Otomorpha. The zygotic stage of newly spawned eggs in *C. heterura* indicates that there is a short period of retention of the fertilized eggs. Fertilization likely occurs after ovulation, in the ovarian lumen, and shortly after the fertilized eggs are released into the external environment, indicating that *C. heterura* (Characidae – Characiformes) is a zygoparous fish.

Zygoparity has been recorded in species of the Cohort Euteleostomorpha, in which the egg retention varies from zygote to embryo (embryoparity), as for example in *Kryptolebias marmoratus* (Poey, 1880), Rivulidae; *Tomeurus gracilis* Eigenmann, 1909, Poeciliidae; *Helicolenus dactylopterus* (Delaroche, 1809) and three species of the genus

Sebastolobus Gill, 1881, Sebastidae (Harrington, 1961; Rosen, Bailey, 1963; Sequeira *et al.*, 2003; Pavlov, Emel'yanova, 2013). In addition, *Oryzias setnai* (Kulkarni, 1940) – Adrianichthyidae – and *Zoarcetes americanus* (Bloch, Schneider, 1801), Zoarcidae, may be zygoparous because copulation and spawning egg in early stages of embryonic development have been observed in both species (Kulkarni, 1940; Pavlov, Emel'yanova, 2013).

Among Characiformes, insemination is found only in Characidae *sensu stricto* (Malabarba, Weitzman, 2003; Oliveira *et al.*, 2011; Mirande, 2018), but is observed in part of the species of all the three main lineages of this family (Javonillo *et al.*, 2010; Oliveira *et al.*, 2011): the Clade A, now Stevardiinae (44 inseminating species listed by Thomaz *et al.*, 2015: Table II); the Clade B, in Cheirodontinae (all species of the tribe Compsurini listed by Malabarba (1998)); and the Clade C, in Stethaprioninae (species of the genera *Hollandichthys* and *Rachoviscus* listed by Quagio-Grassiotto *et al.*, 2012). The presence in different lineages has evidenced the multiple origin of insemination in Characidae. The zigoparity of *C. heterura* (shown here) may be shared among all species of its tribe, but it must be tested. On the other hand, the timing and place of fertilization in the inseminating species of the subfamilies Stevardiinae and Stethaprioninae remain unknown. Since the origin of insemination in these groups is not homologous and arose separately from that of Compsurini, their strategies as inseminating ovuliparous, zygoparous or even embryoparous remains to be investigated.

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AUTHOR CONTRIBUTIONS

Clayton Kunio Fukakusa: Conceptualization, Investigation, Methodology, Project administration, Writing (original draft), Writing (review & editing).

Talita Sarah Mazzoni: Investigation, Methodology, Project administration, Supervision, Visualization, Writing (original draft), Writing (review & editing).

Luiz Roberto Malabarba: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Visualization, Writing (original draft), Writing (review & editing).

Neotropical Ichthyology

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ETHICAL STATEMENT

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COMPETING INTERESTS

Not applicable.

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