EUDRIANO FLORÊNCIO DOS SANTOS COSTA

REPRODUCTIVE STRATEGIES OF MARINE FISHES FROM THE SOUTHWEST ATLANTIC OCEAN: AN APPLICATION OF HISTOLOGICAL AND IMAGE PROCESSING TECHNIQUES

A thesis submitted in partial fulfilment of the requirements of the Instituto Oceanográfico da Universidade de São Paulo for the degree of Doctor in Science, with emphasis in Biological Oceanography.

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SÃO PAULO

2015

UNIVERSIDADE DE SÃO PAULO
INSTITUTO OCEANOGRÁFICO

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CORRECTED VERSION

Evaluated in 29 / 04 / 2015.

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Dedico esta tese aos meus pais, irmãos e sobrinhos.

ACKNOWLEDGEMENTS

(Agradecimentos)

À professora June Dias pela orientação, ensinamentos, amizade, confiança e cumplicidade.

Ao Instituto Oceanográfico (IO) da Universidade de São Paulo pela disponibilidade das embarcações, laboratórios, hospedagens, transportes e outros suportes que foram necessários para realização desta tese.

Ao programa de Pós-graduação em Oceanografia (USP).

À CAPES pelas bolsas concedidas no Brasil e no exterior (processo: 7546/13-0).

À USP, SAS (Superintendência Assistência Social da USP) e a assistente social Neusa Franzoi pelo apoio social e pela concessão de uma vaga no CRUSP (Conjunto Residencial da Universidade de São Paulo).

Aos funcionários das bases de pesquisa do IO, “Clarimundo de Jesus” (Ubatuba) e “Dr. João de Paiva Carvalho” (Cananéia).

Aos motoristas do IO, Roberto, Serginho, Antônio Carlos, Valter e Claúdião pelas conduções para as bases de pesquisa e apoio logístico.

Aos tripulantes dos barcos de pesquisa Veliger II (Daíco, Adriano, Oziel e Seu Sílvio) e Albacora (Amauri, Sérgio, Zeca, Ulisses, "Honhon" e Clovinho) pela execução dos arrastos e ajudas com o material biológico a bordo.

Ao coordenador e funcionários do Laboratório de Instrumentação Oceanográfica (LIO) pelo empréstimo de instrumentos oceanográficos e apoio logístico, em especial ao Henrique e Luíz.

Dr. Hilario Murua jaunari (AZTI-Tecnalia Fundazioa) jasotako zuzenketa, kritika ta iradokizunak eskertu nahi nizkioke. Baita emandako laguntasun eta konfiantza (Ao Dr. Hilário Murua, AZTI-Tecnalia Fundazioa, pelas correções, críticas e sugestões apresentadas. Agradeço também pela amizade e confiança).

AZTI-Tecnalia Fundazioari (Itsas Ikerketa Unitatea) eskeinitako abegikortasunagatik, eta ekipo zein laborategiak euskaragarri jarri izanagatik.Bestalde, bekadun ta futbol taldeko kidei eskeinitako laguntasun ta aisialdi momentu onengatik (AZTI-Tecnalia Fundazioari, Unidade de Investigação Marinha localizada em Pasaia- Espanha, pela receptividade, disponibilidade de equipamentos e laboratórios).

Aos estagiários/ bolsistas do laboratório de Ecologia da Reprodução e do Recrutamento de Organismos Marinhos (ECORREP): Igor, Bruno, Camila, Cecília e Chris pela ajuda durante a triagem do material biológico em campo.

Ao Marcio Ohkawara pela ajuda nos trabalhos de campo e pescarias.

To thank Dr. Holger Haslob from Johann Heinrich von Thünen-Institute (Sea Fisheries) for his availability and help with the grids and ImageJ routine (Ao Dr. Holger Haslob do Instituto do Federal de Pesca Marinha da Alemanha, pela disponibilidade de um "grid" e de uma rotina de processamento de imagens).

Grid-arekin lagundu izana ta behin baino gehiagotan etxera gerturatu izana eskertu nahi nioke Katerin Azpeitia Abarrategi (AZTI-tecnalia/ Euskal herriko Unibertsitatea (A Katerin Abarrategi, AZTI-Tecnalia/ Universidade do País Basco, pela ajuda com o “grid” de 3200 pontos e pelas inúmeras caronas).

Ao Valter Miyagi pela assistência técnica em informática (IOUSP).

Agradezco a Maria y Javier por haber compatido su piso en Donostia con un extranjero brasileño; como también por la amistad, comidas, excursiones y "clases" de español (À Maria Jesus e Javier Berroa por dividirem a sua residência com um estrangeiro brasileiro, em San Sebastián. Agradeço também pela amizade, comidas, passeios e ensinamentos em espanhol).

Aos companheiros do Apartamento 106 do CRUSP, bloco G: Mário, Daniel, Luís e Victor pela amizade e respeito.

Aos companheiros de laboratório e agregados pela amizade e momentos de entretenimento proporcionados: Maria de Lourdes, Ríguel, Wellington (Frango), Jana, Diego, Michel (Migué), Thassya, Isa, Natasha e Cláudia.

À Lourdes e o Professor Mario pela amizade e disponibilidade de materiais de laboratório.

As colegas da República 69: Coelho, Mauro, Sabine, Adriana (Dri), Márcio (Bur), Márcio (mini bur), Caio (Cacipa), Leandro (Lê), Lucas, Robinho e Ermínio pelo abrigo quando cheguei a São Paulo.

Ao Ríguel, Raul, Tamiris e Felipe (Barba) por terem permitido que eu passasse uma temporada morando na sala do apartamento.

Ao Luis, André e Davi pelo apoio logístico.

Luciana Sartori pelo suporte quando cheguei a São Paulo pela primeira vez, amizade e confiança.

As funcionárias da secretária de pós-graduação, Fátima, Ana Paula, Letícia e Silvana pelos serviços acadêmicos.

À Dona Cida, Cidinha e Ruti, do laboratório geral do IO, pela disponibilidade de materiais, equipamentos e lavagem das vidrarias utilizadas na preparação das soluções e durante o procedimento histotécnico.

À Helcy por apresentar os equipamentos utilizados no processamento histológico e ensinamentos durante o meu período de estágio no ECORREP.

Aos professores Frederico Brandini e Eduinetty Ceci pelas experiências proporcionadas durante o estágio PAE (Programa de Aperfeiçoamento de Ensino).

À todos que "fazem" a Biblioteca Professor Dr. Gelso Vazzoler (IO), em especial ao Wagner, Cidinha e Daniel pelas inúmeras ajudas com os livros e revistas.

À Priscila Sartorio pelo companheirismo, carinho, confiança e paciência. Também agradeço a sua família pelo carinho e almoços de domingo.

Aos meus Pais, irmãos e sobrinhos.

“When life gives you lemons, get a bottle of *cachaça*, ice cubes, some sugar and make a caipirinha“

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PREFACE

This thesis contains the result of my research undertaken at the Instituto Oceanográfico da Universidade de São Paulo, Brazil, with financial support of the Coordination for the Improvement of High Level Education Personnel (CAPES, grant number: 7546/13-0). The research was conducted under the supervision of the professor Dr. J.F. Dias. This thesis represents my own original work; I wrote the manuscripts, being the first author and primarily responsible for conceptualisation, sampling strategy, aquisition, analysis and interpretation of the data. However, all co-authors have contributed significantly to the work, participating in drafting and revising critically the intellectual content of each manuscript. Thus, part of the information contained in this Doctoral thesis has been submitted for publication in peer-viewed international journals, as follow:

- **Costa, E. F. S.**; Dias, J. F.; Murua, H. Reproductive strategy and fecundity of six fish species from the southwest Atlantic Ocean: An application of image process techniques. *Submitted.*

- **Costa, E. F. S.**; Dias, J. F.; Murua, H. Reproductive traits of the simultaneous hermaphrodite *Diplectrum radiale* (Quoy & Gaimard, 1824) (Teleostei, Serranidae). *Submitted.*

- **Costa, E. F. S.**; Dias, J. F.; Murua, H. Reproductive strategy and fecundity of the keystone species *Paralonchurus brasiliensis* (Teleostei, Sciaenidae): An image processing techniques application. *Accepted for publication in Environmental Biology of Fishes*

- **Costa, E. F. S.**; Dias, J. F.; Murua, H. Do Congeneric fishes inhabiting different ecosystems exhibit the same oocyte production and recruitment pattern? *Submitted.*

RESUMO

A presente tese testou a hipótese de que espécies que habitam diferentes ambientes, costeiros e estuarinos, apresentam as mesmas estratégias reprodutivas em relação ao desenvolvimento ovariano, recrutamento ovocitário e fecundidade. Desse modo, ovários de *Anchoa filifera*, *Cetengraulis edentulus*, *Citharichthys spilopterus*, *Stellifer brasiliensis*, *S. rastrifer*, *Menticirrhus americanus*, *Paralonchurus brasiliensis* e *Diplectrum radiale* foram amostrados e analisados através de técnicas histológicas e de processamento de imagens. As capturas foram realizadas no período de junho (2012) a maio (2013) na região costeira de Ubatuba e no estuário de Cananéia, São Paulo, Brasil. Os ovários foram removidos, pesados, fixados em solução de formalina e obtidos os preparados permanentes. Todas as secções histológicas foram fotografadas e as imagens analisadas no programa ImageJ. Os resultados revelaram que todas as espécies apresentam desenvolvimento ovócitário do tipo assincrônico. O recrutamento ovocitário ocorre constantemente durante o período de desova das espécies. Os testículos do hermafrodita simultâneo *D. radiale* é do tipo irrestrito com espermatogênese cística. Nessa espécie, a estrutura acessória reprodutiva tem a função de armazenar os ovócitos hidratados até o próximo evento de desova e absorver ovócitos que não foram desovados (atresia). Os ovócitos das espécies iniciam a vitelogênese em diferentes tamanhos. O número total de ovócitos produzidos por fase de desenvolvimento não diferiram significativamente entre as espécies congêneres *S. brasiliensis* e *S. rastrifer*. No entanto, o número de ovócitos em vitelogênese avançada diferiu entre as espécies gonocóricas. A fecundidade média por lote variou de 1.644 em *A. filifera* a 58.884 ovócitos em *M. americanus*, enquanto que a fecundidade relativa variou de 51 a 1.205 ovócitos g^{-1} em *D. radiale* e *C. spilopterus*, respectivamente. O número de lotes potenciais presentes nos ovários também diferiu entre as espécies, variando de 1 em *A. filifera*, *C. edentulus* e *M. americanus* a 4 em *C. spilopterus*. Assim, a hipótese postulada inicialmente foi rejeitada.

Palavras-chave: Biologia reprodutiva. Desenvolvimento ovocitário. Organização ovariana. Fecundidade. Estereologia. Hermafrodita simultâneo.

ABSTRACT

This thesis has hypothesised that species inhabiting different environments such as coastal and estuarine areas exhibit the same reproductive strategies in terms of oocyte development, oocyte recruitment and fecundity. Thus, the ovaries of *Anchoa filifera*, *Cetengraulis edentulus*, *Citharichthys spilopterus*, *Stellifer brasiliensis*, *S. rastrifer*, *Menticirrhus americanus*, *Paralonchurus brasiliensis* and *Diplectrum radiale* were examined using histology and image processing techniques. The fishes were captured bimonthly, from June (2012) to May (2013), in the coastal of Ubatuba and in the estuary of Cananéia, inner shelf of São Paulo State, Brazil. The ovaries were removed, weighed, fixed in formalin and prepared the histological sections. All sections were photographed and the images analysed using the free software ImageJ. The results revealed that all species exhibited asynchronous oocyte development, although some differences in the oocyte development pattern among species have been recorded. The stock of pre-vitellogenic oocytes can develop and be recruited into yolked stock at any time in all species. The simultaneous hermaphrodite *D. radiale* has an unrestricted testicular type with cystic spermatogenesis. In this species, the accessory reproductive structure has the function to store the hydrated oocytes up to the next spawning event and absorb non-spawned oocytes. The size at which the oocytes are recruited to vitellogenesis (ORS) showed difference among species. The total number of oocytes produced per development stage did not differ significantly between the congeneric species *S. brasiliensis* and *S. rastrifer*. However, the number of advanced vitellogenic oocytes varied among gonochoristic species and increased with increasing the ovary weight, total length and weight. The mean batch fecundity ranged from 1,644 in *A. filifera* to 58,884 oocytes in *M. americanus*, whereas the mean relative BF ranged 51 in *D. radiale* to 1,205 oocytes g^{-1} in *C. spilopterus*. The number of potential batches in the ovaries also differed among species, ranging from 1 in *A. filifera*, *C. edentulus* and *M. americanus* to 4 in *C. spilopterus*. Thus, the hypothesis of this thesis was rejected.

Key words: Reproductive biology. Oocyte development. Ovary organisation. Fecundity. Stereology.

STRUCTURE OF THE THESIS

This thesis is structured as follows:

- Section I: **GENERAL INTRODUCTION**. It starts with this section providing an overview and defining the main topics of the study such as the reproductive strategies of the fishes, fecundity, histological methods applied in this type of study and a general description of each studied species. This section also describes both areas of study, the inner shelf of Ubatuba and the estuarine system of Cananéia, in terms of geographical location and oceanographic features.

- Section II: **HYPOTHESIS AND OBJECTIVES.** The hypothesis and objectives of the thesis are detailed in this section.

- Section III: **RESULTS.** In order to answer the hypothesis and to achieve the objectives, the results are arranged in four chapters. Thus, each chapter contains an abstract, introduction, specific objectives, materials and methods, results, discussion and conclusions. However, the conclusions are inserted in the discussion or separately.

  Chapter I: This chapter presents the reproductive strategy of *Anchoa filifera*, *Cetengraulis edentulus*, *Citharichthys spilopterus*, *Stellifer brasiliensis*, *S. rastrifer* and *Menticirrhus americanus* in relation to the oocyte development, ovary organisation, oocyte size-frequency distribution, size at which the oocytes are recruited to vitellogenesis, fecundity and the number of potential batches present in the ovaries. The results may indicate that the reproductive strategy exhibited for different species from distinct environments are similar or not.

  Chapter II: It focused on the reproductive traits of the simultaneous hermaphrodite *Diplectrum radiale* captured in two distinct environments. The ovotestis development and organisation, oocyte size-frequency distribution and fecundity were investigated using histological techniques, as well as the

influence of the total length, weight and body condition (gonadosomatic index, stomach repletion index, Fulton's condition factor and hepatosomatic index) on fecundity. The results are presented and discussed comparing to the general reproductive patterns exhibited by the majority of gonochoristic species.

Chapter III: The reproductive strategy of the keystone species *Paralonchurus brasiliensis* was investigated using histology and advanced image processing techniques in this chapter. The oocyte development, ovary organisation, oocyte size-frequency distribution, oocyte diameter variation, occurrence of the ovary phases during ovarian maturation, quantification of the standing stock of the most advanced yolked oocytes and batch fecundity were evaluated. In this chapter, the vitellogenic oocytes are classified in early (Vtg1) and late vitellogenesis (Vtg2).

Chapter IV: Pre-vitellogenic and vitellogenic oocytes are quantified, the relative production of oocytes, the number of oocytes and batches recruited in spawning capable females of *S. brasiliensis* and *S. rastrifer* are investigated. Results allowed testing the hypothesis that these congeneric species, from two distinct environments, exhibit the same oocyte production and recruitment pattern.

- Section IV: **GENERAL DISCUSSION.** The major findings presented in each chapter are discussed as a whole in this section.

- Section V: **CONCLUSIONS.** This section enumerates the main conclusions, answering the main hypothesis of the study.

- Section VI: **REFERENCES.** The literature revised in the general introduction, areas of study and chapters is enumerated in this section.

- Section VII: **APPENDIX.** Finally, this section presents additional information regarding to the methodological procedures used in this study during the laboratory and field works, although each procedure have already been described in each chapter.

This doctoral thesis was formatted based on the NBRs 6023, 6024, 6027, 6028 and 10520 of the Brazilian Technical Standards Association (ABNT).

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LIST OF ABBREVIATIONS

ANOVA= analysis of variance

ANCOVA= analysis of covariance

Bc= blood cell (erythrocytes)

BF= batch fecundity

Bl= basal lamina

CA= cortical alveolar

ca= cortical alveolus

Cn= chromatin nucleolar

D= individual oocyte diameter

DT= digestive tract

Eg= eosinophilic granules

FBW= ovary-free body weight

Fo= follicle layer

Gc= granulosa cell

GSI= gonadosomatic index

GVM= germinal vesicle migration

HSI= Hepatosomatic index

Hyd= hydration

K= size distribution coefficient of oocytes

K= Fulton's condition factor

LDA= linear discriminant analysis

LGVM= late germinal vesicle migration

Lu=lumen

M_1= mean oocyte diameter

M_3= third moment for the mean oocyte distribution

N= nucleus

N= total number of oocytes

N_a= number of oocytes transected per unit of area in the histological sections.

NB= number of batches

NDO= number of developing oocytes

NPB= number of potential batches

N_{hyd}= number of hydrated oocytes

nu= nucleoli

Nv= density of *i* oocyte stage per unit of volume

O= oogonia

OD= Oil droplets

OD_{ind}= individual oocyte diamer

OD_{vi}= volume-based mean oocyte diameter

OG= Oil globules

Ol= ovarian lamellae

Olu= ovarian lumen

OPD= oocyte packing density

OPD_i= stage-specific oocyte packing density

ORS= oocyte recruitment size

Os= ovarian sinus

Ot= ovary tissue

Ow= ovary weight

Ov= ovary volume

p= significance of probability

Pg1= early primary growth

Pg2= late primary growth

POF= post-ovulatory follicles

r= Pearson's correlation test

r^2= coefficient of determination

r_s= Spearmen's rank correlation test

RI= stomach repletion index

RN= relative number of oocytes per gram of female

RNDO= relative number of developing oocytes

Ss= sperm sinus

Sc=spermatocytes

Sd= sperm duct

Sg= spermatogonia

SI= stomach index

SpCa= spawning capable phase

St= spermatids

Sw= sinus wall

SW= somatic weight

Sz= spermatozoa

T= theca layer

Ta= tunica albuginea

TL= total length

Tt= testicular tissue

TW= total weight

V= vacuoles

Vp= villae-like projections

V_i= oocyte volume fraction

Vtg1= primary vitellogenesis

Vtg2= secondary vitellogenesis

Vtg3= tertiary vitellogenesis

Vtg4= quaternary vitellogenesis

W_{hyd}= weight of the hydrated oocytes

YG= yolk granules

ZR= zona radiata

β= oocyte shape coefficient

General Introduction

1. GENERAL INTRODUCTION

1.1 Fish reproductive strategies

The specific peculiarities of reproduction in every species are an adaptation to the particular conditions of reproduction and the development of its larvae, which provide the replenishment essential for preservation of the species and its abundance (NIKOLSKY, 1963). Reproduction must be timed for species survival, and an appreciation of the ecology of a species depends on understanding reproductive strategy (VLAMING, 1972). Thus, fish follow different strategies and tactics to produce surviving progeny in relation to energy budget and parental life expectancy (WARE, 1984; WOTTON, 1990; PIANKA, 2000; MURUA; SABORIDO-REY, 2003). The reproductive strategy is the overall pattern of reproduction typically shown by individuals in a species, whereas the reproductive tactics are those variations in the typical pattern, which fish make in response to fluctuations in the environment (WOTTON, 1984). However, both strategy and tactical variations are adaptive, and the reproductive success of individual fish over many generations will be dependent on how its reproductive strategy evolved (GARROD; HORWOOD, 1984; STEARNS, 1992). Therefore, the reproductive strategy combines inflexible (e.g. oocyte development and ovary organisation) and flexible traits (e.g. fecundity, length-age at the first maturity, egg production, spawning frequency, and reproductive period) to produce the maximum amount of offspring, allowing the species to persist in different environmental conditions (REAY, 1984; WOOTTON, 1998).

Fish exhibit great diversity in reproductive strategies and associated traits, ranging from gonochorism (separate sexes) to simultaneous hermaphroditism, from species that spawn once and die to multiple spawning, from mass spawning to species with exhibit parental care, and from oviparity to viviparity (WOTTON, 1990; HELFMAN et al., 2009). However, the most species of marine fish are gonochoristic and iteroparous (multiple spawning seasons) with no parental care (HELFMAN et al., 2009) (Table 1).

Table 1. Summary of different reproductive strategies based on different components of breeding systems in marine fishes (Source: MURUA; SABORIDO-REY, 2003; HELFMAN et al., 2009).

I. Number of breeding opportunities
A. Semelparous (spawn once and die): lampreys, capelin, pacific salmons.
B. Iteroparous (multiple breeding seasons): most species (elasmobranchs, perciforms).
II. Type of spawning
A. Total spawners: oocytes are released in a single episode in each season.
B. Batch spawners: oocytes are released in batches over a period that can last days or even months.
III. Mating system
A. Promiscuous (both sexes with multiple partners during breeding season): herrings, greenlings, damselfishes, wrasses, surgeonfishes.
B. Polygamous, including monogamy: sculpins.
IV. Gender system
A. Gonochoristic (sex fixed at maturation): most species (elasmobranchs, clupeiforms, scombroids).
B. Hermaphroditic (sex may change after maturation):
1. Simultaneous (both sexes in one individual): hamlets, *Serranus*, *Diplectrum*.
2. Sequential (individual is first one sex and then changes to the other):
a. Protandrous (male first, change to female): anemonefishes, some moray eels, snooks.
b. Protogynous (female first, change to male): gobies, parrotfishes, angelfishes.
V. Secondary sexual characteristics
A. Monomorphic (no distinguishable external difference between sexes): most species (clupeiformes, mullets, snappers, etc.).
B. Sexually dimorphic:
1. Permanently dimorphic (sexes usually distinguishable in mature individuals): dolphinfishes, parrotfishes, etc.
2. Seasonally dimorphic (including colour change only during spawning act): Pacific salmon, lionfishes.
2. Polymorphic (either sex has more than one form): primary and secondary males in wrasses and parrotfishes.
VI. Spawning site preparation
A. No preparation: most species of broadcast spawners.
B. Site prepared and defended: damselfishes, blennies, gobies.
VII. Place of fertilisation
A. External: most species.
B. Internal: elasmobranchs, some scorpionfishes.
VIII. Embryonic development
A. Oviparity: embryos develop outside the ovary, so oocytes are released at spawning.
B. Viviparity: embryos develop inside the ovary, so embryos or larvae are released at spawning.
IX. Parental care
A. No parental care: most species.
B. Parental care (male, female or bi-parental care): seahorses, rockfishes.

The most studies on reproductive strategies is focused on females because of the control of the number of oocytes produced, and the production of viable offspring is more influenced by egg production than the sperm production (TRIPPEL, 2003; HELFMAN et al., 2009). Central to all reproductive strategies, and fundamental to fecundity estimates, are patterns of oocyte development and recruitment (LOWERRE-BARBIERI, 2009). Thus, oocyte development, ovarian organisation and fecundity regulation strategy are the aspects of reproduction necessary to understand the reproductive strategies in teleosts (MURUA; SABORIDO-REY, 2003; KORTA. 2010; McBRIDE et al., 2013). Moreover, the oocyte development description, identification of the mode by which mature oocytes are developed and spawned is required to correctly estimate of fecundity and reproductive potential of a species (MURUA et al., 2003).

1.1.1 Oocyte development

Teleost oocytes growing within the ovary pass through a series of development stages that eventually culminate in the production of the mature oocyte (CERDÀ et al., 2007). The oocyte growth is a complex process of development and differentiation, which involves:

- Formation of numerous nucleoli, development of lampbrush chromosomes, and inclusion bodies in the nucleus;
- Accumulation of various organelles of diverse morphology, RNA, inclusions in the cytoplasm;
- Formation of various oocyte envelopes.

All these aspects of oogenesis are characterised by complex morphological, biochemical and molecular transformations that lead to the formation of numerous oocytes in the ovary, depending upon the fish species (GURAYA, 1986). Although many differences exist in the maturation of oocytes among teleosts (ROCHA; ROCHA, 2006), four main development stages are generally recognised: primary growth, cortical alveoli, vitellogenic and maturation (WALLACE; SELMAN, 1981; WEST, 1990; McMILLAN, 2011).

Primary growth stage begins after the the trasnformation of oogonia into primary oocytes (WALLACE; SELMAN, 1981, WEST, 1990). Oocytes at this stage are independent of gonadotropin hormone, do not contain yolk, and constitute a "reserve fund" for future spawnings (MURUA; SABORIDO-REY, 2003). On the other hand, cortical alveoli are gonadotropin-dependent (GURAYA, 1986; LUBZENS, 2010). In marine fish, this stage is characterised by appearance of cortical alveoli vesicles and lipid inclusions (marine fishes) in the cytoplasm, which indicates that the oocytes will continue their development within the current spawning season (WALLACE; SELMAN, 1981; TYLER; SUMPTER, 1996; MURUA; SABORIDO-REY, 2003). Moreover, the components of follicle layer (envelope) such as zona radiata, theca and granulosa cells also appear in this stage (WEST, 1990). Cortical alveoli vesicles release their contents into the perivitelline space between the oocyte and the zona radiata during fertilisation (WEST, 1990; PATIÑO; SULLIVAN, 2002). These vesicles are synthesised for later participation in the cortical reaction that takes place after fertilisation as a block against polyspermy (URBATZKA et al., 2011).

An important step in oogenesis process is the transition from pre-vitellogenesis to vitellogenesis. Vitellogenesis is normally a long process during which important and visible changes occur within the oocytes (BROWN-PETERSON et al., 2011). Thus, the main characteristic of the vitellogenic stages is the accumulation of yolk proteins in the cytoplasm, increasing markedly the oocyte size (URBATZKA et al., 2011). This stage ends when the most advanced vitellogenic oocytes reach their maximum size (MASUI; CLARKE, 1979). Moreover, the zona radiata thickens and the oocyte continues to form cortical alveoli (SELMAN; WALLACE, 1986; McMILLAN, 2011). The size and age at the first maturity of females are estimated based on the presence of these vitellogenic oocytes in the ovaries (SABORIDO-REY; KJESBU, 2005; McBRIDE et al., 2013).

The first visible event of the maturation stage is the breaking and migration of the germinal vesicle (nucleus) toward the micropyle, for the region so-called animal pole (MURUA; SABORIDO-REY, 2003; McMILLAN, 2011). The envelope of the germinal vesicle breaks down during the first meiotic division, and the nucleus contents blend with the surrounding cytoplasm (McMILLAN, 2011). The coalescence of the lipid droplets and yolk proteins occur during this event (WALLACE; SELMAN,

1981). During the terminal event of the oocyte maturation, many oocytes enlarge again in size due to rapid water uptake (hydration) prior to the next spawning event (WALLACE; SELMAN, 1981; TYLER; SUMPTER, 1996; URBATZKA et al., 2011). This process is critical in marine teleosts with pelagic eggs to ensure specific buoyancy in seawater (WALLACE; SELMAN, 1981; CERDÀ et al., 2007; URBATZKA et al., 2011). Thus, the hydrated oocytes are detached from the ovarian lamellae, released into the ovarian lumen and evacuated through the gonoduct in most species (URBATZKA et al., 2011). The remaining follicle layers is termed post-ovulatory follicle and degenerates through several stages up to complete absorption (GURAYA, 1986; HUNTER; MACEWICZ, 1985b; GANIAS, 2013) (Figure 1). The completion of the second meiotic division of the mature oocytes occur only after fertilisation and results in the formation of a haploid egg and a polar body, with the haploid hydrated oocyte fusing the haploid sperm (URBATZKA et al., 2011).

1.1.2 Ovarian organisation

Based on the description of the oocyte development stage is possible to classify the ovary dynamics in three types of ovarian development organisation: synchronous, group-synchronous and asynchronous (MARZA, 1938; WALLACE; SELMAN, 1981; MURUA; SABORIDO-REY, 2003). A synchronous ovary is typical of species that spawn once and then die; all oocytes formed grow and spawn at the same time, i.e. further replenishment of one stage by an earlier stage does not take place. The frequency distribution of the oocyte diameter represented by a single bell curve, i.e. only one cohort progress from primary growth to maturation stage (Figure 2a).

In many matured fishes, a reserve of pre-vitellogenic oocytes persist form year to year, from which the vitellogenic oocytes develop and mature on a seasonal basis (McBRIDE et al., 2013). In group-synchronous type, at least two populations of oocytes, separated by a hiatus, may be distinguished in mature females: a synchronous population of vitellogenic oocytes (defined as a "clutch") and a more heterogeneous population of pre-vitellogenic oocytes from which the clutch is recruited. The former are the oocytes to be spawned during the current spawning season, whereas the latter are those to be spawned in future spawning seasons (MURUA; SABORIDO-REY, 2003) (Figure 2b). On the other hand, oocytes of all

development stages are present without dominant populations in asynchronous ovary. This ovary appears to be a random mixture of oocytes in every conceivable stage. The overlapping size cohorts of pre-vitellogenic and vitellogenic oocytes persist throughout the spawning period; however, a clear separation between the cohorts of the most advanced vitellogenic oocytes and the matured oocytes may appears (Figure 2c). In an asynchronous ovary organisation, two or three groups of vitellogenic oocytes are clearly visible in the ovary during the vitellogenesis (PAVLOV et al., 2009).

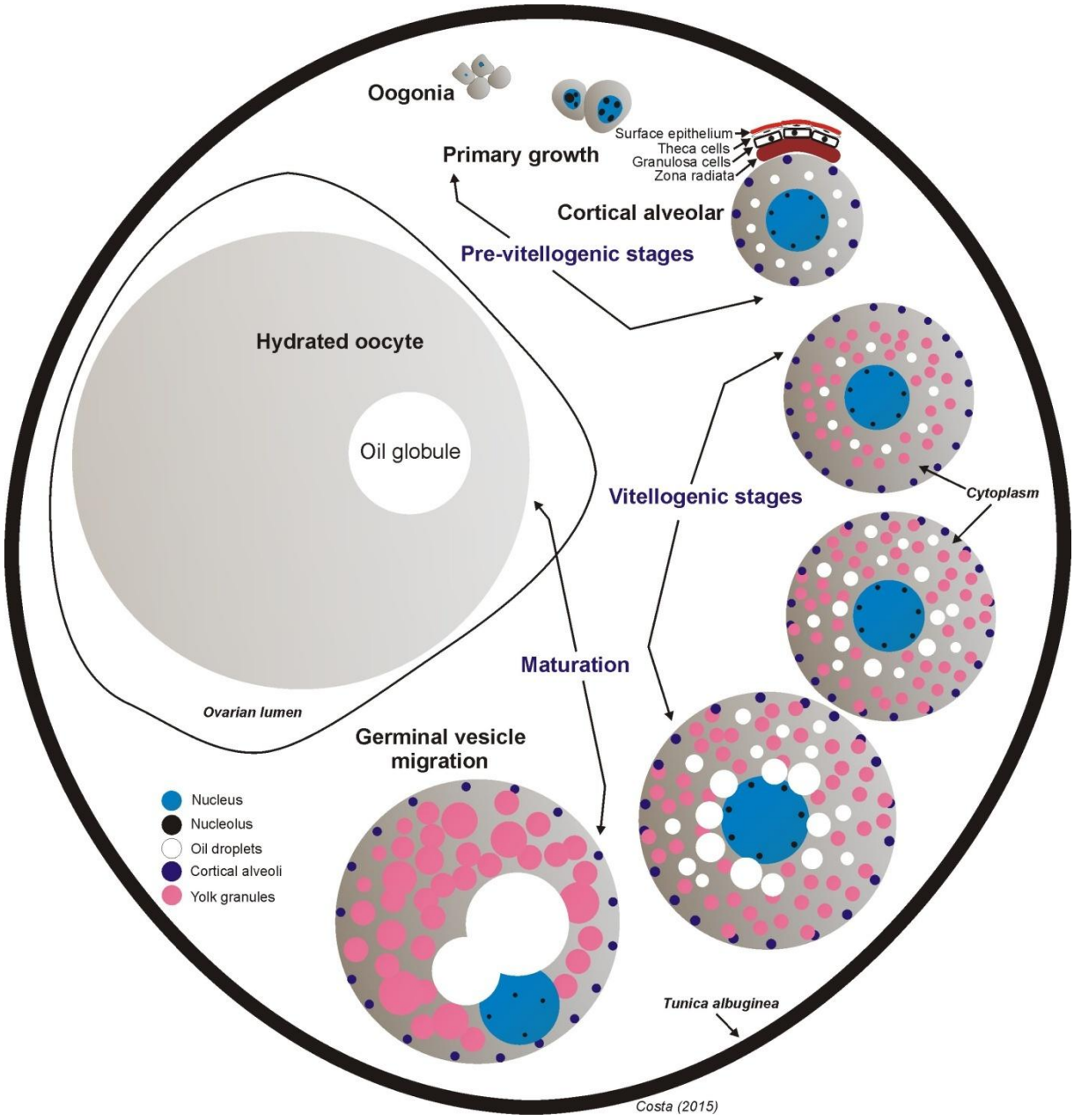


Figure 1. Illustrating the oocyte maturation in marine fishes.

1.1.3 Fecundity regulation strategy

Two fecundity regulation strategies have been identified in regard to the strategy by which oocytes are recruited and developed from pre-vitellogenic to vitellogenic stock: indeterminate and determinate oocyte recruitment (HUNTER et al., 1992; MURUA; SABORIDO-REY, 2003; GANIAS et al., 2014). Synchronous ovarian organisation is indicative of semelparous species, i.e. determinate fecundity, whereas species with asynchronous ovarian organisation may exhibit either determinate or indeterminate fecundity (Figure 2). Thus, in indeterminate fecundity species, the potential annual fecundity is not fixed before the onset of spawning because pre-vitellogenic oocytes are recruited into vitellogenic stock during the spawning season (*de novo* vitellogenesis) (HUNTER et al., 1992; MURUA, SABORIDO-REY, 2003). Thus, potential fecundity in this species should be estimated multiplying the batch fecundity, the spawning fraction and the duration of the spawning season (HUNTER; MACEWICZ, 1985b). In contrast, the total fecundity measured prior to the onset of spawning is considered to be equivalent to the potential annual fecundity in fish species with determinate fecundity, because the recruitment of oocytes to the vitellogenic pool ended before the spawning season (MURUA; SABORIDO-REY, 2003; GANIAS et al., 2014).

Four lines of evidence have been reported to distinguish between species that exhibit determinate or indeterminate fecundity regulation strategy in marine fish species (HUNTER et al., 1992; MURUA; SABORIDO-REY, 2003; Ganias et al., 2014). The first evidence is to investigate the stage-specific and monthly-specific variation of oocyte size frequency distribution. A gap separating the vitellogenic oocyte stock from the reservoir of pre-vitellogenic oocytes, typical of synchronous and group-synchronous species, will indicate that the annual fecundity is indeterminate, whereas the absence of this gap may indicate that the annual fecundity is indeterminate. However, the lack of hiatus does not necessarily imply that the fecundity is indeterminate. The second evidence is to analyse the seasonal variation in the percentage of different oocyte classes during the spawning season. In indeterminate fecundity species, the percentage throughout the spawning season of pre-vitellogenic and vitellogenic oocytes is similar, whereas in determinate species

this percentage decreases as the spawning season progress because the standing stock of oocytes diminishes, as they are not replaced during the spawning season.

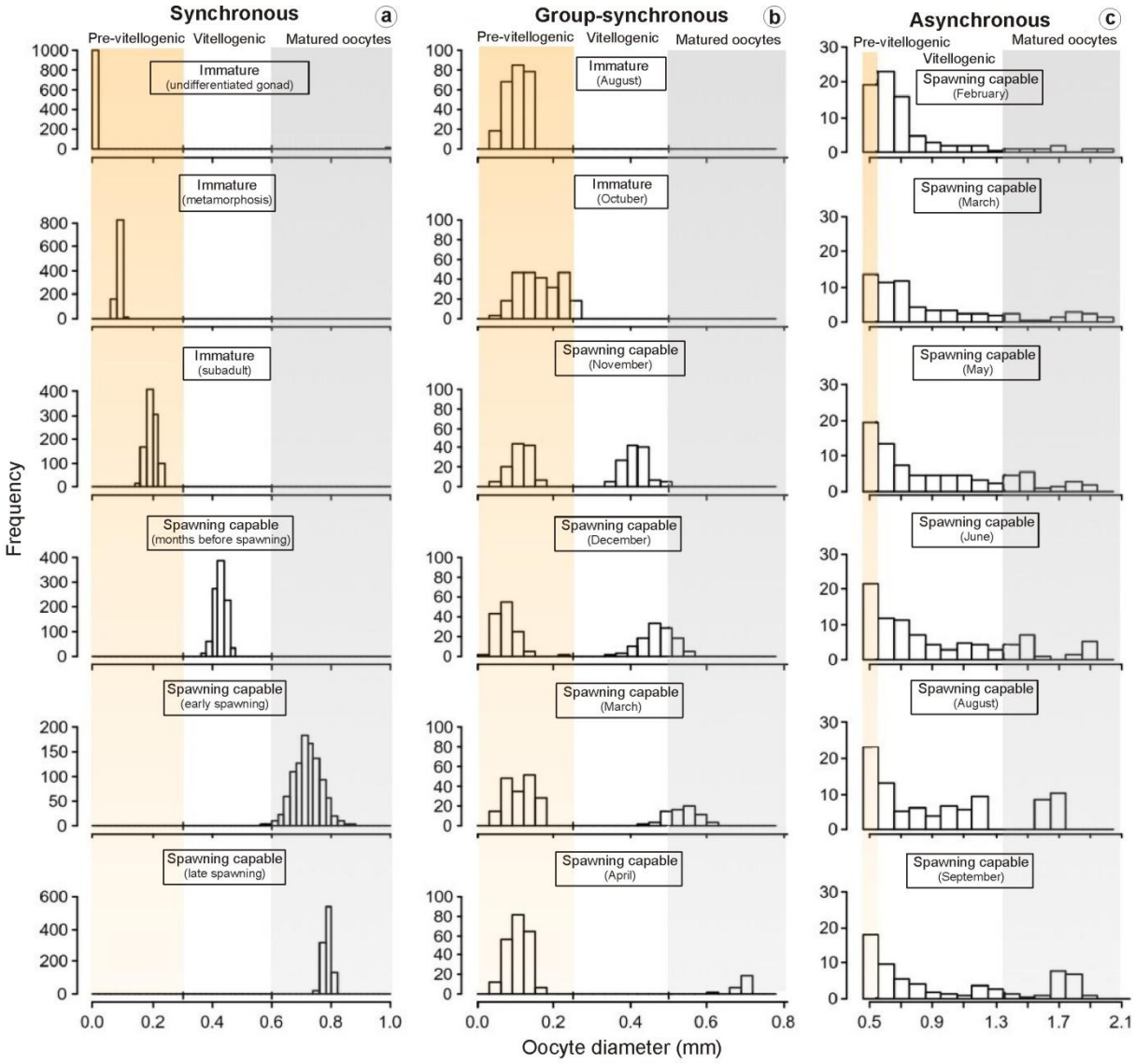


Figure 2. Examples of three types of ovarian development organisation in relation to the ovary phase and sampled period. (a) Sea lamprey, *Petromyzon marinus* (Petromyzontiformes, Petromyzontidae), a semelparous fish with synchronous oocyte development (determinate fecundity); (b) winter flounder, *Pseudopleuronectes americanus* (Pleuronectiformes, Pleuronectidae), an iteroparous fish with group-synchronous oocyte development (determinate fecundity); (c) Mummichog, *Fundulus heteroclitus* (Cyprinodontiformes, Fundulidae), an iteroparous fish with asynchronous oocyte development (indeterminate fecundity). Source: McBRIDE et al. (2013) modified.

Thirdly, a seasonal decrease in the mean oocyte diameter of advanced vitellogenic stage as the spawning season progress may be expected in indeterminate fecundity. This occurs, due to the recruitment of newly formed early vitellogenic oocytes into the stock of the most advanced vitellogenic oocytes. On the other hand, a seasonal increase in the mean diameter of the advanced vitellogenic oocytes, over the spawning season, may be observed in determinate fecundity, because no vitellogenic oocytes are recruited to replace those oocytes that have been spawned during the current spawning season.

Finally, indeterminate fecundity species show an increase in prevalence and intensity of atresia at the end of the spawning period. A low incidence of atresia occurs throughout the spawning season, but becomes marked when approach the end of the spawning season, and the remaining production of oocytes in the ovary needs to be absorbed (HUNTER; MACEWICZ, 1985b; WEST, 1990; MURUA; MOTOS, 2006). On the other hand, in determinate fecundity fishes the level of atresia during the spawning season and at the end of this season is very low (HUNTER et al., 1992; GANIAS et al., 2015).

In addition, a new line of evidence based on the relationship between the duration of spawning and oocyte growth rate have been reported by GANIAS et al. (2014b). Thus, populations, which have oocyte growth period less than the spawning period, exhibit indeterminate fecundity strategy. On the other hand, fishes with determinate fecundity strategy have the oocyte growth period greater than the spawning period (GANIAS et al., 2015).

1.2 Fish fecundity

Fecundity is a quantitative variable, and not a qualitative or subjective indicator of individual reproductive success, which express the number of mature oocytes spawned by a female during the spawning season, or event of spawning (DeMARTINI et al., 2006; GANIAS et al., 2014). This reproductive trait is often considered the most important component of a reproductive strategy in marine fishes (LOWERRE-BARBIERI, 2009). The total number of oocytes produced is typical of a species, although the oocyte size, female size/age, intraspecific genetic variation, environmental factors, fishing pressure and nutrition condition may increase the

variance in the final number of oocytes released into the environment (JEFFRY; LEWIS, 1989; TYLER; SUMPTER, 1996; DENNEY et al., 2002; LAMBERT et al., 2003; LAMBERT, 2008; DOMÍNGUEZ-PETIT; SABORIDO-REY, 2010).

Data on marine fish fecundity are scarce worldwide, mainly for those species with indeterminate fecundity inhabiting tropical and subtropical regions (TOMKIEWICZ et al., 2003; LOWERRE-BARBIERI et al., 2011b; FITZHUGH et al., 2012). However, the knowledge of fecundity is very important for estimating the individual reproductive potential, population dynamics (stock-recruitment models) or productivity (spawning stock biomass), and for characterising specific populations, subpopulations, and stocks of fishes (KRAUS et al., 2002, DeMARTINI et al., 2006; LAMBERT, 2008; ARMSTRONG; WITTHAMES, 2012). Moreover, these studies also provide information useful for monitoring population in relation to climate changes and fishing pressure (resilience) (SADOVY, 2001; PANKHURST; KING, 2010; STRÜSSMAN et al., 2010). Thus, there are several ways of measuring fecundity in fish, such as (HUNTER et al., 1992; MURUA; SABORIDO-REY, 2003):

Annual realised fecundity: The real number of oocytes finally spawned by a female per year.

Potential annual fecundity: The total number advanced vitellogenic oocytes matured per year, uncorrected for atretic losses.

Total fecundity: The total number of vitellogenic oocytes or standing stock of advanced vitellogenic at any time in the ovary.

Batch fecundity: The number of hydrated released in one event of spawning. Therefore, the sum of batch fecundities represents the annual realised fecundity.

Potential fecundity: The number of developing oocytes present in the ovary at a certain time.

Relative fecundity: Fecundity divided by female weight.

1.3 Histological and stereological analysis in fish reproduction

Histology is considered the most accurate and reliable method to assess the reproductive strategy of fish species, although is an expensive and time-consuming technique (WEST, 1990; KJESBU et al., 2009; LOWERRE-BARBIERI, 2011a). The latest reproductive studies have been applied histological methods for assessing the oocyte development, ovary organisation, oocyte recruitment patterns, spawning season, spawning fraction, spawning intervals, fecundity down-regulation and skipped spawning in fishes with indeterminate and determinate fecundity from several regions (Table 2). Therefore, there is a need to review gonad histology techniques for a wide range of species and regions, to standardize histological indicators and terminology, and to present new applications of these techniques to address emerging issues in fish reproductive biology (LOWERRE-BARBIERI et al., 2011a).

Stereology is the three-dimensional interpretation from measurements made on two-dimensional sections (WEIBEL, 1979). Stereological methods provide quantitative information about the entire cells and tissues based on photomicrographs of sections, taking into account geometry of the cells and probability statistics (WEIBEL, 1979; SUNDBERG, 1992; JENSEN 1998). In fish reproductive biology studies, stereometry is one of the most precise and accurate method to estimate fecundity from images of the histological sections of the ovaries (EMERSON et al., 1990; ARAGÓN et al., 2010; GONZÁLEZ-RUFINO et al, 2013). Thus, the majority of these studies have been carried out based on the equation proposed by Weibel and Gomez (1962) and Weibel et al. (1966). This equation depends on assumptions about the oocyte shape and size distribution (so-called model-based stereology), and is based on the Delesse Principle which states that the fractional volume of a component in a material is proportional to its fractional area (DELESSE, 1847; EMERSON et al., 1990).

The stereological procedures allow a complete analysis of the reproductive status of the females including spawning condition, atresia, fecundity, oocyte size and quantification of pre-vitellogenic oocytes (MURUA et al., 2003). So far, the model-based stereology is the most common procedure to estimate the number of oocytes in fish (GANIAS et al., 2014). This includes oocytes in primary growth,

vitellogenesis and atresia, as well as post-ovulatory follicles (ISAAC-NAHUM et al., 1988; GREER WALKER et al., 1994; MACCHI; WÖHLER, 1994; BROMLEY et al., 2000; COWARD; BROMAGE, 2002; MEDINA et al., 2002; COOPER et al., 2005; MEDINA et al., 2007; ALONSO-FERNÁNDEZ et al., 2008; SABER et al., 2011; HASLOB et al., 2013; GARCÍA-SEOANE et al., 2014; KNAPP et al., 2014; NEVES et al., 2014; VILLEGAS-RÍOS et al., 2014). Although, it should not be used to count cells of irregular shape and/or highly variable size, e.g. atretic oocytes and post-ovulatory follicles (GANIAS et al., 2014).

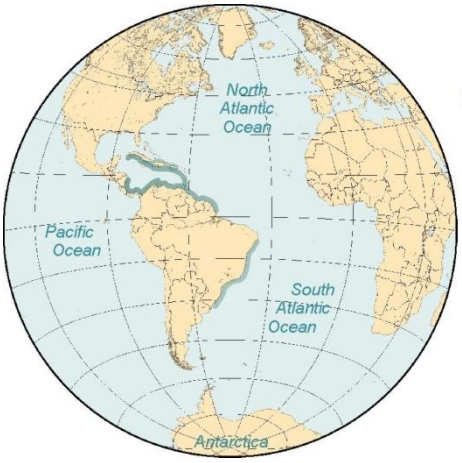
1.3 Species of study

The gonochorists *Anchoa filifera*, *Cetengraulis edentulus*, *Citharichthys spilopterus*, *Stellifer brasiliensis*, *S. rastrifer*, *Menticirrhus americanus*, *Paralonchurus brasiliensis* and the simultaneous hermaphrodite *Diplectrum radiale* are found inhabiting coastal and estuarine environments in the central western and southern Atlantic Ocean (CARPENTER, 2002a, 2002b). These species are commonly captured as bycatch by shrimp fishery trawl in the south-eastern and southern Brazil (COELHO et al., 1986; PAIVA-FILHO; SCHMIEGELOW, 1986; VIANNA et al., 2004; VIANNA; ALMEIDA, 2005; BRANCO; VERANI, 2006; GOMES; CHAVES, 2006; PINA; CHAVES, 2009); showing little importance as a fishery resource but participating in the annual Brazilian fishery production, with exception of *D. radiale* and *C. spilopterus* (MPA, 2011). Although they have an important ecological role participating in the structure of the ecosystem, little is known about their reproductive strategy and fecundity.

Table 2. Examples of application of histological techniques for investigating reproductive strategies in marine teleost fishes.

Species	Histological Application	References
Pseudopleuronectes americanus, Paralichthys lethostigma, Acanthocybium solandri, Kajikia audax, Thunnus albacares, Katsuwonus pelamis, Lophius budegassa, Diplectrum formosum.	Oocyte development description and ovarian development phase for estimating the size at first maturity.	BUBLEY; PASHUK (2010), KOPF et al. (2012), MIDWAY; SCHARF (2012), COLMENERO et al. (2013), McBRIDE et al. (2012), ZISCHKE et al. (2013); ZUDAIRE et al. (2013b), GRANDE et al (2014).
Thunnus albacares, Nezumia aequalis, Coelorinchus mediterraneus, Benthosema glaciale, Clupeonella cultriventris, Mugil liza, Thunnus obesus.	Identification and description of oocyte development for assessing the ovary organisation dynamics (asynchronous or synchronous).	FERNANDEZ-ARCAYA (2013), KARIMI et al. (2013), SUN et al. (2013), ZUDAIRE et al. (2013a), GARCÍA-SEOANE et al. (2014), LEMOS et al. (2014).
Alosa sapidissima, Serranus atricauda, Lophius budegassa, Thunnus albacares, Sprattus sprattus balticus, Mugil liza	Identification and description of oocyte development stages to investigate the oocyte size-frequency distribution.	HASLOB et al. (2012), ZUDAIRE et al. (2013a), HYLE et al. (2014), NEVES et al. (2014).
Acanthocybium solandri, Kajikia audax, Serranus atricauda, Lophius budegassa, Benthosema glaciale, Sprattus sprattus balticus.	Assigning the active spawning females for estimating the spawning season.	KOPF et al. (2012), COLMENERO et al. (2013), HASLOB et al. (2013), ZISCHKE et al. (2013), GARCÍA-SEOANE et al. (2014), NEVES et al. (2014).
Alosa sapidissima, Paralichthys olivaceus, Thunnus alalunga, Thunnus orientalis, Engraulis japonicus.	Description of oocyte development stages and post-ovulatory follicle degeneration to estimate the spawning intervals.	FARLEY et al. (2013), YASUDA et al. (2013), YONEDA et al. (2013), HYLE et al. (2014), ASHIDA et al. (2014).
Engraulis encrasicolus, Merluccius merluccius, Thunnus alalunga, T. thynnus, T. obesus, Sardinops sagax, Trachurus mediterraneus, Engraulis ringens.	Description of oocyte development stages and post-ovulatory follicle degeneration to assign the females to a daily spawning class for estimation of the spawning fraction and spawning frequency.	MURUA; MOTOS (2006), PEREA et al. (2011), URIARTE et al. (2012), DEMIREL; YÜKSEK (2013), FARLEY et al. (2013), SUN et al. (2013), ASHIDA et al. (2014), DORVAL et al. (2014), KNAPP et al. (2014).
Clupea harengus, Platichtys flesus, Pleuronectes platessa, Pseudopleuronectes americanus.	Identification and description of different stages of atresia and post-ovulatory follicles for investigating fecundity down regulation by resorption (atresia) of vitellogenic oocytes, and skipped spawning females.	KURITA et al. (2003), KENNEDY et al. (2007), KENNEDY et al. (2011), van DAMME (2013); NISSLING et al. (2014), PRESS et al. (2014).

1.3.1 *Anchoa filifera* (Fowler, 1915)



Class: Actinopterygii

Order: Clupeiformes

Family: Engraulidae

Genus + Species:

Anchoa filifera (Fowler, 1915)

Costa (2015)

Common name: Longfinger anchovy, Manjuba (Portuguese)

Geographical distribution: Western and South Atlantic: Antilles, Trinidad and south to Brazil; also Venezuela to Panama and Honduras.

Habitat: Pelagic species inhabiting marine and estuarine areas.

Biology: To 130 mm in standard length; a plankton-feeding occuring in large schools in depths up to 25 m.

Length at first maturity (L_{50}): ?

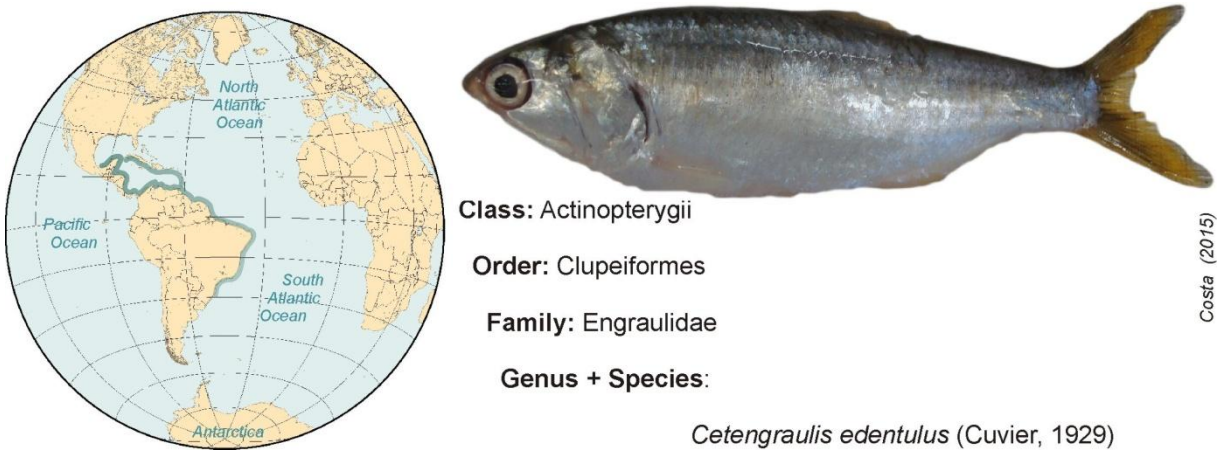
Spawning season: ?

Fecundity: ?

Fisheries: Captured ocasionally by beach seines, contibuting to the total clupeoid catches, but not as a special fishery. In Brazil, *A. filifera* is comercialised along with other engraulid species, participating in the annual Brazilian fishery production.

References: WHITEHEAD et al. (1988), CERVIGÓN et al. (1993), CARPENTER (2002a), MPA (2011), CONTENTE (2013).

1.3.2 *Cetengraulis edentulus* (Cuvier, 1829)



Common name: Atlantic anchoveta, Manjubão (Portuguese)

Geographical distribution: Western central and South Atlantic: Antilles from Cuba southward; Costa Rica south and east to Colombia and Venezuela, Trinidad south to Santa Catarina, Brazil.

Habitat: Pelagic species inhabiting marine and estuarine areas.

Biology: Maximum length ≈166 mm in total length (common to 150 mm); feeding on planktonic organisms (presumably both animal and plant); occurrs in large schools near the surface, but also down to depths of 25 m. The oocytes are oval, small, ranging in volume from 0.18 to 0.34 mm^3. Spawning at 02.30 and 05.00 hours, hatching about 20 to 24 hours later.

Length at first maturity (L_{50}): 97.5-118 mm in total length for females from Santa Catarina, Brazil, and Terminos Lagoon, Mexico, respectively.

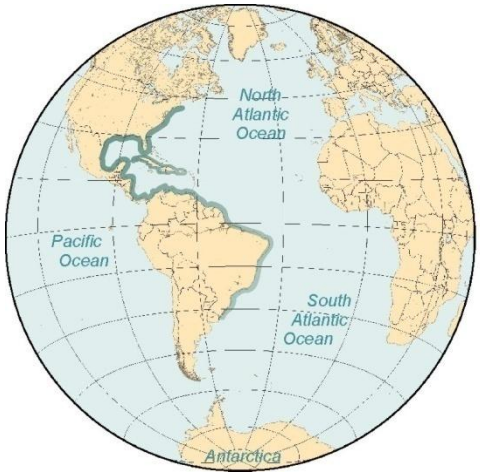
Spawning season: Spawning may occurs throughout the year. However, the peaks of spawning have been recorded from October to February in the coastal of Santa Catarina, Brazil, and from October to January in Araya Peninsula, Venezuela.

Fecundity[1]**:** The Relative fecundity has been estimated at 585.5 oocytes per gram of fish weight in the Terminos Lagoon, Mexico.

Fisheries: *C. edentulus* captured by beach seines and cast nets. Little used for human consumption, but valued as a source of fish meal for animals in Venezuela. In Brazil, this species is also captured as bycatch and participates in the annual Brazilian fishery production, being commercialised mainly by artisanal fishermen.

References: CERVIGÓN (1966), WHITEHEAD et al. (1988), CERVIGÓN et al. (1993), CARPENTER (2002a), SOUZA-CONCEIÇÃO et al. (2005), MPA (2011).

1.3.3 *Citharichthys spilopterus* Günther, 1862



Class: Actinopterygii

Order: Pleuronectiformes

Family: Paralichthyidae

Genus + Species:

Citharichthys spilopterus Güther, 1862

Costa (2015)

Common name: Bay whiff, Linguado (Portuguese)

Geographical distribution: Western and South Atlantic: coast of United States; West Indies; Carribean Sea; Tobago; coastal of South America to lagoa dos Patos, Rio Grande do Sul, Brazil.

Habitat: Demersal species usually found in coastal**,** estuaries and hypersaline lagoons.

Biology: Maximum length of 180-200 mm (common to 150 mm); inhabits shallow bottoms up to 75 m; an ambush predator feeding mainly on fishes and crustaceans.

[1] The method adopted for fecundity estimation has not been detailed in the work.

Its spawning period coincides with increasing temperature and decreasing salinity, being also considered a stuarine-dependent species

Length at first maturity (L_{50}): ?

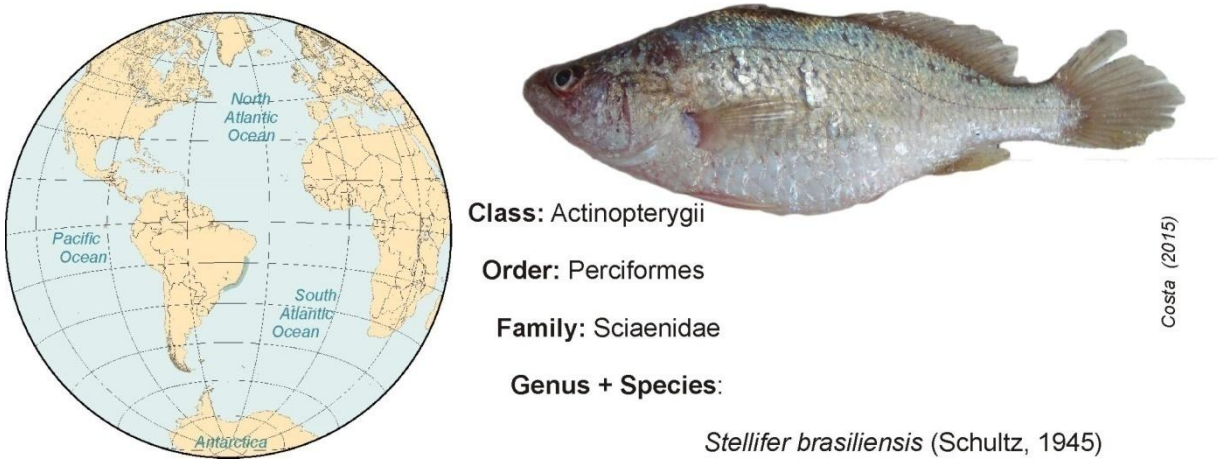
Spawning season[2]**:** Spring and summer in Guratuba Bay, Paraná, and Mamanguá Inlet, Rio de Janeiro, Brazil.

Fecundity[3]**:** The batch fecundity has been ranged from 7000 to 61 000 oocytes and the relative batch fecundity from 253 to 1,552 oocytes g^{-1} in the Mamanguá Inlet, Rio de Janeiro, Brazil. .

Fisheries: Usually captured by artisanal fishermen using a beach seine, but it has a little commercial importance as a fishery resources.

References: CERVIGÓN et al. (1993), CHAVES; VENDEL (1997a), CASTILLO-RIVERA et al. (2000), MENEZES; FIGUEIREDO (2000), CARPENTER (2002b), MENEZES et al. (2003), DIAS et al. (2005), SÁNCHEZ-GIL et al. (2008).

1.3.4 *Stellifer brasiliensis* (Schultz, 1945)



Common name: Cangoá, Canganguá (Portuguese)

[2] The Spawning season was estimated based on the seasonal variation in the gonadosomatic index.
[3] Estimated for eight specimens using germinal vesicle migration and hydrated oocytes. However, some ovaries were with sign of spawning, i.e. post-ovulatory follicle.

Geographical distribution: South-west Atlantic: from the coast of Bahia to Santa Catarina, Brazil.

Habitat: Demersal species usually found in coastal and estuarine areas.

Biology: Maximum length ≈180 mm; inhabits shallow waters over sand and muddy bottoms, feeding mainly on crustaceans.

Length at first maturity (L_{50}): 133 mm for females.

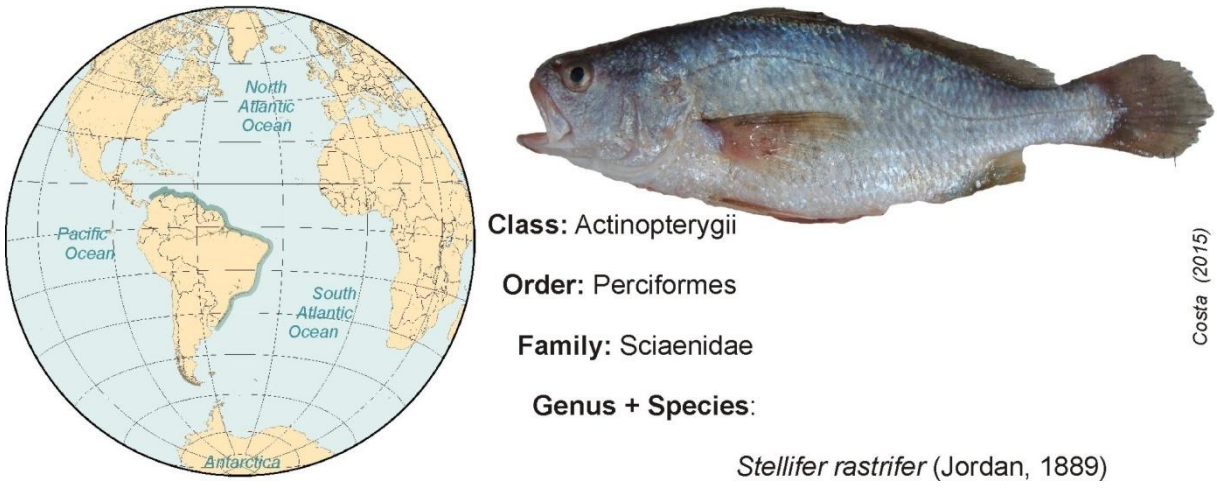
Spawning season: The peak of spawning probably occurs during the spring in the Santos Bay, Brazil.

Fecundity: ?

Fisheries: No commercial importance as a fishery resources.

References: MENEZES; FIGUEIREDO (1980b), MENEZES et al. (2003), SCHMIDT (2011), POMBO et al. (2013).

1.3.5 *S. rastrifer* (Jordan, 1889)



Common name: Rake stardrum**;** Cangoá and Canganguá (Portuguese)

Geographical distribution: Western and south-west Atlantic: along Caribbean coast, from Panama to Uruguayan Coast.

Habitat: Demersal species usually found in coastal and estuarine areas.

Biology: Maximum length 212 mm (common to 120 mm); most abundant in warm waters and over sandy and muddy bottoms; spawning occurs specially in estuarine areas; feeds mainly on polychaetes and crustaceans.

Length at first maturity (L_{50}): 136.3 mm for females.

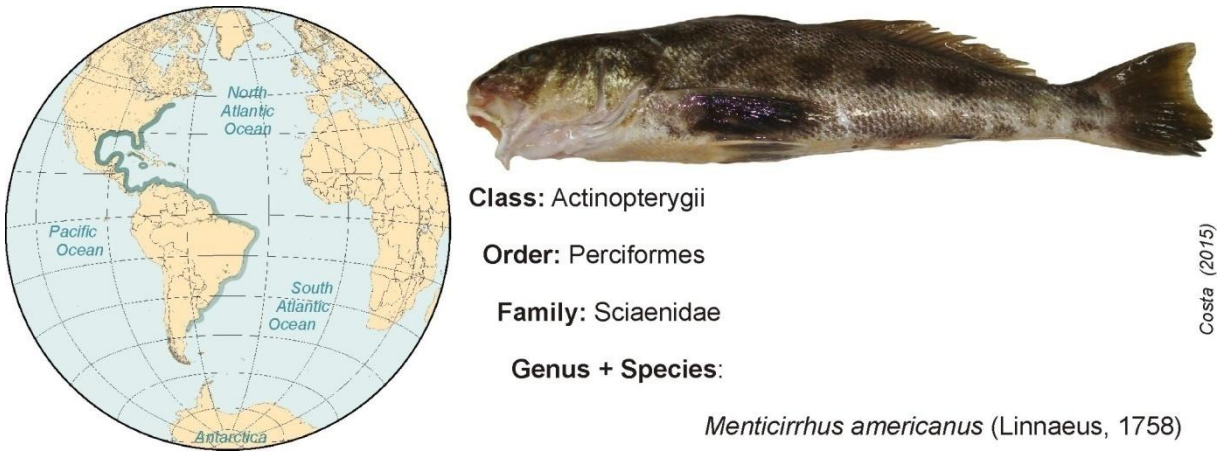
Spawning season[4]: The spawning may occurs throughout the year. However, peaks of spawning have been recorded during the spring and winter in the Guaratuba Bay, Paraná, and during the autumn and summer in the Santos Bay, Brazil.

Fecundity: ?

Fisheries: No a target species, but very abundant as bycatch in shrimp trawl fisheries off Guyana and south-eastern Brazil. Usually not marked for human comsuption, but used in manufacture of fisheries by-products. The larger ones are comsumed in some regions.

References: MENEZES; FIGUEIREDO (1980b), GIANINI; PAIVA-FILHO (1990), CERVIGÓN et al. (1993), CHAVES; VENDEL (1997b), CARPENTER (2002a), PINA; CHAVES (2009); SEGURA et al. (2009), SCHMIDT (2011), POMBO et al. (2013).

1.3.6 *Menticirrhus americanus* (Linnaeus, 1758)



Common name: Southern kingcroacker**;** Betarra and Papa-terra (Portuguese)

[4] The Spawning season was estimated based on the seasonal variation in the gonadosomatic index.

Geographical distribution: Western and south-west Atlantic: from United State Atlanc coast, Gulf of of Mexico, Carribean Sea, to Argentina. Few records from Antilles and none from the lesser Antilles.

Habitat: Demersal species found in coastal and estuarine areas.

Biology: Maximum length ≈ 600 mm (common to 350 mm); found over sandy mud to hard sand bottoms, as weel as in the surf zone; juveniles are more abundant in estuarine areas; feeds mainly on bottom-dwelling organisms such as crustaceans and polychaetes.

Length at first maturity (L_{50}): 150-240 mm for males and 180-240 mm for females in total length.

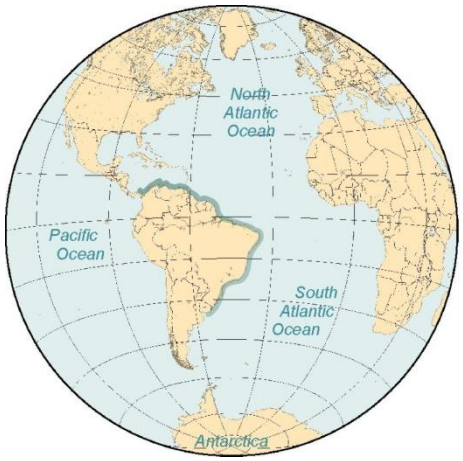
Spawning season: The spawning may occurs throughout the year. Peaks of spawning during the spring, summer and autumn have been reported for specimens captured in the coasts of Paraná and Santa Catarian, Brazil.

Fecundity: The batch fecundity estimated for eight hydrated females from Rio de la Plata estuary, Argentina, ranged from 45,115 to 273,188 hydrated oocytes (relative batch fecundity from 143 to 374 oocytes g^{-1}).

Fisheries: Artisanal and industrial. An excellent foodfish, usually captured with otter trawls, pound net and seine. In Brazil, is very common as bycatch in the shrimp trawl fisheries.

References: CERVIGÓN et al. (1993), CARPENTER (2002a), SANTOS (2006), RONDINELI et al. (2007), MUNIZ; CHAVES (2008), HALUCH et al. (2009), HALUCH et al. (2011), MILITELLI et al. (2013).

1.3.7 *Paralonchurus brasiliensis* (Steindachner, 1875)



Class: Actinopterygii

Order: Perciformes

Family: Sciaenidae

Genus + Species:

Paralonchurus brasiliensis (Steindachner, 1875)

Costa (2015)

Common name: Banded croacker**;** Maria-luísa (Portuguese)

Geographical distribution: Western and south-west Atlantic: Caribean and Atlantic coats of South America from Panama to Argentina.

Habitat: Demersal species found in coastal and estuarine areas.

Biology: Maximum length of 300 mm (common to 250 mm); found over muddy bottoms in coastal areas in depths up to 100 m; the juveniles entering in estuarine areas; feeds mainly on bottom-dwelling organisms such as crustaceans and polychaetes.

Length at first maturity (L_{50}): 148-170 mm for males, 148-180 mm for females and 145-175 mm for grouped sexes.

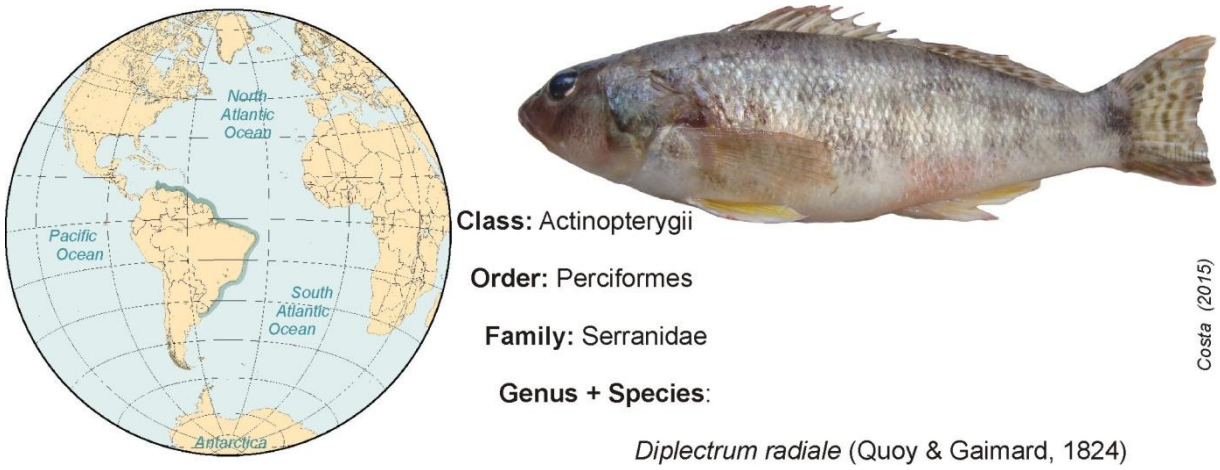
Spawning season: The spawning may occurs throughout the year. Peaks of spawning during the spring, autumn and winter have been reported for specimens captured in the coastal waters of Paraná and São Paulo, Brazil.

Fecundity: The batch fecundity estimated for hydrated females from Rio de la Plata estuary, Argentina, ranged from 5,560 to 32,400 hydrated oocytes (relative batch fecundity from 58 to 320 oocytes g^{-1}).

Fisheries: Of little comercial importance, It usually captured as a bycatch in shrimp trawl fisheries and beach seines.

References: PAIVA-FILHO et al.(1976a), PAIVA-FILHO et al.(1976b), MENEZES; FIGUEIREDO (1980b), CERVIGÓN et al. (1993), CUNNINGHAM; DINIZ-FILHO (1995), CARPENTER (2002a), LEWIS; FONTOURA (2005), BRANCO et al. (2005), ROBERT et al. (2007), SCHMIDT (2011), MILITELLI et al. (2013).

1.3.8 *Diplectrum radiale* (Quoy and Gaimard, 1824)



Common name: Pond perch**;** Michole (Portuguese)

Geographical distribution: Western and south-west Atlantic: Venezuela to Uruguay.

Habitat: Demersal species found in coastal and estuarine areas.

Biology: reach a reasonable large size of 260 mm in total length (common to 200 mm); commonly in coastal areas in depths up to 50 m, also entering in the estuaries; inhabiting over soft substrates and soft corals, but not a typical coral-reef species; consumes mainly crustaceans and small fish.

Length at first maturity (L_{50}): 100-131 mm

Spawning season: The peak of spawning occurs during the spring and summer.

Fecundity: ?

Fisheries: Artisanal; flesh of good quality; usually captured by hook-and-line, traps and as bycatch by shrimp trawl fisheries;.

References: CERVIGÓN (1966), BORTONE (1977b), MENEZES; FIGUEIREDO (1980a), DIAS (1989), CERVIGÓN et al. (1993), MAGRO (1996), CARPENTER (2002a), MEURER; ANDREATA (2002), MENEZES et al. (2003).

1.4 Areas of study

The inner shelf of Ubatuba and estuarine system of Cananéia are located in a transition from tropical to subtropical region, South Atlantic Ocean, on the coast of São Paulo state, Brazil (SCHAEFFER-NOVELLI et al., 1990; AIDAR et al., 1993; PIOLA et al., 2000) (Figure 3). Both areas were established as MPA (Marine Protected Area) by the Brazilian Ministry of the Environment for their sustainable use, preservation and conservation of the marine living resources (Proclamation No. 53525 and 53527, on 8 October 2008).

1.4.1 Ubatuba

This area is located in the northern coast of the São Paulo State, and it is strongly influenced by three water masses: Tropical Waters (TW) with high temperature and salinity (T > 20 °C and S > 36); the South Atlantic Central Water (SACW) with low temperature and salinity (T < 20 °C and S < 36), and the Coastal Water (CW) with high temperature and low salinity (T > 20 °C and S < 36) (CASTRO-FILHO; MIRANDA, 1998). However, the seasonal presence of the SACW during the summer and spring months is the main factor influencing the environmental conditions in this area (PIRES-VANIN; MATSUURA, 1993). A strong thermocline is established during the SACW intrusion in isobaths from 10 to 50 m (PIRES-VANIN AND MATSUURA, 1993). On the other hand, during the autumn and winter, the SACW retreats toward the margin of the continental shelf and is replaced by the CW (CASTRO-FILHO; MIRANDA, 1998). Thus, these water masses affect the nourishment condition and the reproduction of species inhabiting in this ecosystem (MATSUURA, 1990; MATSUURA et al., 1992; KATSURAGAWA et al., 1993; MYASHITA et al., 2011)

1.4.2 Cananéia

The Cananéia estuarine system is located in the southern coast of the São Paulo State, near the latitudinal limit for mangroves (25° S). This area is shaped

mainly by the Cardoso, Cananéia and Comprida islands, which are separated from each other by channels and rivers with a maximum depth of 12 m that flow into the ocean (SCHAEFFER-NOVELLI et al., 1990). The tides are semidiurnal (with diurnal inequality) with mean amplitude of 0.82 m, and 1.25 m for spring tides (MESQUITA; HARARI, 1983). The environment is controlled mainly by the intrusion of the tides, freshwater runoff and pluvial input. Thus, the salinity and temperature ranges from approximately 16 to 34 and 19 to 28 °C, respectively. The mean water tempretarure is approximately 21°C with a mean daily variation of 7.9 °C (SCHAEFFER-NOVELLI et al., 1990; BÉRGAMO, 2000). The cumulative monthly rainfall is never less than 80 mm showing peaks during the summer (267 mm) and minima during the winter (95 mm) (SILVA, 1989; SCHAEFFER-NOVELLI et al., 1990; BÉRGAMO, 2000).

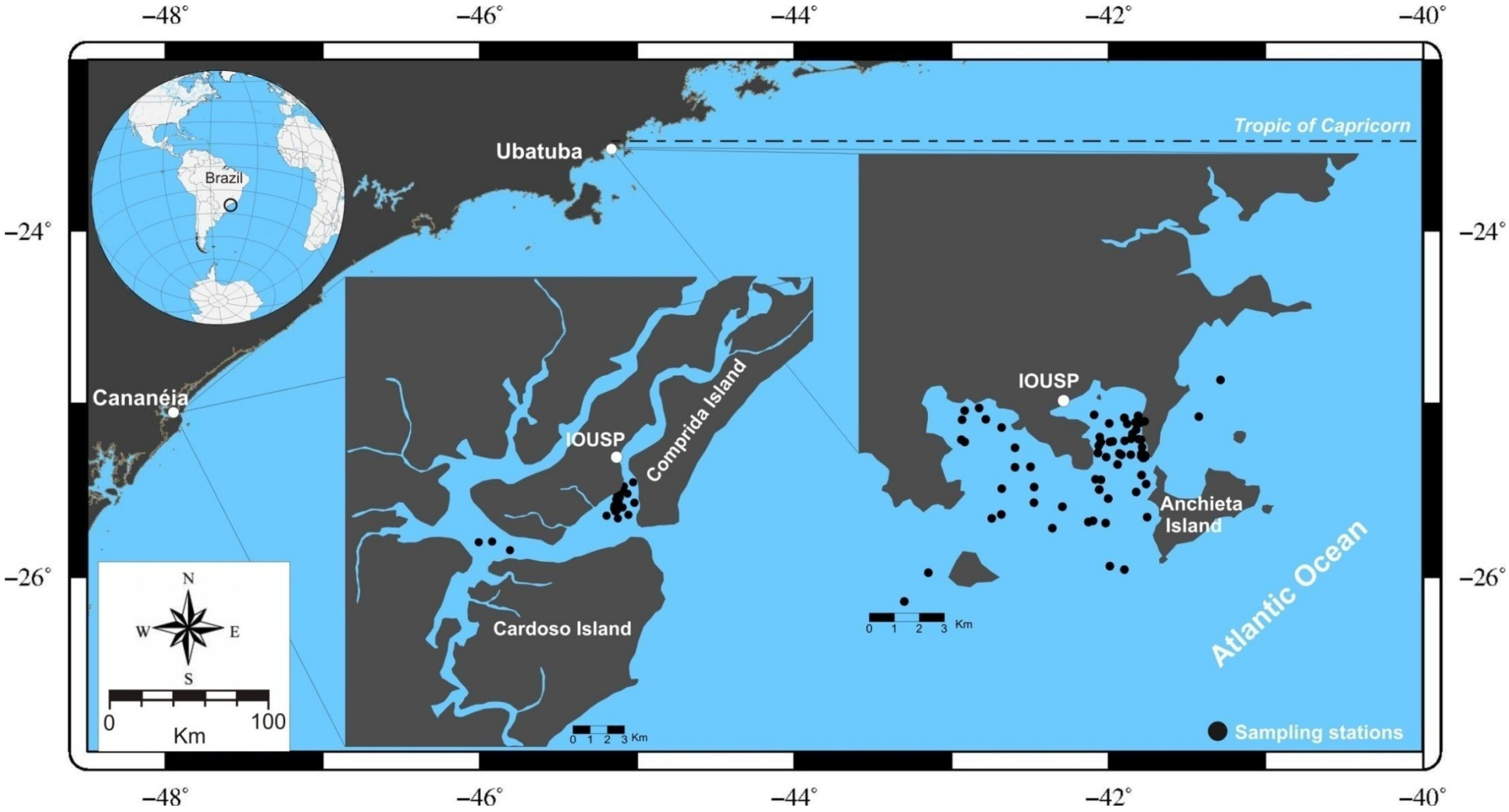


Figure 3. Map showing the inner shelf of Ubatuba and the estuarine system of Cananéia, São Paulo State, Brazil. IOUSP: Research base of the Instituto Oceanográfico da Universidade de São Paulo.

1.5 Guiding questions

The teleosts *Anchoa filifera*, *Cetengraulis edentulus*, *Citharichthys spilopterus*, *Stellifer brasiliensis*, *S. rastrifer*, *Menticirrhus americanus*, *Paralonchurus brasiliensis* and *Diplectrum radiale* are very important structural and seasonal species of coastal ecosystems (ROSSI-WONGTSCHOWSKI; PAES, 1993) and for artisanal fishery. However, the most of these species, in particular Sciaenidae, Paralichthyidae and Serranidae, are captured in high number as bycatch by shrimp fishery trawl in the south-eastern and south Brazil (COELHO et al., 1986; PAIVA-FILHO; SCHMIEGELOW, 1986; VIANNA et al., 2004; VIANNA; ALMEIDA, 2005; BRANCO; VERANI, 2006; GOMES; CHAVES, 2006; PINA; CHAVES, 2009; BERNARDO et al., 2011). For instance, the species *S. brasiliensis*, *S. rastrifer*, *M. americanus*, *P. brasiliensis* are among the most abundant species in the incidental fishing, mainly because their distribution coincides with the distribution of many species of shrimps such as *Farfantepenaeus brasiliensis*, *F. paulensis*, *Litopenaeus schimitti* and *Xiphopenaeus kroyeri* (SOUZA et al., 2008). Studies on species captured as bycatch are scarce worldwide. Many scientists are now urging fishery management to expand their entire focus from only the species being fished to consideration of the entire habitat (GELPKE et al., 2013). Thus, information on species captured as bycatch are essential for the sustainability of the commercial stocks, marine ecosystem and fisheries management (GRAÇA-LOPES et al., 2002; BRANCO; VERANI, 2006; RODRIGUEZ-FILHO et al., 2011).

In addition, the majority of studies on reproductive biology of the marine fish in Brazil have been focused mainly in estimating the size at which the species reach the first sexual maturity (L_{50}), sex ratio and spawning season by analysing the seasonal variation in gonadosomatic index and condition factor (BERNARDES; DIAS, 2000; ARAUJO; CHELLAPPA, 2002; ANDRADE et al., 2003; FAVARO et al., 2005; FAVARO et al., 2007; SILVA et al., 2005; BESSA et al., 2007; ARAÚJO et al., 2008; ALBIERI; ARAÚJO, 2010; OLIVEIRA; FÁVARO, 2010; MARQUES; FERREIRA, 2011; OLIVEIRA; FÁVARO, 2011; FERNANDES et al., 2012; CASSACA et al., 2012; BRANCO et al., 2013; BEZERRA et al., 2013; FERNANDEZ; DIAS, 2013; CONDINI et al., 2014; LEMOS et al., 2014; OLIVEIRA; CHELLAPPA, 2014). Thus, the application of histological methods are still scarce, and some of these authors have

been applied this important tool only to assess the ovarian development and to validate the estimation of the L_{50}. Furthermore, data on fish fecundity are still rare, even for species with economic importance. Therefore, the application of histology and advanced methods for fecundity estimation could contribute to a better understanding of the population dynamics of target and non-target fishes in the coastal and estuarine systems of Brazil.

HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

This thesis has hypothesised that different species inhabiting coastal and estuarine ecosystems will exhibit the same reproductive strategies in terms of ovarian development, oocyte recruitment and fecundity. Thus, main objective of this thesis is to investigate the reproductive strategies of *Anchoa filifera*, *Cetengraulis edentulus*, *Citharichthys spilopterus*, *Stellifer brasiliensis*, *S. rastrifer*, *Menticirrhus americanus*, *Paralonchurus brasiliensis* and *Diplectrum radiale* from the inner shelf of Ubatuba and estuarine system of Cananéia, southeastern Brazil, using histological, stereological and image processing techniques. To achieve this objective and test the hypothesis, ten specific objectives have been addressed:

1. Describe the oocyte development and ovary organisation (chapters I, II and III).

2. Analysis the oocyte size-frequency distribution (chapters I, II and III).

3. Estimate the size at which the oocytes are recruited to vitellogenesis (chapter I).

4. Estimate the batch and the relative batch fecundity (chapter I, II and III).

5. Verify the number of potential batches present in the ovaries (chapters I III and IV).

6. Investigate the influence of the ovary weight, fish size and weight on the batch fecundity (chapters I and III).

7. Investigate the influence of the fish size, weight and the body condition (gonadosomatic index, stomach repletion index, condition factor and hepatosomatic index) on the relative batch fecundity (chapter II).

8. Analysis the seasonal variation in oocyte diameter (chapter III).

9. Analysis the occurrence of the ovary phases during ovarian maturation (chapter III).

10. Investigate the oocyte production and recruitment patterns in two congeneric species inhabiting different ecosystems (chapter IV).

RESULTS

Chapter I

Reproductive strategy and fecundity of six fish species from the southwest Atlantic Ocean: An application of image process techniques

3.1 Abstract

The aims of this study was investigate, for the first time, the reproductive strategy of six marine fish species using histology and advanced image processing techniques, as well to estimate their fecundity and the number of potential batches present in the ovaries. Specimens of *Anchoa filifera*, *Cetengraulis edentulus*, *Citharichthys spilopterus*, *Stellifer brasiliensis*, *S. rastrifer* and *Menticirrhus americanus* were captured bimonthly, from June (2012) to May (2013), in the coast of São Paulo State, southeastern Brazil. The mature ovaries were removed, weighed, fixed in formalin and carried out the histological preparations. All histological sections were photographed and the images analysed using free software to assess the aspects of the reproductive strategy and fecundity. The results revealed that all species showed asynchronous oocyte development with a continuous size-frequency distribution showing no gap between pre-vitellogenic and vitellogenic oocytes. Differences in the size at which the oocytes are recruited to vitellogenesis were observed among species and regions comparing ovaries in developing phase. The total and relative number of oocytes in the most development stages (NDO-RNDO) ranged from 422 to 157,361 oocytes and from 60 to 2,974 oocytes g^{-1} (female ovary-free body weight), respectively. A significant relationship of the NDO with the variables ovary weight, total length and total weight was also recorded. On the other hand, batch fecundity ranged from 1,225 oocytes in *A. filifera* to 93,649 oocytes in *M. americanus*. The lowest value of relative batch fecundity was registered for *C. spilopterus* (110 oocytes g^{-1}) and the highest for *S. rastrifer* (677 oocytes g^{-1}). In general, the approximation number of potential batches that will be released in the current reproductive cycle ranged from one to four batches.

Key words: Reproduction. Oocyte development. Oocyte recruitment. Indeterminate fecundity. Histology. Stereology. Marine fishes.

3.2 Introduction

Studies on marine fishes captured as bycatch are scarce worldwide mainly due to their low economic value. Lately, however, it has been indicated the interest of moving from focusing only on the species with economic importance to understand how the ecosystem and stocks of other fish species in the same region interact as stated by the concept of ecosystem-based fishery management (EBFM) (GELPKE et al., 2013). As such, studies on reproductive strategy of fishes, both target and non-target, generate important knowledge on the population resiliency to exploitation by fisheries or perturbation caused by human activities, providing valuable advice for fisheries management (MORGAN, 2008; MORGAN et al. 2009).

An accurate evaluation of the ovary development is crucial to describe the maturation process, timing of reproduction and oocyte recruitment patterns (MURUA; SABORIDO-REY, 2003). Thus, oocyte development, ovarian organization, oocyte size-frequency distribution and intensity of oocyte atresia have been stated as the main goals in comprehending the fish reproductive strategy, being essential to assess the fecundity type (i.e. indeterminate or determinate fecundity regulation) of any fish species (MURUA et al., 2003; PLAZA et al., 2007; GRANDE et al., 2012; ALONSO-FERNÁNDEZ et al., 2013; GARCÍA-SEOANE et al., 2014). The results from these studies are very important and determine the type of method of choice for estimating fecundity and the egg production method for the assessment of the spawning stock biomass (ARMSTRONG; WITTHAMES, 2012; GANIAS, 2013), as well for understanding fish phenology in a climate changing world (SORIA et al. 2008; MIRANDA et al., 2009; STRÜSSMANN et al., 2010). Moreover, fecundity can also be used as a reference point for management fisheries at sustainable levels; however this information comes from a relatively small number of species, the majority with a commercial importance (HUNTER et al., 1992, TYLER; SUMPTER, 1996).

The teleosts *Anchoa filifera* (Fowler, 1915) (Engraulidae), *Cetengraulis edentulus* (Cuvier, 1829) (Engraulidae), *Citharichthys spilopterus* Günther, 1862 (Paralichthyidae), and the sciaenids *Stellifer brasiliensis* (Schultz, 1945), *S. rastrifer* (Jordan, 1889), and *Menticirrhus americanus* (Linnaeus, 1758) are found inhabiting coastal and estuarine environments in the central western and southern Atlantic

ocean (CARPENTER, 2002a, 2002b). These species are commonly captured as bycatch by shrimp fishery trawl in the southeastern and southern coast of Brazil (VIANNA et al., 2004; VIANNA; ALMEIDA, 2005; BRANCO; VERANI, 2006; GOMES; CHAVES, 2006); however, they have little importance as a fishery resource (MPA, 2011). Moreover, they also have an important ecological role participating in the functional structure of the coastal ecosystems.

Marine fishes exhibit wide heterogeneity in reproductive strategies and associated traits such as fecundity, time of spawning, etc. (HELFMAN et al., 1997; MURUA; SABORIDO-REY, 2003). Nevertheless, there are much more data available on other life history parameters of the fishes than on reproduction and fecundity (TOMKIEWICZ et al., 2003). In this context, the aims of the present study was to investigate, for the first time, the reproductive strategy of six marine fish species captured in inner shelf of southeastern of Brazil using histology and advanced image processing techniques, as well to estimate their fecundity and the number of potential batches present in the ovary. The reproductive strategy was studied in relation to the following aspects: (1) oocyte development and ovary organisation, (2) oocyte size-frequency distribution and (3) size at which the oocytes are recruited to vitellogenesis.

3.3 Materials and Methods

3.3.1 Areas of study and sampling strategy

The areas of study are located in the inner shelf of Ubatuba and in the estuarine system of Cananéia, São Paulo State, southeastern Brazil (Figure 1). The fishes were captured bimonthly, from June (2012) to May (2013), during research cruises using an otter trawl. To capture females in spawning condition, trawls were carried out twice per day (between 08:00–10:30 am and 13:30–17:00 pm) on three consecutive days in each sampled month. Each station was trawled for a 15 min at depths that ranged from 1.9 m in the estuary to 20.6 m in Ubatuba. After trawling, the specimens were immediately taken to the laboratory to measure their total length (to the nearest millimetre), and weight (to the nearest gram, 0.01 g), identify the maturity phase by macroscopic examination, and remove their ovaries. Then, the ovaries were weighed and fixed in 10% formalin buffered with sodium phosphate monobasic

($NaH_2PO_4•H_2O$) and dibasic ($Na_2H_2PO_4$) to ensure the adequate preservation of the oocytes (HUNTER, 1985).

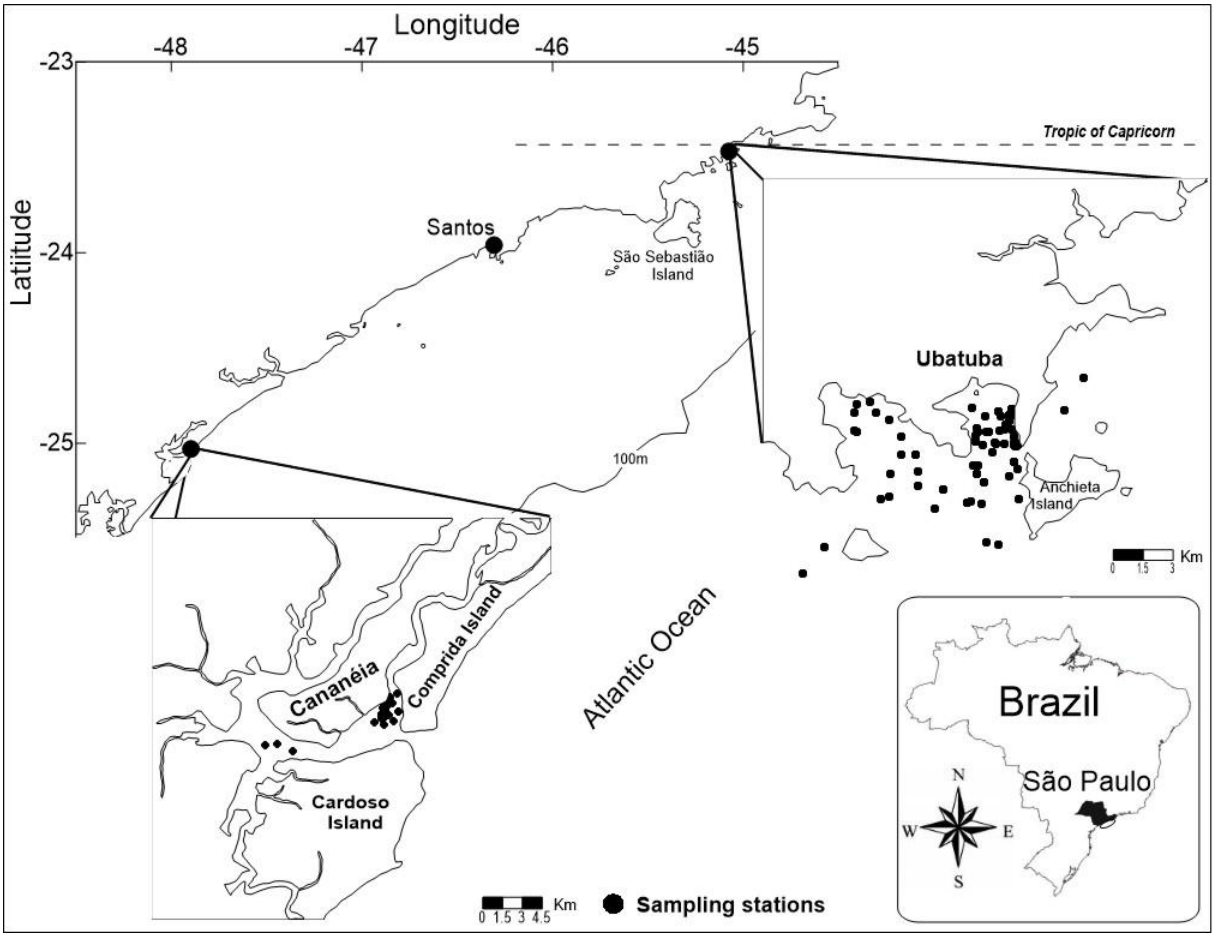


Figure 1. Areas of study showing the sampled stations.

3.3.2 Histological analysis

All histological preparations were carried out on three sub-samples from the right ovary (anterior, middle and posterior portion), containing the whole cross-section (covering the thickness of the ovarian wall). For doing histology, the tissue were dehydrated in alcohol series, cleared in xylol, embedded in paraffin blocks, sectioned at 3 µm (*A. filifera* and *C. edentulus*) and 5 µm (*C. spilopterus*, *M. americanus*, *S. brasiliensis* and *S. rastrifer*) and stained with Harris' hematoxylin and Puttis' eosin. Periodic Acid-Schiff's, counterstained with Hematoxylin and metanil yellow (PAS-MY), was also performed for correct identification of cortical alveolus (GARCÍA-SEOANE et al., 2014).

3.3.3 Oocyte and ovarian classification

The oocyte stages were classified based on the histological criteria established by Wallace and Selman (1981) and Tyler and Sumpter (1996). However, oocytes in primary growth were classified as early and late primary growth (Pg1 and Pg2) and those oocytes in vitellogenic growth (Vtg) were divided in four development stages (Vtg1, Vtg2, Vtg3 and Vtg4). On the other hand, the ovarian phases were classified based on Brown-Peterson et al. (2011). In this study were used only females being capable of spawning within the current reproductive cycle owing to advanced oocyte development (BROWN-PETERSON et al., 2011). The descriptions of each oocyte stage and phases are detailed in the results.

3.3.4 Oocyte size-frequency distribution

At least 50 oocytes per development stages, from one to three females with similar size, were selected to measure the individual oocyte diameter and to estimate the size at which the oocytes are recruited to vitellogenesis (ORS). Thus, the number of oocytes counted was based on the work published by Aragón et al. (2010) which observed that the oocyte size distribution became stable after 40 measurements. The oocyte diameters were calculated by the arithmetic mean of the longest (L) and shortest (S) diameter measured in the histological sections using the free software ImageJ (RASBAND, 1997-2009). Then, the oocyte diameters were grouped in classes of 40 µm and plotted to analyse the size frequency distribution.

3.3.5 Fecundity

The number of developing oocytes (NDO) was estimated stereometrically and defined as the standing stock of the advanced yolked oocytes present in the ovaries in spawning capable phase containing Vtg3 or Vtg4 oocytes (MURUA; SABORIDO-REY 2003), without considering the atretic oocytes. The relative number of developing oocytes (RNDO) was also calculated dividing the absolute NDO value by the female ovary free weight. NDO was estimated by applying model-based methodology described in Emerson et al. (1990) and Murua et al. (2003).

The total number of the most advanced vitellogenic oocytes (NDOn) was estimated following the equation proposed by Weibel et al. (1966):

$$NDO = Ov \times \frac{\mathrm{K}}{\beta} \times \frac{N_a^{3/2}}{V_i^{1/2}}$$

where Ov = ovary volume (cm³); K = size distribution coefficient; *β* = is a shape coefficient; N_a = number of advanced vitellogenic oocytes (LVO) transected per unit area and V_i = volume fraction occupied by LVO in the histological section.

The ovary volume was calculated applying the methodology proposed by Scherle (1970). K was estimated by following the equations proposed by Williams (1977):

$\mathrm{K} = \left[\frac{M_3}{M_1}\right]^{3/2}$ where M_1 is the mean oocyte diameter, i.e. $M1 = [(D1 + D2 + Dn)/n]$ and M_3 is the third moment about the mean of oocyte distribution, i.e. $M_3 = \left[\frac{(D_1)^3 + (D_2)^3 + (D_n)^3}{n}\right]^{1/3}$; $OD_{\text{ind.}}$ is the individual oocyte diameter calculated as described previously and n the number of LVO oocytes counted. However, *β* was calculated by the ratio between the longest and shortest axis of each oocyte transected (COWARD; BROMAGE, 2002). A Weibel grid (WEIBEL et al., 1966) with 256 test point and an area of 0.050 cm² was utilised to calculate N_a and V_i (Figure 2). Images from the non-overlapped area in the same histological section were taken. The number of images taken varied with the ovary volume and ranged from four in *A. filifera* and *C. spilopterus* to 22 in *M. americanus*. Then, a routine developed by Haslob et al. (2013) to overlay histological images with a grid was applied using the software ImageJ version 1.44r (Figure 2) (RASBAND, 1997-2009)..

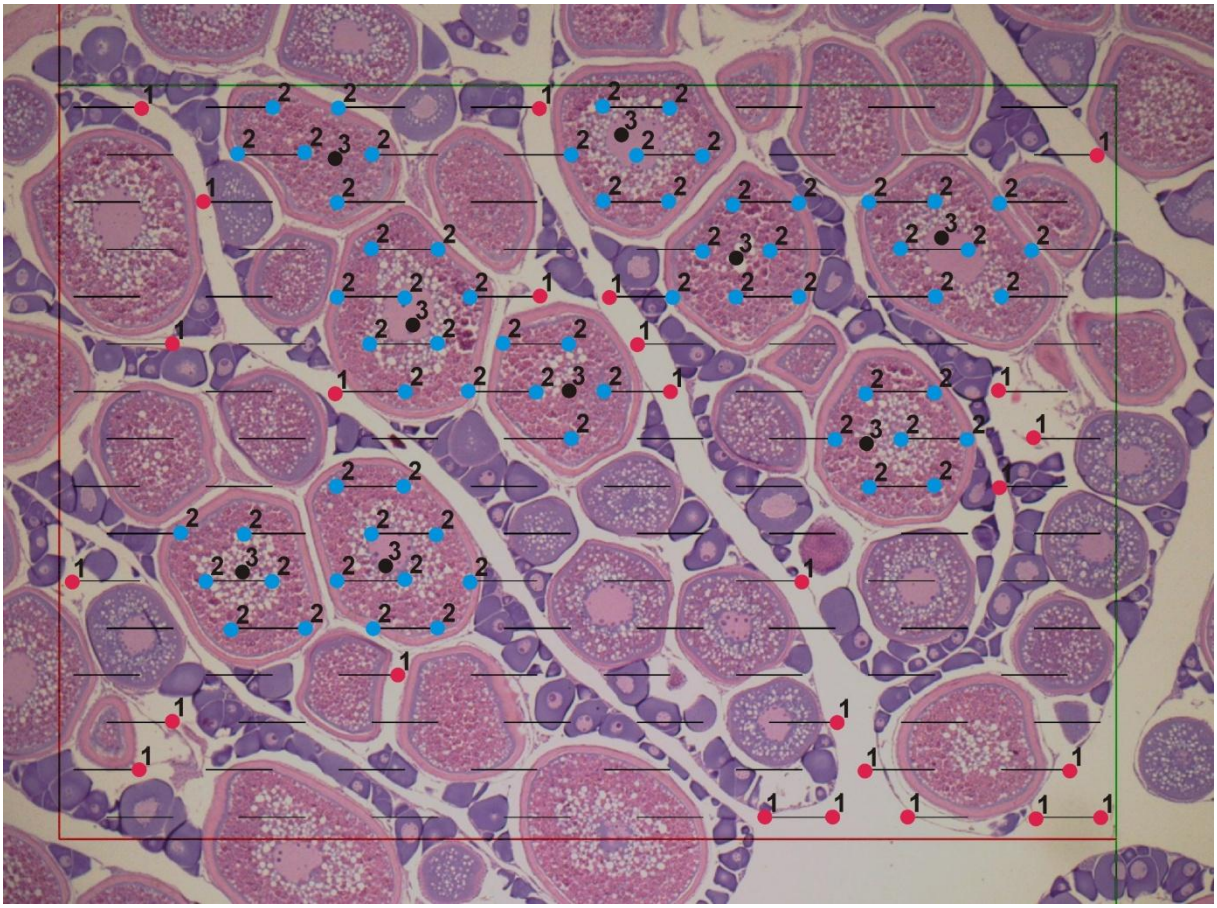


Figure 2. Illustrative image after ImageJ application showing a Weibel grid overlaid on a histological section with the test point. There are 128 bars in this grid and the end of each bar represents a test point (n = 256). N_a was calculated dividing the number of oocytes/ profiles in Vtg3 stage (3) by the frame area, i.e. N_a = 9/ 0.05 = 180 profiles/ cm², whereas V_i was calculated dividing the number of points laid on Vtg3 (2) by the total points subtracted by negative grid (1), i.e. V_i = 63/ (256-26) = 0.274. Only oocytes overlapping the green borders (upper and right borders) of the grid were included in the analyses.

Batch fecundity (BF) defined as the total number of oocytes released per batch was estimated by counting the number of oocytes in germinal vesicle migration (GVM) or in hydration (Hyd). Only ovaries with no sign of spawning (post-ovulatory follicle) were used to estimate BF. Thus, three subsamples ranging from 10 to 525 mg each from the anterior, middle and posterior position of the left ovary were taken and weighed to the nearest 0.0001 g. To separate the oocytes from the connective tissues, each sub-sample was sieved (300–500 µm mesh sieve) and sprayed with high pressure water using a pipette filled with distillated water. Then, the oocytes

were stained with Rose Bengal, placed in a Petri dish and photographed with a digital camera. All GVM/Hyd oocytes present in each image were counted and measured using the automated method proposed by Ganias et al. (2010). Finally, BF was estimated as the weighted mean density of the three subsamples multiplied by the ovary total weight. The relative BF (RBF) was also calculated dividing the absolute BF value by the female ovary free weight.

The number of potential batches (NPB) within the ovaries that will be released in the current reproductive cycle was estimated by the ratio NDO/BF and defined as the number of batches that will be recruited to the final maturation (GVM/ Hydration). Thus, NPB was estimated dividing the mean NDO by the mean BF in a given female size class (GARCÍA-SEOANE et al., 2014). NDO and BF vary with the female total length and due to a low number of ovaries with oocytes in GVM/ Hyd stages showing no sign of spawning; the females were grouped into class intervals of 20 mm.

3.3.6 Data analysis

The tests of Kolmogorov-Smirnov and Levene were applied prior any statistical test application to verify the normality distribution and homogeneity of variances, respectively (ZAR, 1999). Thus, linear and exponential regressions were performed to analyse the relationship among the oocyte stages and their diameters by species, as well as between their ovary volume (Ov) and ovary weight (Ow). However, the correlation between NDO and the female parameters (Ow, TL and TW) was evaluated through application of the Spearman's Rank Correlation test (r_S).

Logistic regression models were fitted to calculate the probability at which the oocytes are recruited to vitellogenesis (ORS). All regressions were performed using bias-reduction GLM with binomial family and "logit" canonical link as link function (KOSMIDIS, 2007). Then, the oocytes by development stage were inserted as binary response variable (pre-vitellogenic oocyte= 0 and vitellogenic oocyte= 1) and their diameters as predicted variable. The logistic regression model followed:

$$P(Y = 1|OD) = \frac{1}{1 + e^{\beta_0 + \beta_1 * OD}}$$

where P(Y= 1|OD) is the probability of the oocyte being in vitellogenesis at size OD, β_0 is the intercept and β_1 the slope, respectively. ORS was estimated dividing $-\beta_0$ by β_1. The method of bootstrapping with 1000 interactions was applied to place 95% confidence intervals around ORS (MANLY, 1991). Then, the ORS values were compared among species and ovarian phases analyzing visually the overlap of the confidence intervals. All analyses were performed at level of 0.01 or 0.05 when appropriate using the free software R (R DEVELOPMENT CORE TEAM, 2009).

3.4 Results

3.4.1 Oocyte development and ovarian organisation

In general, the analyses of histological sections of the ovaries of all species suggested ten oocyte development stages, as follow: early primary growth (Pg1) and late primary growth (Pg2) as oocytes in pre-vitellogenesis; cortical alveolar (CA) as earliest sign of oocyte maturation, and primary vitellogenesis (Vtg1), secondary vitellogenesis (Vtg2), tertiary vitellogenesis (Vtg3) and quaternary vitellogenesis (Vtg4) as oocytes in vitellogenesis; and germinal vesicle migration (GVM), late germinal vesicle migration (LGVM) and Hydration (Hyd) as final maturation (Figure 3). The presence of cortical alveolus was also observed at the periphery of the oocyte cytoplasm in Vtg1 and Vtg2 in all species. Moreover, CA stage in *A. filifera* and *C. edentulus* had already started the vitellogenesis and for this reason, this stage was not considered separated in these species. The descriptions of each oocyte development stage are given in table 1.

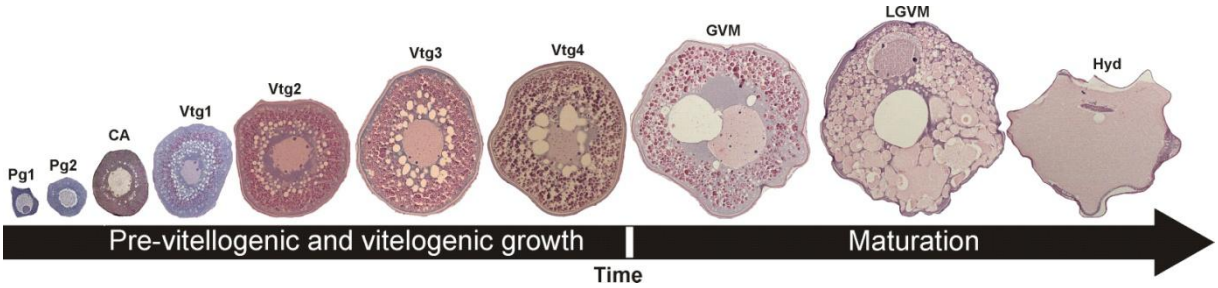


Figure 3. General oocyte development progression based on the histological description of six species of fishes captured in inner shelf of Ubatuba and estuarine system of Cananéia, São Paulo state, southeastern Brazil. Pg1, Pg2= early and late primary growth; CA= cortical alveolar; Vtg1, Vtg2, Vtg3, Vtg4= primary, secondary,

tertiary and quaternary vitellogenesis; GVM= germinal vesicle migration; LGVM= late germinal vesicle migration; Hyd= hydration.

The classification of the ovaries into ovarian phases was carried out based on the presence of the most advanced oocytes types such as Vtg 3-4, GVM-LGVM, Hyd and post-ovulatory follicle (POF) and following Brown-Peterson et al. 2010. Thus, four ovarian phases were described, as follow: spawning capable, spawning capable subphase with germinal vesicle migration (GVM), spawning capable subphase with hydrated oocytes (hydration) and spawning capable with POFs. The following oocyte stages Pg1, Pg2, CA, Vtg1, Vtg2 and Vtg3 were observed in all ovarian phases with exception of CA in the species of the family Engraulidae species. Females in spawning capable and spawning capable with POFs showed oocytes in vitellogenic growth as the most advanced stage, being Vtg3 and Vtg4 in the former and Vtg3 in the later phase. The presence of the POFs in the spawning capable phase with POFs indicated that the females have already spawned at least one time in the current reproductive cycle. Ovaries with oocytes in GVM and LGVM were ready to spawn in few hours and were classified as spawning capable subphase with GVM. The other spawning capable subphase was characterised by the presence of hydrated oocytes (hydration) which indicated that the females were developmentally and physiologically able to spawn. Figure 4 shows the ovarian development phases according to the different oocyte development stages.

All sampled species showed ovaries in spawning capable, spawning capable subphase with GVM and spawning capable with POFs. However, hydrated females were only registered for *S. brasiliensis* and *M. americanus*.

Table 1. General histological description of the oocyte development stages for six fish species captured in the inner shelf of Ubatuba and in the estuarine system of Cananéia, southeastern Brazil. Between brackets are given the mean of oocyte diameter ± standard deviation and the total number of oocyte measured (n).

Oocyte development stages	Histological description
Pre-vitellogenic oocyte growth	
Early primary growth (Pg1)	Unshaped and unyolked oocytes with a basophilic cytoplasm stained with Haematoxylin and nucleus enlarged showing nucleolus arranged at the periphery with at least one nucleoli larger than others. (86.1 µm ± 19.6, n=1641).
Late primary growth (Pg2)	Regular shape in comparison with Pg1, unyolked oocytes with a basophilic cytoplasm stained with Haematoxylin and nucleus showing nucleolus arranged at the periphery. Lipid inclusions can be observed in some species. (120.6 µm ± 29.4, n=1209).
Cortical alveolar (CA)	Small oil droplets and cortical alveolus begin to accumulate dispersed in the cytoplasm. The zona radiata, theca and granulosa cells become visible from this stage, however, yolk granules are still absent. (143.2 µm ± 31.9, n=1073).
Vitellogenic oocyte growth	
Primary vitellogenesis (Vtg1)	The eosinophilic protein granules start to fill the cytoplasm. Small oil droplets are more abundant and cortical alveolus can be visible arranged at the periphery of the oocyte. (207.4 µm ± 42.5, n=1495).
Secondary vitellogenesis (Vtg2)	The protein granules are arranged as a ring in the cytoplasm and oil droplets start to arrange around the nucleus. (275.9 µm ± 49.2, n=1150).
Tertiary vitellogenesis (Vtg3)	Yolk granules increase in number and fill the cytoplasm. The oil droplets increase in size and are distributed around the nucleus.(368.4 µm ± 60.6, n=1417).
Quaternary vitellogenesis (Vtg4)	The cytoplasm is filled with yolk granules and large oil globules are arranged around the nucleus. (356.1 µm ± 41.2, n=147).
Oocyte maturation	
Germinal vesicle migration (GVM)	The nucleus (germinal vesicle) starts to migrate toward the animal pole at the periphery of the oocyte, yolk granules and oil droplets start to coalesce and rapidly increase in size. An oil globule larger than others is apparent in this stage.(429.1 µm ± 58.2, n=306).
Late germinal vesicle migration (LGVM)	GVM with yolk coalescence. Moreover, an oil globule and the nucleus are visible in this stage. (473.7 µm ± 58.2, n=119).
Hydration (Hyd)	Oocyte enlarges rapidly due to intake of water and become translucent. The cytoplasm become homogeneous which difficult the identification of the cell structures. Due to histological process, the oocytes appear misshapen and stained lightly with eosin. (704.2 µm ± 53.3, n=752).

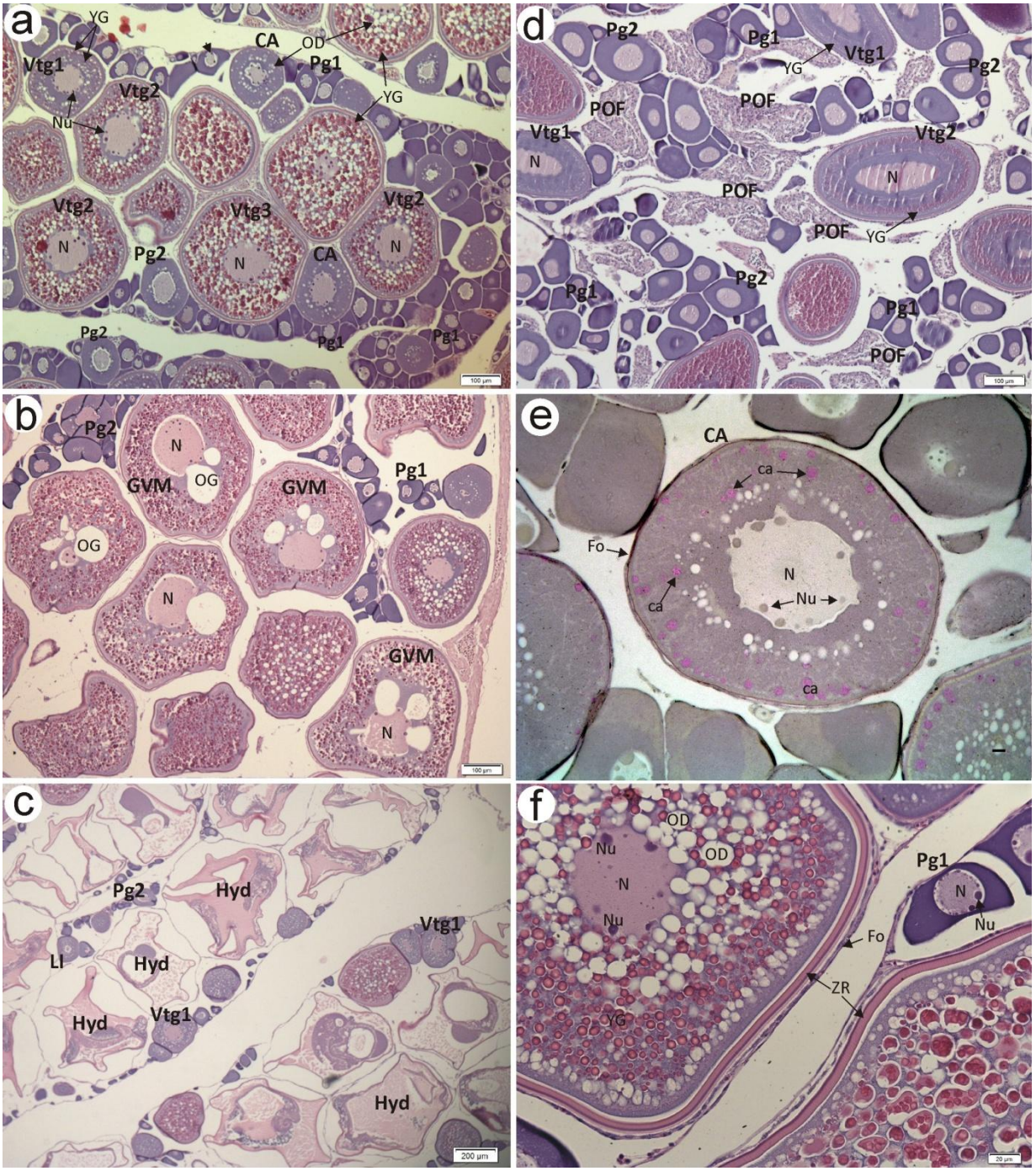

a
YG
Vtg1
Vtg2
Nu
CA
OD
Pg1
YG
Vtg2
Vtg3
Vtg2
N
Pg2
N
CA
N
Pg2
Pg1
Pg1
d
Pg2
Pg1
YG
Vtg1
Pg2
POF
POF
Vtg1
Vtg2
N
N
POF
POF
YG
Pg2
Pg1
Pg1
POF
b
Pg2
N
GVM
OG
GVM
Pg1
OG
N
GVM
N
e
CA
ca
Fo
N
ca
Nu
ca
c
Pg2
Hyd
Vtg1
LI
Hyd
Hyd
Vtg1
Hyd
f
OD
Nu
OD
N
Pg1
N
Nu
Nu
Fo
ZR
YG

Figure 4. Photomicrography of the histological sections of fishes captured in the inner shelf of Ubatuba and estuarine system of Cananéia, São Paulo state, southeastern Brazil, showing different ovarian phases and oocyte development stages. (a) Spawning capable- *Stellifer rastrifer* (Pg1-Pg2= early and late primary growth; CA= cortical alveolar; Vtg1-Vtg2-Vtg3= early, secondary and tertiary vitellogenic stages; (b) spawning capable subphase with germinal vesicle migration (GVM)- *S. brasiliensis*; (c) spawning capable subphase with hydrated oocytes (Hyd)- *Menticirrhus americanus*; (d) spawning capable with post-ovulatory follicles (POF)- *Cetengraulis edentulus*; (e) CA stage showing cortical alveolus (ca); (f) Follicle layer (Fo) and zona radiata (ZR). N = nucleus, nu = nucleoli; YG = yolk granules; OG = oil globules; OD = Oil droplets.

3.4.2 Oocyte size-frequency distribution

The oocyte diameter measured ranged from 34.4 µm in *A. filifera* to 827.2 µm in *S. brasiliensis*. The relationship between the oocyte stages and their sizes was described by linear and exponential regressions, showing a significant increase in oocyte size along with the oocyte development in spawning capable ovaries. The exponential relationship registered for *C. edentulus*, *C. spilopterus*, *S. brasiliensis*, *S. rastrifer* and *M. americanus* also indicated a major yolk accumulation increasing drastically the oocyte sizes during the latest stages of vitellogenic growth. However, a linear relationship found in *A. filifera* could indicate that the pre-vitellogenic and vitellogenic oocytes have a similar growth rate (Figure 5).

The oocyte size distribution estimated for females in spawning capable phase showed a continuous diameter progressing with no gap between pre-vitellogenic and vitellogenic oocytes. Moreover, these analysis also showed that at least one cohort of the most advanced oocyte stage (Vtg3 and Vtg4), develop from Vtg2 increasing their diameters to form the batch to be spawned. However, the oocytes that will form the final batch in *A. filifera* are clearly recruited from Vtg1 (Figures 6 and 7).

3.4.3 Size at which the oocytes are recruited to vitellogenesis (OSR)

Differences in the size at which the oocytes are recruited to vitellogenesis (ORS) were observed among species and regions when compared ovaries in different developing phase. Thus, the highest ORS value (205.8 µm) was estimated for specimens of *M. americanus* captured in Ubatuba and the smallest one (146.1 µm) for *C. spilopterus* from Cananéia estuary. On the other hand, no significant differences were found between the engraulids species and between *S. brasiliensis* and *S. rastrifer*. In addition, the ORS result for *M. americanus* indicated that oocytes from ovaries sampled in Ubatuba are recruited to vitellogenesis in larger size than those from Cananéia (Table 2).

Comparing ORS among all ovarian phases by species; in *A. filifera*, ORS in spawning capable was significantly larger than those in spawning capable subphase with GVM and spawning capable with POFs. A similar tendency was also observed in *C. edentulus* from Cananéia. *S. brasiliensis* captured in Ubatuba and *S. rastrifer* from Cananéia revealed a similar oocyte recruitment pattern, with a large ORS in

spawning capable with POF and a smaller size in ovaries with GVM; although there was no significant difference among phases in the latter species. The oocytes from *M. americanus* in spawning capable, spawning capable subphase with GVM and spawning capable with POFs are recruited to vitellogenesis in a similar size in Cananéia; however, a significant difference was registered in specimens in hydration. Concerning *M. americanus* captured in Ubatuba, the ORS estimated in spawning capable phase departed significantly from those in spawning capable subphase with GVM and those in hydration.

Photomicrography of the histological sections showing oocytes in different stages in developing phase, oocyte size frequency distribution and the logistic curves of oocyte maturation (ORS) are given in the figures 6 and 7.

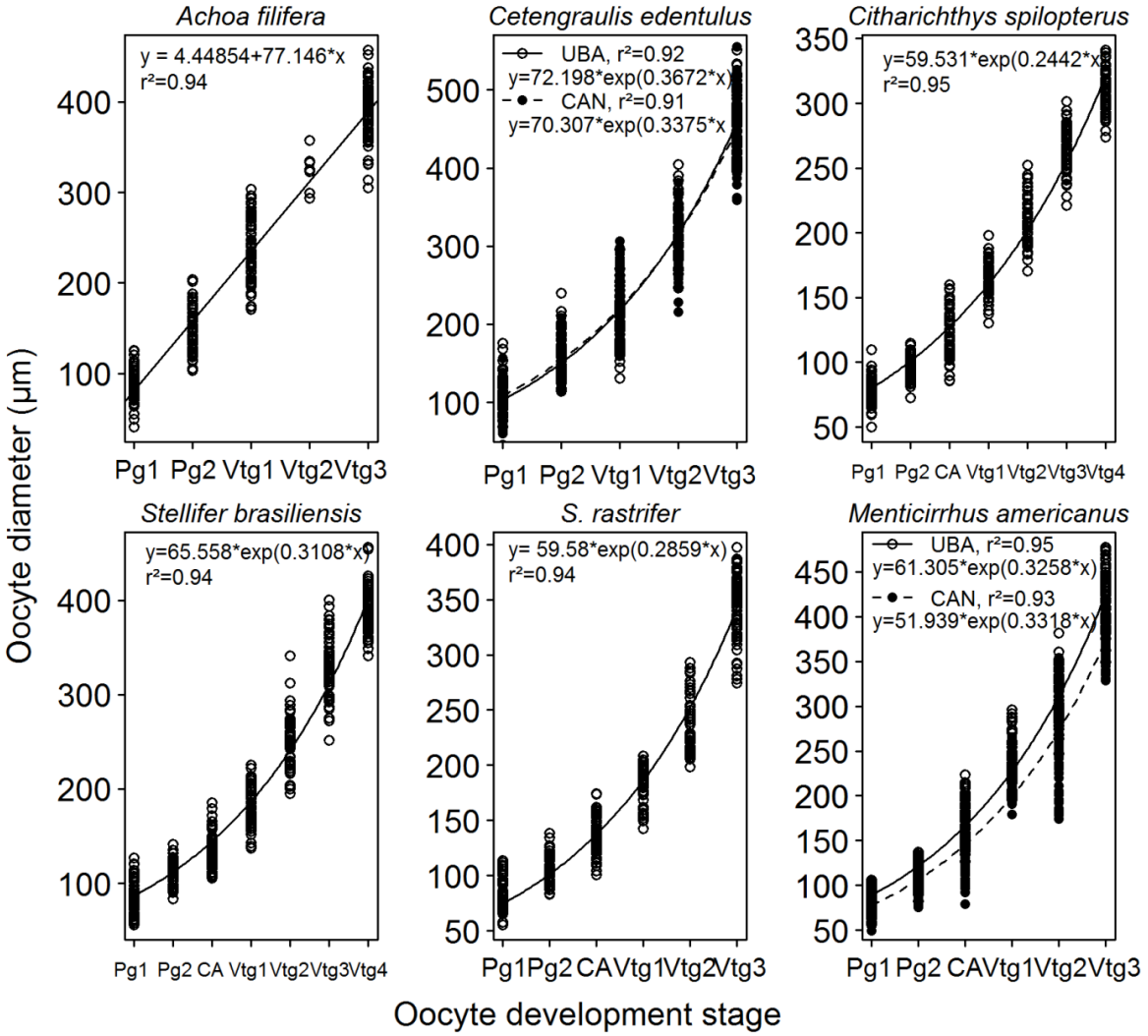


Figure 5. Relationship between oocyte diameter and oocyte stages in ovaries in spawning capable phase for six species in the inner shelf of Ubatuba and estuarine system of Cananéia, São Paulo State, southeastern Brazil. Early primary growth (Pg1), late primary growth (Pg2), cortical alveolar (CA), primary vitellogenesis (Vtg1), secondary vitellogenesis (Vtg2), tertiary vitellogenesis (Vtg3), quaternary vitellogenesis (Vtg4).

Table 2. Estimation of the equation parameters and oocyte recruitment size (ORS) by ovary phase for all species captured. n: total number of oocytes measured, β_0: intercept, β_1: slope, *C.I.*: 95% lower and 95% upper confidence interval. Different letters indicate significant differences in ORS among species in spawning capable phase. The values of ORS in bold indicate significant differences between regions for a same species in relation to each ovary phase.

Species	Ovary phase	Ubatuba							Cananéia						
		n	$ORS_{\mu m}$ (*C.I.*)		β_0(*C.I.*)		β_1 (*C.I.*)		*n*	$ORS_{\mu m}$ (*C.I.*)		β_0(*C.I.*)		β_1 (*C.I.*)	
Anchoa filifera	SpCa	268	194.4[ab]	(184.47 — 205.68)	-9.57	(-15.54 — - 7.39)	0.05	(0.04 — 0.08)							
	SpCa (GVM)	284	164.0	(156.45 — 173.66)	-22.10	(-21.58 — - 7.47)	0.13	(0.04 — 0.13)							
	SpCa (POF)	294	164.0	(160.26 — 169.73)	-22.10	(-44.06 — - 15.23)	0.13	(0.09 — 0.27)							
Cetengraulis edentulus	SpCa	289	181.0[b]	(171.43 — 190.63)	-9.62	(-13.04 — - 7.83)	0.05	(0.04 — 0.07)	282	191.6	(183.72 — 198.99)	-16.28	(-24.18 — - 13.25)	0.08	(0.07 — 0.12)
	SpCa (GVM)	382	137.7	(133.74 — 141.69)	-18.42	(-25.66 — - 14.99)	0.13	(0.11 — 0.19)							
	SpCa (POF)	291	173.1	(166.34 — 180.36)	-14.42	(-21.24 — - 11.38)	0.08	(0.06 — 0.12)	312	173.1	(166.33 — 180.78)	-14.42	(-21.37 — - 11.64)	0.08	(0.07 — 0.13)
Citharichthys spilopterus	SpCa								427	146.1[d]	(141.67 — 150.41)	-22.32	(-32.02 — - 18.16)	0.15	(0.13 — 0.22)
	SpCa (GVM)								352	138.8	(134.70 — 143.38)	-21.82	(-38.12 — - 16.54)	0.16	(0.12 — 0.27)
	SpCa (POF)								400	133.0	(128.38 — 137.82)	-14.08	(-23.42 — - 10.55)	0.11	(0.08 — 0.17)
Stellifer brasiliensis	SpCa	385	158.4[c]	(152.09 — 165.43)	-14.71	(-20.99 — - 12.02)	0.09	(0.08 — 0.13)							
	SpCa (GVM)	403	147.7	(142.76 — 152.88)	-14.96	(-23.34 — - 11.70)	0.10	(0.08 — 0.16)							
	SpCa (Hyd)	316	171.1	(163.44 — 179.73)	-11.29	(-15.11 — - 9.55)	0.07	(0.06 — 0.16)							
	SpCa (POF)	316	182.9	(177.20 — 188.42)	-20.77	(-39.36 — - 15.69)	0.11	(0.06 — 0.21)							
S. rastrifer	SpCa								368	159.2[c]	(153.63 — 163.90)	-21.75	(-31.12 — - 18.01)	0.14	(0.11 — 0.20)
	SpCa (GVM)								311	156.2	(146.58 — 168.37)	-12.01	(-17.89 — - 9.39)	0.08	(0.06 — 0.12)
	SpCa (POF)								394	165.5	(159.57 — 171.29)	-16.32	(-20.83 — - 13.93)	0.10	(0.08 — 0.13)
Menticirrhus americanus	SpCa	375	**205.8**[a]	(200.56 — 211.23)	-27.20	(-43.07 — - 21.01)	0.13	(0.10 — 0.21)	376	**185.4**	(177.79 — 193.17)	-17.12	(-28.02 — - 13.73)	0.09	(0.07 — 0.15)
	SpCa (GVM)	345	**234.2**	(224.19 — 246.44)	-15.40	(-34.65 — - 11.07)	0.07	(0.05 — 0.15)	392	**176.9**	(169.42 — 185.09)	-18.84	(-43.12 — - 14.37)	0.11	(0.08 — 0.25)
	SpCa (Hyd)								336	220.9	(211.97 — 229.82)	-14.37	(-22.41 — - 11.32)	0.07	(0.05 — 0.10)
	SpCa (POF)	345	**221.4**	(213.02 — 229.49)	-16.43	(-32.87 — - 12.43)	0.07	(0.06 — 0.14)	342	**184.2**	(177.09 — 191.54)	-16.24	(-24.46 — - 13.12)	0.09	(0.07 — 0.13)

SpCa= Spawning capable females showing oocytes in vtg3/vtg4 as the most advanced vitellogenic stages;

SpCa (GVM)= Spawning capable females with oocytes in germinal vesicle migration;

SpCa (Hyd)= Spawning capable females with hydrated oocytes;

SpCa (POF)= Spawning capable females with post-ovulatory follicles.

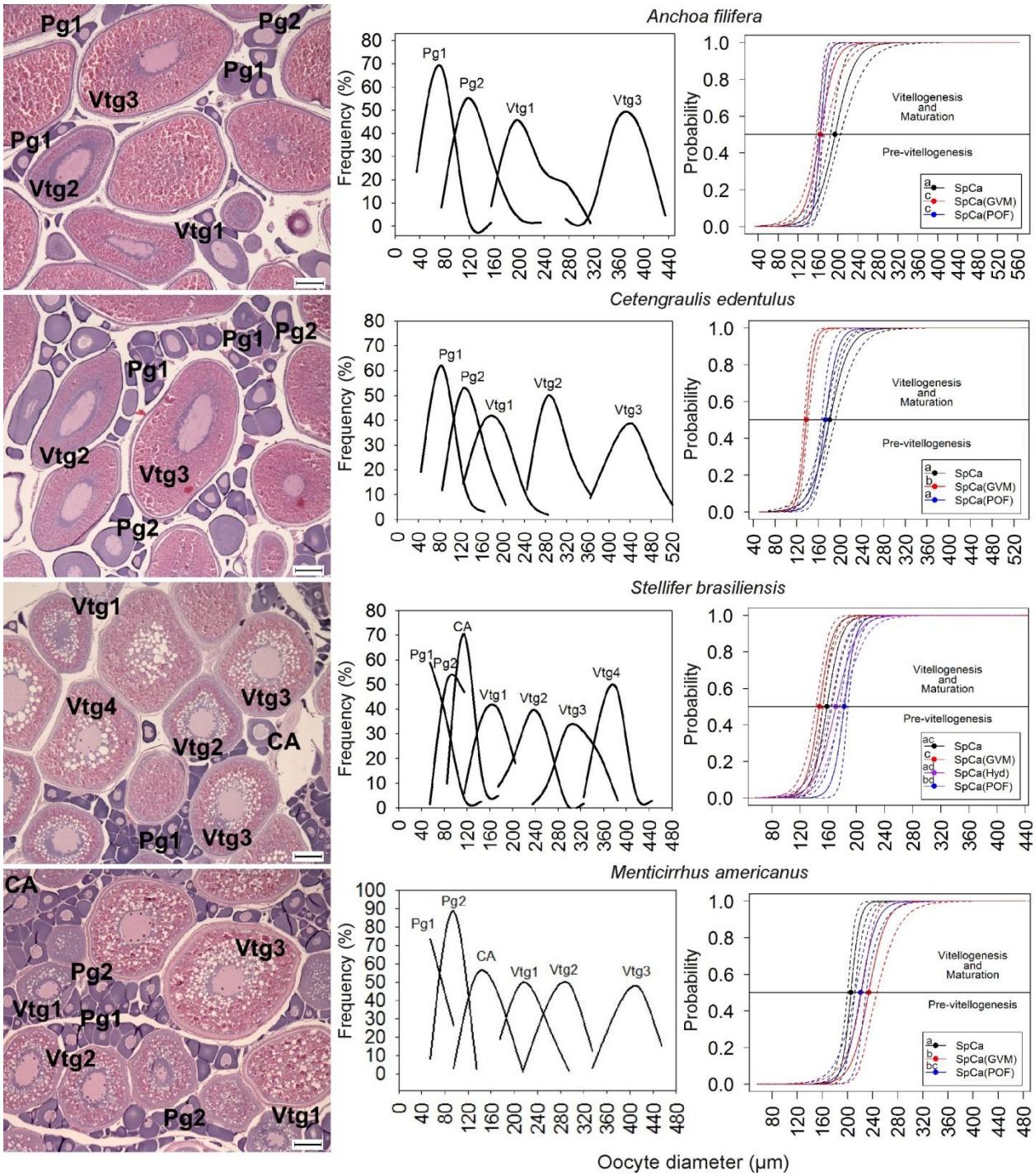

Anchoa filifera
Cetengraulis edentulus
Stellifer brasiliensis
Menticirrhus americanus
Frequency (%)
Probability
Vitellogenesis and Maturation
Pre-vitellogenesis
SpCa
SpCa(GVM)
SpCa(Hyd)
SpCa(POF)
Oocyte diameter (µm)

Figure 6. Photomicrography of the histological section and oocyte size frequency distribution for ovaries in spawning capable phase (SpCa), and logistic curves of the oocyte maturation for all species sampled in the inner shelf of Ubatuba. Different letters indicate significant differences among oocyte recruitment sizes (circles) in relation to ovary phase. Early primary growth (Pg1), late primary growth (Pg2), cortical alveolar (CA), primary vitellogenesis (Vtg1), secondary vitellogenesis (Vtg2), tertiary vitellogenesis (Vtg3), quaternary vitellogenesis (Vtg4), germinal vesicle migration (GVM), hydration (Hyd). Different letters indicate significant differences. Scale bar= 100 µm.

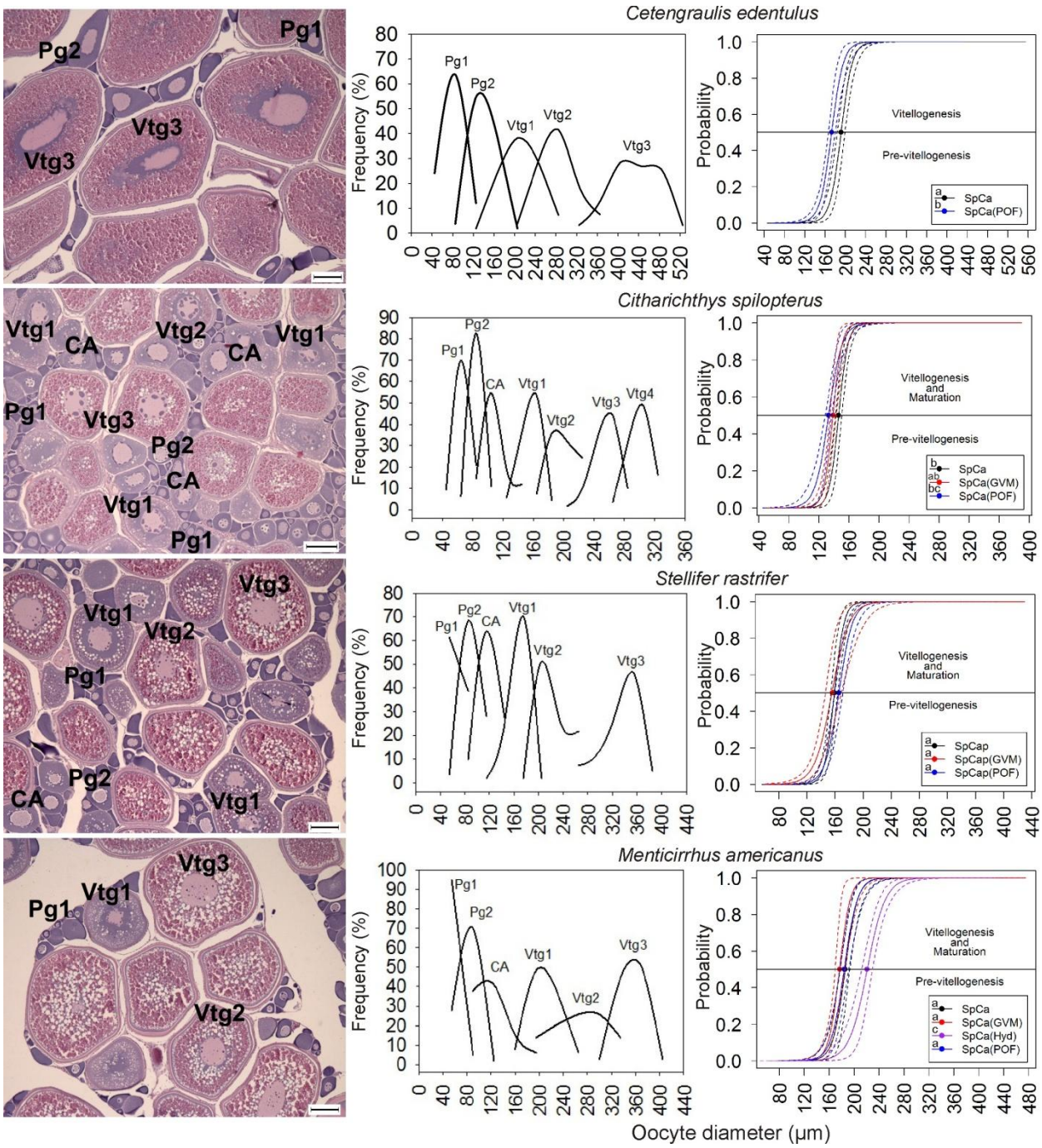

Cetengraulis edentulus
Citharichthys spilopterus
Stellifer rastrifer
Menticirrhus americanus
Frequency (%)
Probability
Vitellogenesis
Pre-vitellogenesis
Vitellogenesis and Maturation
SpCa
SpCa(POF)
SpCa(GVM)
SpCap
SpCap(GVM)
SpCap(POF)
SpCa(Hyd)
Oocyte diameter (μm)
Pg1
Pg2
CA
Vtg1
Vtg2
Vtg3
Vtg4

Figure 7. Photomicrography of the histological section and oocyte size frequency distribution for ovaries in spawning capable phase (SpCa), and logistic curves of the oocyte maturation for all species sampled in the estuarine system of Cananéia. Different letters indicate significant differences among oocyte recruitment sizes (circles) in relation to ovary phase. Early primary growth (Pg1), late primary growth (Pg2), cortical alveolar (CA), primary vitellogenesis (Vtg1), secondary vitellogenesis (Vtg2), tertiary vitellogenesis (Vtg3), quaternary vitellogenesis (Vtg4) germinal vesicle migration (GVM), hydration (Hyd). Different letters indicate significant differences. Scale bar= 100 µm.

3.4.4 Fecundity

The relationship between the ovary weight and ovary volume for the six species were well described by linear regression models ($p<0.05$), estimated with the intercept forced through the origin. In general, the r^2 ranged from 0.95 in *A. filifera* to 0.99 in *S. brasiliensis*, *S. rastrifer* and *M. americanus*. The number of oocytes in Vtg3 and Vtg4 used to estimate the coefficient of oocyte size distribution (K) and oocyte shape (β) ranged from 58 to 133 oocytes. The minimum and maximum values for K were 0.98 and 104 whereas, for β were 1.16 and 1.81, respectively (Table 3).

Table 3. Linear equations calculated to estimate the ovary volume (Ov), coefficient of oocyte size distribution (K) and oocyte shape coefficient (β) for the six species captured in the the inner shelf of Ubatuba and the estuarine system of Cananéia, southeastern Brazil. Ow: ovary weight; r^2: coefficient of determination; n_1: total number of ovaries used to estimate the volume which include ovaries no analysed histologically; n_2: total number of oocytes measured used to estimate K and β.

Species	n_1	Equations	r^2	n_1	K	β
Anchoa filifera	35	Ov= 1.0783×Ow	0.95	65	1.01	1.76
Cetengraulis edentulus	26	Ov= 0.88×Ow	0.98	133	1.01	1.81
Citharichthys spilopterus	64	Ov= 0.9713×Ow	0.97	58	0.99	1.23
Stellifer brasiliensis	66	Ov= 1.0695×Ow	0.99	68	1.02	1.19
S. rastrifer	31	Ov= 1.0848×Ow	0.99	82	0.98	1.16
Menticirrhus americanus	35	Ov= 1.0975×Ow	0.99	133	1.04	1.27

Due to small number of females in spawning capable phase, species with specimens captured in both regions were combined to estimate NDO, as follow: *C. edentulus* (n_{Ubatuba}= 12, $n_{\text{Cananéia}}$= 7) and *M. americanus* (n_{Ubatuba}= 5, $n_{\text{Cananéia}}$= 13). Thus, NDO and RNDO ranged from 422 to 157,361 oocytes and from 62 to 3,178 oocytes g^{-1} (female ovary free weight), respectively. The lowest value of NDO was registered in *A. filifera* and the highest in *M. americanus*. The lowest value of RNDO was also observed in *A. filifera*, but the highest in *C. spilopterus*. Spearman's Rank correlation test indicated a significant relationship of the NDO with the variables ovary weight (Ow), total length (TL) and total weight (TW). However, the ovary weight

explained better NDO variation than others variables as indicated by r_s values and the confidence intervals (Figures 8-9). The equations that describe the relationship between NDO and Ow and descriptive statistics of the NDO, RNDO, TL and TW for females sampled are summarized in table 4.

The batch fecundity (BF) ranged from 1,225 to 93,649 germinal vesicle migration/hydrated oocytes, with lowest and largest values for *A. filifera* and *M. americanus*, respectively. The lowest value of relative batch fecundity (RBF) was estimated for *C. spilopterus* (110 oocytes g^{-1}) and the highest for *S. rastrifer* (677 oocytes g^{-1}) (Table 5).

The number of potential batches (NPB) that will be spawned during the current spawning season, evaluated by the ratio NDO/BF, revealed an estimation of one batch in *A. filifera*, *C. edentulus* and *M. americanus*, and between two and four batches in *C. spilopterus*. On the other hand, two-three potential batches were registered within the ovaries of *S. brasiliensis* and *S. rastrifer* (Table 5).

Table 4. Ovary weight (Ow) relationship equations, number of developing oocytes (NDO) and descriptive statistics for NDO, relative NDO (RNDO) and female parameters (total length and total weight) of six species captured in inner shelf of Ubatuba and the estuarine system of Cananéia, southeastern Brazil. n: total number of ovaries analysed, r_S: Spearman's Rank Correlation value.

Species	*n*	Linear equation	r_s	NDO		RNDO		Total length (mm)		Total weight (g)	
				mean ± s.d.	min-max	mean ± s.d.	min-max	mean ± s.d.	min-max	mean ± s.d.	min-max
Anchoa filifera	19	*NDO* = 5356.4* *OW* - 288.8	0.80	1445 ± 857	422-3531	176 ± 87	60-370	101.5 ± 4.9	94-112	7.9 ± 1.2	6.1-10.7
Cetengraulis edentulus	19	*NDO* = 351.3* *OW*+ 265.2	0.80	5487 ± 2197	2702-9632	152 ± 46	82-263	151.7 ± 12	125-172	36.0 ± 9	20.9-58.5
Citharichthys spilopterus	24	*NDO* = 34431* *OW* - 2806	0.95	35571 ± 21267	7019-91417	1205 ± 607	227-2974	145.3 ± 14.1	114-169	29.2 ± 8.3	13.9-44.9
Stellifer brasiliensis	14	*NDO* = 18801* *OW*+ 257.1	0.98	40505 ± 22198	2067-81441	885 ± 376	65-1369	155.4 ± 11	139-176	43.9 ± 11.3	30.0-69.7
S. rastrifer	29	*NDO* = 26468* *OW* - 6829	0.97	24523 ± 17611	4970-65687	825 ± 441	251-1744	129.7 ± 11.7	110-154	28.2 ± 8.4	16.4-44.6
Menticirrhus americanus	23	*NDO* = 8969.5* *OW* - 1025.8	0.94	49683 ± 43746	8094-157361	484 ± 264	115-1058	228.2 ± 43.3	153-325	143.1 ± 79.9	37.1-385

[1] Given as the number of oocytes per female ovary free weight (g^{-1})

Table 5. Number of potential batches within the ovaries according to the female total length (TL), batch fecundity (BF) and relative batch fecundity (RBF- number of oocytes per female ovary free weight) for six marine fish species captured in the captured in the inner shelf of Ubatuba and the estuarine system of Cananéia, southeastern Brazil. The number of females in spawning capable and germinal vesicle migration/ hydration is given between brackets, respectively. BF and RBF mean ± standard deviation and their ranges are also given.

TL (mm)	*Anchoa filifera*	*Centengraulis edentulus*	*Citharichthys spilopterus*	*Stellifer brasiliensis*	*Stellifer rastrifer*	*Menticirrhus americanus*
94 ⊢114	1 (15-4)					
114 ⊢ 134			2 (1-2)	2 (0-5)	2 (19-1)	
134 ⊢ 154		1 (9-1)		2 (6-5)	3 (6-2)	
154 ⊢ 174		1 (7-1)	4 (7-2)	3 (4-12)		
174 ⊢ 194				3 (2-2)		
214 ⊢ 234						1 (2-1)
234 ⊢ 253						1 (4-1)
274 ⊢ 294						1 (1-1)
BF	1644 ± 653	9608 ± 2008	11119 ± 3919	12379 ± 6015	14094 ± 11085	58884 ± 32720
(oocytes)	1225 - 2606	8189 - 11028	6009 - 15559	2136 - 25171	6926 - 26862	28691 - 93649
RBF	215 ± 83.2	255 ± 45	319 ± 154	306 ± 89	444 ± 203	385 ± 115
(oocytes g^{-1})	152 - 333	223 - 287	110 - 460	150 - 497	304 - 677	256 - 475

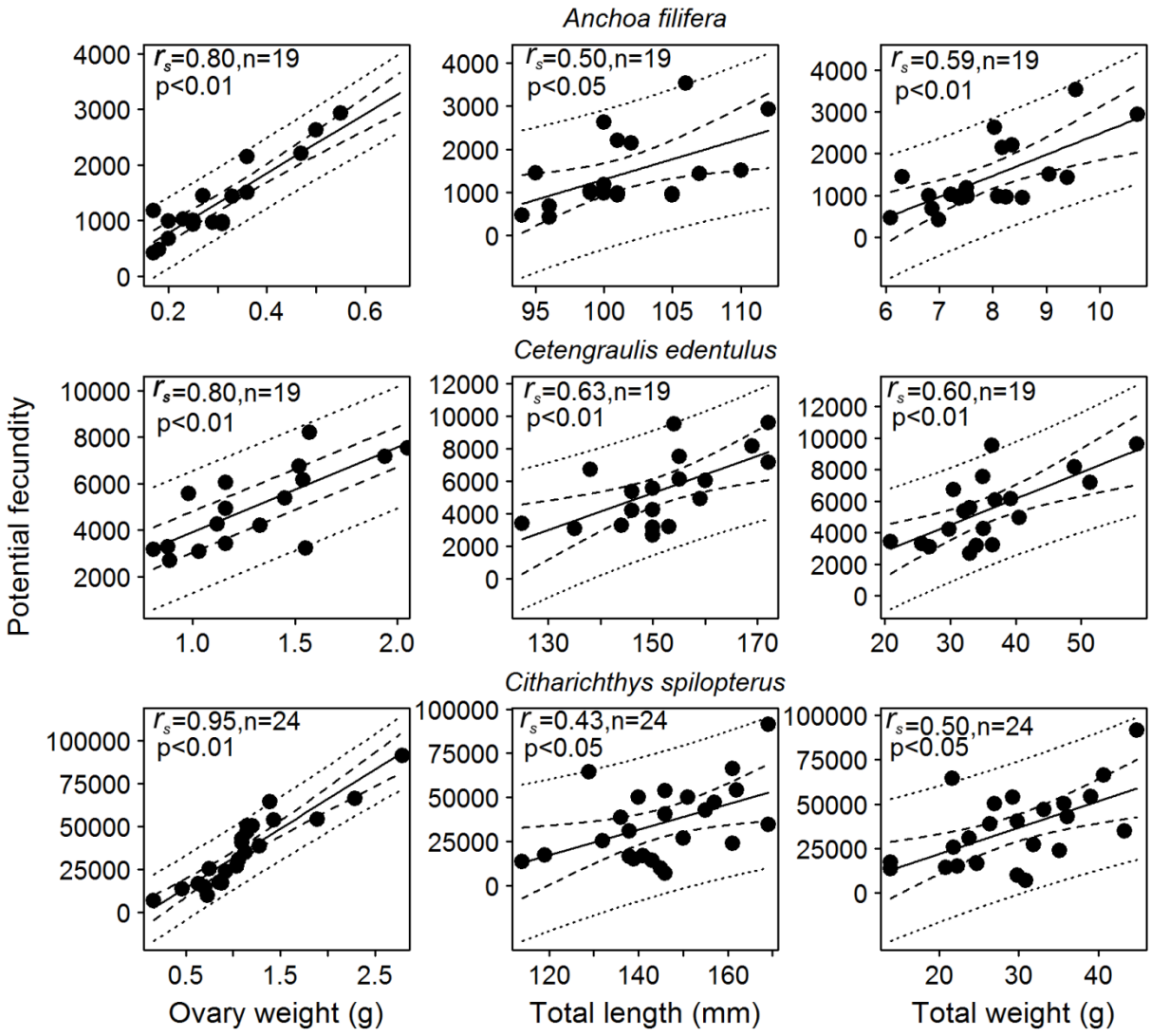


Figure 8. Relationship between the number of developing oocytes (NDO) and female parameters (ovary weight, total length and total weight) for *Anchoa filifera*, *Cetengraulis edentulus* and *Citharichthys spilopterus*. 95% confidence interval (---), 95% prediction interval (···), p-value, number of ovaries sampled (n) and Spearman's Rank Correlation (r_S) are also given.

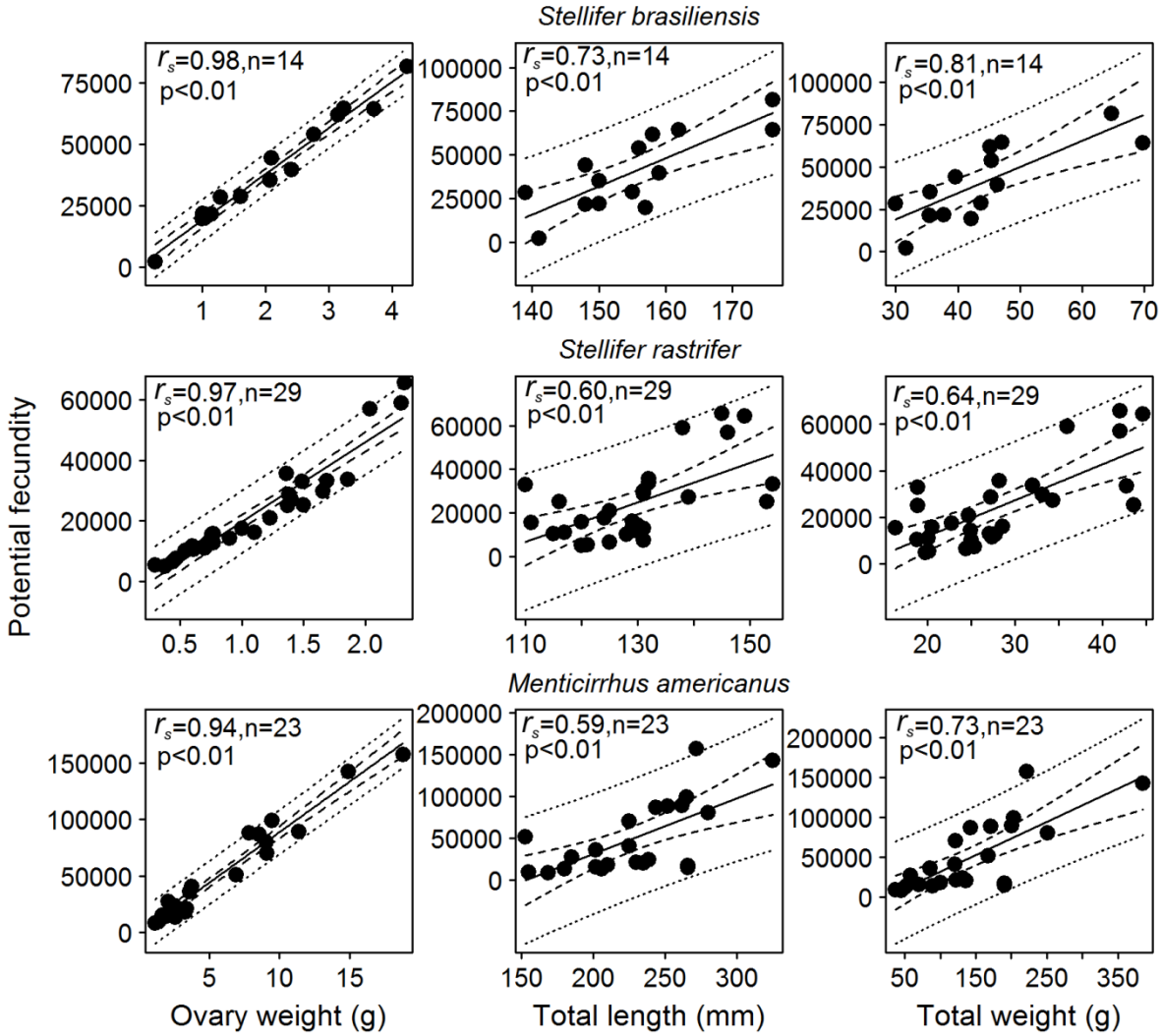


Figure 9. Relationship between the number of developing oocytes (NDO) and female parameters (ovary weight, total length and total weight) for *Stellifer brasiliensis*, *S. rastrifer* and *Menticirrhus americanus*. 95% confidence interval (---), 95% prediction interval (···), p-value, number of ovaries sampled (n) and Spearman's Rank Correlation (r_S) are also given

3.5 Discussion

In the present study, the reproductive strategy of six marine fish species was examined and their fecundity estimated using histology and advanced image processing techniques. This kind of study is essential for understanding fish population dynamics and generates critical information for designing and implementing effective fisheries management strategies (LOWERRE-BARBIERI, 2009; BROWN-PETERSON et al., 2011).

3.5.1 Oocyte development and ovarian organisation

A comprehensive description and knowledge of the oocyte history is necessary to understand the reproductive strategy of the fish species (KORTA, 2010). In general, the ovaries of the all six species revealed a pattern of oocyte development stage commonly found in other teleosts, such as: primary growth, cortical alveolar, vitellogenesis and final maturation with germinal vesicle migration and hydration (WALLACE; SELMAN 1981; WEST 1990; TYLER; SUMPTER 1996; MURUA; SABORIDO-REY 2003). However, variability in oocyte development among fishes may be occurring due to their great reproductive diversity (LUBZENS et al. 2010). Thus, the absence of cortical alveolar stage in *A. filifera* and *C. edentulus* may indicate that this stage has a short period of time, followed by a rapid vitello accumulation, or the cortical alveolus follows a different development pattern in these species.

All females studied also have a simultaneous presence of oocytes in several development stages without dominant population, independent of their ovarian phase. Such result reveals a continuous type of oogenesis with an asynchronous ovarian organisation (TYLER; SUMPTER, 1996; MURUA; SABORIDO-REY, 2003). This type of ovary organisation is typical of batch spawning species with a very protracted spawning season, usually in those species from low latitudes, and represents a strategy for investment of larger number of smaller eggs when resources are patchy on a relatively large spatial scale (OVEN, 1976; KJESBU, 2009, PAVLOV, et al., 2009). Similar results have been recorded for other species in reproductive studies carried out by Grande et al. (2012), Zudaire et al. (2013a) and García-Seoane et al. (2014).

3.5.2 Oocyte size-frequency distribution

Assessing the type of fecundity regulation is necessary to choice the correct method for fecundity estimation. The analysis of the oocyte size-frequency distribution has been widely applied for investigating the fecundity type of many fishes based on the presence/ absence of a hiatus between pre-vitellogenic and vitellogenic modes (GRANDE et al., 2012; GANIAS, 2013, ZUDAIRE et al., 2013a). According to Hunter et al. (1992), fecundity in batch spawners may be assigned as

indeterminate or determinate depending on the strategy of oocyte recruitment into vitellogenesis. Thus, the standing stock of pre-vitellogenic oocytes can develop and be recruited into yolked oocyte stock any time in indeterminate fishes (HUNTER; GOLDBERG, 1980; KJESBU, 2009). On the other hand, in those fishes with determinate fecundity, the potential fecundity is fixed prior to the onset of spawning, i.e. the standing stock of yolked oocyte is not replaced during the spawning season (HUNTER et al., 1992; MURUA, SABORIDO-REY, 2003). Therefore, the oocyte size-frequency distributions with several modes and a clear overlap suggest an indeterminate fecundity for all species studied.

Tropical and subtropical fishes have a faster oocyte development rate than those inhabiting coldwater regions. Thus, many batch spawners from warmwater habitats exhibit continuous oocyte recruitment, repeatedly recruiting oocytes from pre-vitellogenic to vitellogenic growth (LOWERRE-BARBIERI et al., 2011b). The linear and exponential relationships between the oocyte diameter and their respective stage must be related with the oocyte recruitment pattern in these species. The increase in oocyte size during the oocyte maturation occurs mainly due to the inclusion of vitellogenin, resulting in formation of yolk granules and increasing the oocyte size (WALLACE; SELMAN, 1981; TYLER; SUMPTER, 1996; PATIÑO; SULLIVAN, 2002). However, this relationship can also indicates that the oocytes are continuous recruited to form the next group of development stage in the current reproductive cycle; corroborating with the results discussed previously (ovary organisation and oocyte-size distribution).

3.5.3 Size at which the oocyte are recruited to vitellogenesis (ORS)

Oocyte growth in teleosts is regulated mainly by the environmental events and hormonal mechanisms (WALLACE; SELMAN, 1981). Moreover, the oocyte maturation rate may vary among several species, even in specimens of the same phylogenetic group (MASUI; CLARKE, 1979). However, the results of the present study revealed that the oocytes, measured in spawning capable females, begin the vitellogenesis (ORS) with a similar size in *A. filifera* and *C. edentulus* and in *S. brasiliensis* and *S. rastrifer* even captured in distinct environments. The rate at which these oocytes are recruited to vitellogenesis was not evaluated in the present study,

and such similarity must be related with phylogenetic class of these species or other factor not investigated in this study.

In multiple spawning fishes, the egg size vary significantly among batches, and it usually decreases as spawning proceeds (DeMARTINI, 1991; KJESBU et al., 1992; HSIAO et al., 1994). Murua and Motos (2006) and Zudaire et al. (2013a) also observed this variation in yolked oocyte size as spawning season proceeds in *Merluccius merluccius* and *Thunnus albacores*, respectively. These authors stated that such decrease in oocyte size is related with the fecundity type of these species, being an evidence of indeterminate fecundity; this due to replenishment of newly formed oocytes to the standing stock of the advanced vitellogenic oocytes (HUNTER et al., 1992). Thus, differences in ORS-values among phases by species may be considered as evidence for indeterminate fecundity, indicating that the size at which the oocytes begin the vitellogenesis and are recruited to form the new development group is ovary-phase dependent and it is related with the time of spawning in these species. This idea is supported by the tendency of females in spawning capable phase show the largest ORS-values, i.e. females that have not been spawned in the current reproductive cycle yet, while those in spawning capable phase in GVM and with POFs has lower values of ORS indicating that recruitment of pre-vitellogenic oocytes is occurring.

The use of the ORS analysis in spawning capable females would provide an object basis for comparison of oocyte maturation in relation to species, population and habitat. However, the influence of some factors such as female length, age, condition, time of spawning and environmental factors must be investigated.

3.5.4 Fecundity

Stereometry is a powerful procedure for quantifying particles in three-dimensional structures from two-dimensional sections (Aragón et al., 2010). Thus, stereological methods have been widely applied in fishery science as an accurate tool for estimating the number of vitellogenic oocytes/ fecundity from ovarian histological preparations (COWARD; BROMAGE, 2002; MEDINA et al., 2007). Model-based stereology was the stereological procedure adopted, in the present study, for quantifying the number of developing oocytes in the most advance stage

(NDO) within the ovaries. This method takes into account the oocyte shape (*β*) and size distribution (K) (EMERSON et al., 1990). Both coefficients depend on the oocyte development stage: *β*-values have been ranged from 1.00 to 1.46 and K from 0.85 to 1.56 (EMERSON et al., 1990; COWARD; BROMAGE, 2002; MEDINA et al., 2002; ARAGÓN et al., 2010; HASLOB et al., 2013). In general, the values of *β* and K found in the present study lie within the range reported previously. However, the highest values of *β* found in *A. filifera* and *C. edentulus* can be attributed to the ellipsoidal shape of engraulid oocytes. Model-based stereology has been proved to be a valuable tool for estimating fish fecundity and it has been applied in other species with indeterminate fecundity, such as *Sardinella brasiliensis* (ISAAC-NAHUM et al., 1988), *Thunnus thynnus* (MEDINA et al., 2002), *Merluccius merluccius* (DOMÍNGUEZ-PETIT, 2007) and *Sprattus sprattus balticus* (HASLOB et al., 2013).

Fecundity is usually proportional to fish size, weight and condition, being the key parameters to assess fecundity at the population level (HUNTER; GOLDBERG, 1980; MURUA et al., 2003). According to Boehlert et al. (1982), both linear and potential models may be used to examine fecundity-length relationships. Thus, NDO estimated for all species was linearly related to ovary weight, total length and total weight. Although all the relationships were significant, the ovary weight explained most of the NDO variability (80-98%), indicating that ovary weight and gonadossomatic index (GSI) in these species could be applied as an indicator of reproductive condition. Similar pattern has been reported by Domínguez-Petit (2007) for *Merluccius merluccius*, Fernandez-Arcaya et al. (2012) for *Trachyrincus scabrous* and Fernandez-Arcaya et al. (2013) for *Nezuma aequalis*.

On the other hand, estimating fish fecundity using intermediate modes of oocyte development stage, i.e. the standing stock of yolked oocytes, have been used to estimate batch fecundity (BF) in indeterminate fishes; however, the usefulness of this estimation needs to be investigates species specifically and the results, interpreted with caution because oocytes are continuously recruited to form the next mode. Thus, the total number of vitellogenic oocytes estimated must not represent the number of hydrated oocytes that will be spawned in the next batch (HUNTER; GOLDBERG, 1980; LOWERRE-BARBIERI et al., 2011b). For this reason, only oocytes in the most advanced development stages were quantified and then,

estimated the approximate number of potential batches (NPB) within the ovaries diving NDO by BF. Thus, examining NPB in *A. filifera*, *C. edentulus* and *M. americanus* was observed that NDO was very close to BF. These results indicate that NDO could be used as an indicator of BF; however the number of oocytes lost by atresia must be taken into account.

NDO estimated for *A. filifera* (422 to 3,531 oocytes) in this study was close to other species of the same genus inhabiting tropical waters; such as *A. naso* (990 to 4,409 oocytes) (JOSEPH, 1963) and *A. parva* (450 to 2,250 oocytes) (OSORIO; ACERO, 1996). Moreover, the mean number of hydrated oocytes spawning per batch was also similar to found by Bassista and Hartman (2005) for *A. mitchilli* (between 1,233 and 1,508 hydrated oocytes) in the Hudson River estuary. However, *A. filifera* revealed that both RNDO and RBF were lower than observed for *A. mitchilli* (506 oocytes g^{-1}). On the other hand, the NDO estimated for *C. edentulus* from Cananéia estuary departed from that reported by Peterson (1961) for *C. mysticetus* (9,810 to 59,090 oocytes and relative fecundity of 863 oocytes g^{-1}) from the Gulf of Panama. However, differences between the NDO values for the engraulids of the present study and those reported previously, must be also attributed to that fact that these authors estimated NDO by counting the total number of yolked oocytes within the ovaries, and in the present study were counted only those oocytes in the most development stage detected by histological examination.

BF and RBF for *C. spilopterus* from Cananéia estuary was lower than that reported by Dias et al. (2005) for the population of Mamanguá inlet, Rio de Janeiro, Brazil. BF and RBF estimated by these authors ranged from 7,000 to 61,000 oocytes and from 253 to 1,552 oocytes g^{-1}, respectively. Although the low number of GVM/ Hydrated females sampled in both studies, such information are the unique available for *C. spilopterus* up to date.

Considering the NDO was close to BF in *M. americanus*, in general, the RBF among *M. americanus*, *S. brasiliensis* and *S. rastrifer* were similar, ranging from 306 to 484 oocytes g^{-1}. Militelli et al. (2013) studying the reproductive biology of the sciaenids in the Río de la Plata and Buenos Aires Coastal zone, reported that the species *Micropogonias furnieri*, *Cynoscion guatucupa*, *Macrodon ancylodon*, *Umbrina canosai*, *P. brasiliensis* and *M. americanus* revealed a similar RBF with a

mean value ranging between 150 and 200 hydrated oocytes per ovary-free body weight. Thus, the highest values of RBF fecundity found in the present study could be attributed to the low number of GVM/hydrated female sampled, condition of the females, sampled period and the differences in the oocyte sizes between the distinct sampled areas, once the mean diameter of the hydrated oocytes measured by Militelli et al. (2013) were larger than measured in this study. Moreover, fecundity in teleosts, as well the final number of developing oocytes, could be affected by food availability, female condition, sampled year, female size and environmental conditions (LAMBERT et al., 2003; MURUA; MOTOS, 2006, DOMÍNGUEZ-PETIT; SABORIDO-REY, 2010).

In conclusion, the species *Anchoa filifera*, *Cetengraulis edentulus*, *Citharichthys spilopterus*, *Stellifer brasiliensis*, *S. rastrifer* and *Menticirrhus americanus* have indeterminate fecundity with asynchronous ovarian organisation. The size at which the oocytes are recruited into vitellogenesis differs among species, indicating differences in oocyte recruitment strategies. The total number of developing oocytes (NDO) in the ovaries will generates from one (*A. filifera*, *C. edentulus* and *M. americanus*) to four (*C. spilopterus*) batches of matured oocytes. Small females with low ovary weight produce low NDO, which could affect the total number of oocytes released in the spawning season. Therefore, the knowledge generated by this study contributes to a better understanding of the reproduction of species inhabiting different ecosystems.

Chapter II

Reproductive traits of the simultaneous hermaphrodite *Diplectrum radiale* (Quoy & Gaimard, 1824) (Teleostei, Serranidae)

4.1 Abstract

The present study generates, for the first time, information on the reproductive traits of the simultaneous hermaphrodite *Diplectrum radiale* captured in the inner shelf and in an estuarine system of southeastern Brazil. The specimens were captured bimonthly, from June (2012) to May (2013), using an otter trawl. The mature ovotestes were removed, preserved in formalin and examined using histological techniques. The results revealed an indeterminate fecundity with an asynchronous ovarian organisation. The entire spermatogenesis occurs within the cysts and the spermatogonial distribution indicated an unrestricted testicular type. The testicular and ovarian tissues were separated from each other by different ducts, indicating no possibility of internal self-fertilisation. The hydrated oocytes were stored in the accessory reproductive structure for at least 24h. The presence of atretic hydrated oocytes within this structure and the positive PAS reaction of the villae-like projections revealed the function of the ovary sinus absorbing unspawned oocytes. The relative batch fecundity ranged (RBF) from 20 to 82 oocytes g^{-1} (51 ± 16) for specimens from the coastal, and from 67 to 113 oocytes g^{-1} (90 ± 19) for those sampled in the estuary. The RBF was negatively affected by the fish size, weight and condition, revealing that smaller specimens with a better condition produce more hydrated oocytes per gram of body.

Key words: Reproductive strategy. Ovarian development. Fecundity. Brazil.

4.2 Introduction

Fish exhibit a diversity of sexual patterns and the majority has separated sexes (gonochoristic). The hermaphrodite fishes function both as male and female, and the mature testiticular and ovarian tissues may occur either simultaneously or sequentially in the same individual (SADOVY; LIU, 2008). Simultaneous hermaphrodite species are uncommon, and the Serranidae is one of the four shallow water families (Muraenidae, Rivulidae and Gobiidae) in which is found this sexual pattern (HELFMAN et al., 2009).

The serranids of the genus *Diplectrum* are simultaneous hermaphrodites inhabiting tropical and subtropical ecosystems (BORTONE, 1977b). The main characteristic of this genus is the presence of an accessory reproductive structure (ovarian sinus) whose function is to store hydrated oocytes. This structure has been reported for *D. rostrum*, *D. pacificum*, *D. bivittatum* and *D. formosum* (BORTONE, 1974, 1977a, 1977b; TOUART; BORTONE, 1980; BUBLEY; PASHUK, 2010). The species *D. radiale* is found inhabiting coastal and estuarine areas of eastern and northeast coasts of South America in depths up to 60 m, may reach 250 mm in total length and feeds on crustaceans and small fish, playing an important role in the structure of the ecosystems (BOTONE, 1977b; CARPENTER, 2002a). Moreover, this species is also important for artisanal fisheries as food resource because of the good quality of the flesh; the specimens are usually captured with hook-and-line, traps and as a bycatch by shrimp trawlers (CERVIGÓN et al., 1993).

Knowledge on the reproductive traits of the fishes such as ovary development, oocyte recruitment and fecundity are essentials for understanding their reproductive strategy (HUNTER et al., 1992; MURUA; SABORIDO-REY, 2003; MURUA; MOTOS, 2006). The results from these studies are widely applied in fisheries science for estimation of the spawning stock biomass, for understanding the resilience of fish population to fishing and fish phenology in a climate changing world, as well as an important component to marine reserve design (MORGAN, 2008; PANKHURST; KING, 2010; STRÜSSMANN et al., 2010; ARMSTRONG; WITTHAMES, 2012; GANIAS, 2013). Owing to the rarity of synchronous hermaphrodite's species, studies on their reproduction are scarce. Thus, the present study generates, for the first time, information on the reproduction of the simultaneous hermaphrodite *D. radiale*,

captured in two distinct environments (coastal and estuarine areas) in the southeastern Brazil. The following aspects of the reproduction were studied: (1) ovotestis development and organisation, (2) oocyte size-frequency distribution and (3) fecundity (relative batch fecundity). Moreover, the influence of the fish size, weight and body condition (gonadosomatic index, stomach repletion index, Fulton's condition factor and hepatosomatic index) on fecundity was also investigated.

4.3. Materials and Methods

4.3.1 Areas of study and sampling strategy

The inner shelf of Ubatuba and the estuarine system of Cananéia are located in the São Paulo State, southeastern Brazil (Figure 1). Specimens of *D. radiale* were captured bimonthly, from June (2012) to May (2013), during research cruises using an otter trawl. To capture specimens in spawning condition, trawls were carried out twice per day (between 08:00-09:30 am and 15:30-17:00 pm) on three consecutive days in each sampled month. Each station was trawled over a 15 min in depths that ranged from 20.6 m in the coastal of Ubatuba to 1.9 m in the estuary. After trawling, the specimens were immediately taken to the laboratory to measure the total length (TL, to the nearest millimetre) and to weigh the total weight (TW), somatic weight (SW), gonad weight (GW), digestive tract weight (DT) and the liver weight (LW) (to the nearest gram, 0.01 g). Then, the ovotestes were fixed in 10% formalin buffered with sodium phosphate monobasic ($NaH_2PO_4 \bullet H_2O$) and dibasic ($Na_2H_2PO_4$) to ensure the adequate preservation of the oocytes (HUNTER, 1985).

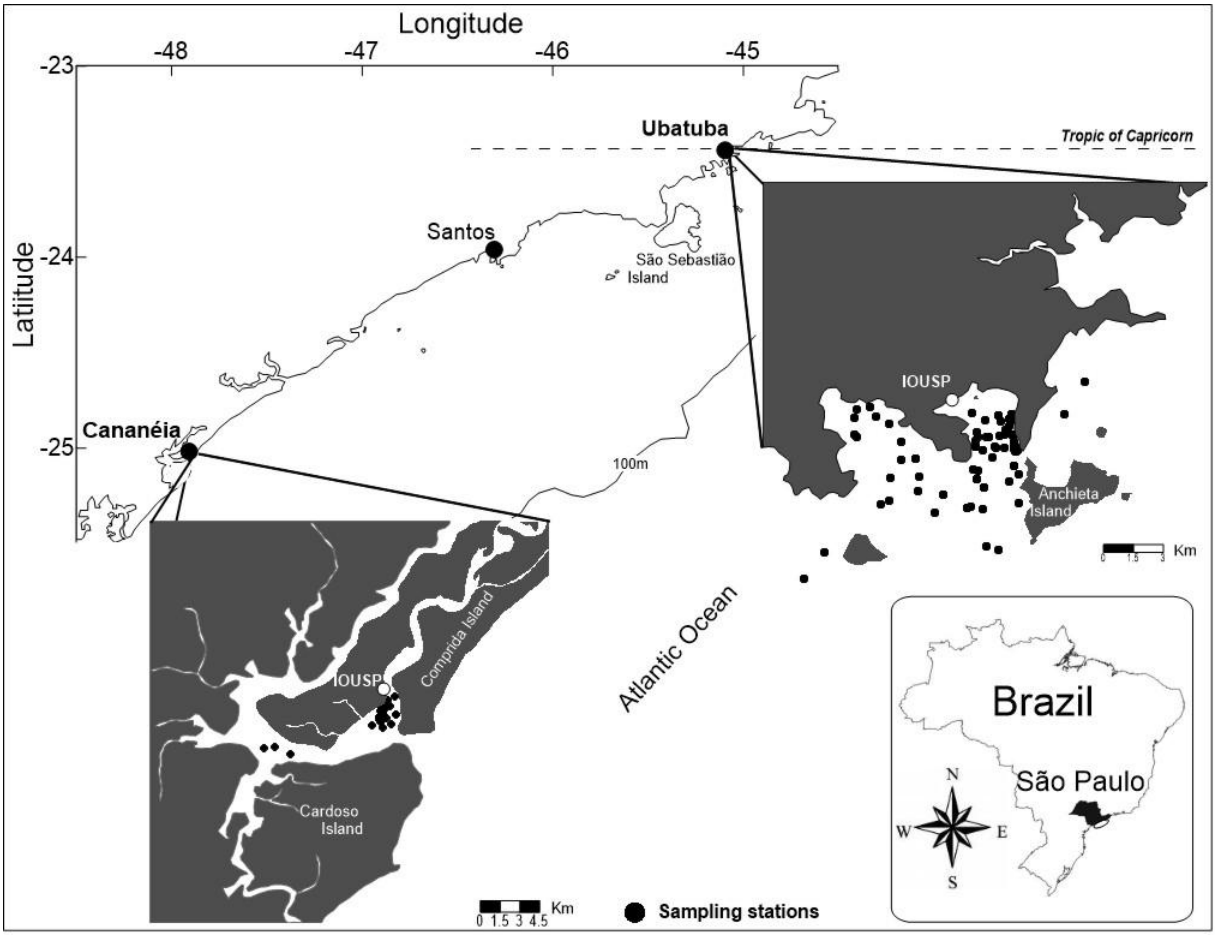


Figure 1. Areas of study showing the sampled stations. IOUSP= research bases of the Instituto Oceanográfico da Universidade de São Paulo, Brazil.

4.3.2 Histological analysis

All histological preparations were carried out with three to four sub-samples from the anterior, middle and posterior portion, containing the whole thickness of the ovotestis wall. Sampled tissues were dehydrated in alcohol, cleared in xylol, embedded in paraffin blocks, sectioned at 5 µm and stained with Harris' Haematoxylin and Putti's Eosin. Periodic Acid–Schiff (PAS), counterstained with Haematoxylin, was also used to stain the internal structures of the ovarian sinus and to identify cortical alveolus (BORTONE, 1977a; GURAYA, 1986).

4.3.3 Ovotestis classification

The oocyte stages were classified based on the histological criteria established by Wallace and Selman (1981) and Tyler and Sumpter (1996). However, oocytes in primary growth were classified as early and late primary growth (Pg1 and

Pg2), and those oocytes in vitellogenic growth was divided into four development stages (Vtg1, Vtg2, Vtg3 and Vtg4). On the other hand, the testicular germ cells were classified according to Grier (1981). The terminology used in this study was those proposed by Brown-Peterson et al. (2011). The atretic oocytes were indentified based on the zona radiata fragmentation, appearance of vacuoles, disintegration of the nucleus and fragmentation of the basophilic cytoplasm in pre-vitellogenic oocytes (HASTING, 1981; HUNTER; MACEWICZ, 1985a).

4.3.4 Post-ovulatory follicles classification

The post-ovulatory follicles were assigned according to their histological and morphological characteristics. Only ovotestes sampled in Ubatuba were used for this purpose. Thus, the histomorphological features used for POF classification were the size, shape, dimension of the lumen and the state of the follicle layers (granulosa and theca cells) (GANIAS et al., 2007; HASLOB et al., 2012; GANIAS, 2012). To improve the POF staging, the cross-sectional area (µm²), aspect ratio (the ratio between the shortest and longest extremes of the POF) and the circularity $\left(4\pi \times \frac{Area}{(perimeter)^2}\right)$ were estimated for the whole POF located along the epithelium of the lamellae (GANIAS et al., 2007). All measurements were made on the images of the histological sections using the free software image J (RASBAND, 1997–2009). Moreover, the approximate age of the POFs, i.e. the elapsed time from spawning (6h, 12h and 24h), was estimated based on a study carried out by Macchi et al. (2003) in the same range of temperature where the specimens of *D. radiale* were sampled (20-25 °C).

4.3.5 Oocyte size-frequency distribution

One to two subsamples of ≈ 200 mg from the middle and posterior position of ten ovotestes (five from each region) with hydrated oocytes were taken to estimate the individual oocyte diameter. Only oocytes larger than 100 µm were measured, including pre-vitellogenic, vitellogenic and hydrated oocytes. To separate the oocytes from each other, each sub-sample was sieved (using a 100, 300 and 500 µm mesh sieve) and sprayed with high-pressure water using a pipette filled with distilled water. Then, the oocytes were stained with Rose Bengal, placed in a Petri dish and

photographed with a digital camera. All oocytes present in each image were counted and measured using the automated method proposed by Ganias et al. (2010). All measures were performed using the free software ImageJ (RASBAND, 1997–2009). The oocyte diameter was calculated by the arithmetic mean of the longest and shortest diameter measured automatically and then, grouped into classes of 50 µm to analyse the size frequency distribution.

4.3.6 Condition indices

Four condition indices, i.e. gonadosomatic index (GSI), stomach repletion index (RI), Fulton's condition factor (*K*) and hepatosomatic index (HSI), were estimated, as follow: $GSI= (GW/SW) \times 10^2$, $RI= (DT/SW) \times 10^2$, $K= (SW/TL^3) \times 10^3$ and $HSI= (LW/SW) \times 10^2$.

4.3.7 Fecundity: relative batch fecundity (RBF)

The batch fecundity was not estimated because of the presence of both tissues, female and male, in a single gonad became difficult to separate the ovarian sinus with hydrated oocytes from the whole gonad. Moreover, all ovotestes used to estimate the RBF were with sign of spawning (POF). For this reason, It was assumed that the presence of POFs in the ovarian tissues a long with hydrated oocytes, do not imply that the mature oocytes were released to the environment, i.e. these oocytes were conducted to the ovarian sinus and stored up to the next spawning event. Thus, a subsample of ≈ 250 hydrated oocytes present in the ovarian sinus was removed manually and sieved with 500 µm mesh, following the same methodology used for analysis of the oocyte size-frequency distribution. Then, these hydrated oocytes were stained with Rose Bengal, weighed (to the nearest milligram, 0.0001 mg), photographed, counted and measured using the software ImageJ (RASBAND, 1997–2009). Thus, the relative batch fecundity (RBF), defined as the number o hydrated oocytes (Nhyd) per gram of gonad-free body weight (FBW) was calculated as follow: $RBF= (N_{hyd}/ W_{hyd}) \times FBW$ where N_{hyd} and W_{hyd} correspond to the number of hydrated oocytes in each subsample and the weight of these hydrated oocytes, respectively.

4.3.8 Data analysis

The tests of Kolmogorov-Smirnov and Levene were applied prior any statistical test application to verify the normality distribution and homogeneity of variances, respectively (ZAR, 1999). The relationship between the POF diameter and POF area was described by a potential regression. On the other hand, linear regressions and Pearson's correlation coefficient (*r*) were performed to evaluate the relationship between the RBF and the variables TW, TL, GSI, RI, *K* and HSI; however, the TW and TL were transformed prior to natural logarithm (ln) for normalization and linearity. Moreover, 95% confidence and 95% prediction intervals were also estimated and plotted to improve the visualisation of the data. The t-test was applied to compare the diameter of the hydrated oocytes between areas. Linear discriminant analysis (LDA) was performed to validate the classification of the POF stages, identifying the groups (stages) of degeneration using the POF size (area) and the shape descriptors (circularity and aspect ratio). The LDA significance was evaluated by application of the Wilks' Lambda test. All analyses were performed at level of 0.01 or 0.05 when appropriate. The mean ± standard deviation is given between brackets.

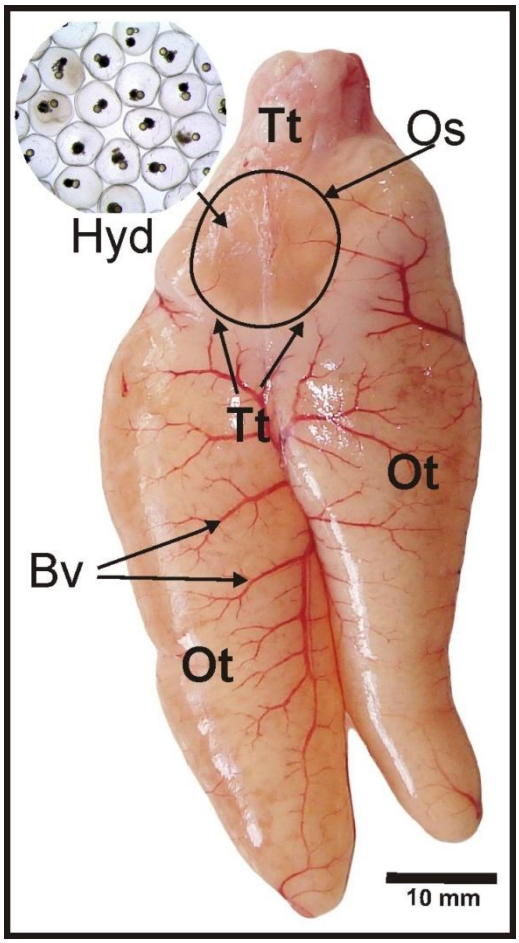


Figure 2. Ovotestis of the simultaneous hermaphrodite *Diplectrum radiale* showing the ovarian sinus (Os) filled with hydrated oocytes (Hyd). Ot= ovarian tissue, Tt= testicular tissue, Bv= blood vessels.

4.4 Results

A total of 108 mature ovotestes were examined using histological methods, being 54 (TL, 130–212 mm) from Ubatuba and 54 (TL, 133–234 mm) from Cananéia.

4.4.1 Ovotestis development and organisation

In general, the left lobe of the gonad of *D. radiale* was larger than the right in mature

specimens. All individuals examined macroscopically and histologically showed the presence of both ovarian and testicular tissues. Female and male tissues were clearly differentiated from each other because the gonad was predominantly ovarian and the testicular tissue was always white. The male tissue was restricted to the posterior ventral region of the gonad surrounding the ovarian sinus, so-called the accessory reproductive structure (Figure 2). Specimens with hydrated oocytes and testes containing spermatozoa were recorded simultaneously in both regions.

4.4.1.1 Ovarian tissue

The histological examination revealed that the oocytes mature from the posterior to anterior portion of the ovotestis with all oocytes developing inside the ovarian lamellae. However, the final maturation, i.e. hydration, seems to occur outside the ovarian lamellae because all hydrated oocytes were found in the ovarian sinus and in the central lumen of the ovary. Thus, the late germinal vesicle migration oocytes were the most developed stage observed inside the ovarian lamella. The Figure 3 shows the progression of the ovarian maturation with pre-vitellogenic oocytes in the anterior portion of the ovary and those more developed/ matured in the posterior portion, as well as the positive PAS reaction of the villae-like projections in the ovarian sinus (Figure 3)**.**

Specimens in spawning capable phase revealed the presence of ten stages of oocyte development, beginning with pre-vitellogenic and ending with the final maturation, including the degeneration of non-spawned oocytes (atretic oocytes) which may occur at any development stage. All stages will derive from oogonia which were arranged isolated on the periphery of the ovarian lamellae in *D. radiale*. Thus, the pre-vitellogenic growth, i.e. development of unyolked oocytes, was divided into chromatin nucleolar, primary growth (perinucleolar) and cortical alveolar stages:

– *Stages I-II: Chromatin nucleolar and primary growth (Pg)*. Oocytes in theses stages have a basophilic cytoplasm, densely stained with Haematoxylin and a large nucleus with several nucleoli inside. However, the nucleus to cytoplasm ratio increase showing a conspicuous nucleus in the former stage whereas in the latter, this ratio decrease and the nucleus appear with several nucleoli arranged at the periphery. The Pg stage can also be subdivided into early and late primary growth

(Pg1 and Pg2) based on the shape and size of the oocytes which were more regular and larger in Pg2.

– *Stage III: Cortical alveolar (CA)*: Small oil droplets and cortical alveolus begin to accumulate in the cytoplasm. The follicle components such as zona radiata, theca and granulosa cells become visible at this stage.

On the other hand, the vitellogenic growth begins with the appearance of yolk granules in the oocyte cytoplasm and was divided into primary, secondary, tertiary and quaternary vitellogenesis. However, the final maturation was characterised by the presence of germinal vesicle migration (GVM), late germinal vesicle migration (LGVM) and hydrated (Hyd) oocytes:

– *Stage IV: primary vitellogenesis*. The eosinophilic protein granules start to fill the cytoplasm. Small oil droplets are more abundant around the nucleus or arranged at the periphery of the oocyte.

– *Stage V: Secondary vitellogenesis*. The protein granules are arranged as a ring in the cytoplasm and the oil droplets located around the nucleus start to increase in size.

– *Stage VI: Tertiary vitellogenesis*. Yolk granules increase in number and fill the cytoplasm. The fusion of the oil droplets into oil globules start in this stage.

– *Stage VII: Quaternary vitellogenesis*. The cytoplasm is filled with yolk granules and large oil globules are arranged around the nucleus.

– *Stage VIII: Germinal vesicle migration (GVM)*. The nucleus starts to migrate toward the animal pole at the periphery of the oocyte; yolk granules and oil droplets coalesce and rapidly increase in size. Three to one oil globules larger than others are visible in this stage.

– *Stage IX: Late germinal vesicle migration (LGVM)*. GVM with yolk coalescence. Moreover, an oil globule and the nucleus with several nucleoli are easily observed in this stage.

– *Stage X: Hydration (Hyd)*. The oocyte enlarges rapidly due to the intake of water and becomes translucent. The cytoplasm becomes homogeneous, which makes it difficult to identify the cell structures. Due to the histological process, the oocytes appear misshapen and stained lightly with eosin. All stages of oocyte development can be seen in figure 3.

The analysis of the histological sections also revealed the degeneration of pre-vitellogenic (Pg1, CA), vitellogenic (Vtg1, Vtg2, Vtg3 and GVM) and hydrated oocytes. In general, the prevalence of atresia in Ubatuba and Cananéia were 51.9% and 46.3%, respectively. The disintegration of nucleus was the first sign of atresia followed by fragmentation of the basophilic cytoplasm in Pg1, and the zona radiata in CA, vitellogenic and GVM stages. On the other hand, atretic hydrated oocytes were only visualised under stereomicroscopic examination; they were lightly black and reduced in size with a conspicuous oil globule (Figure 4).

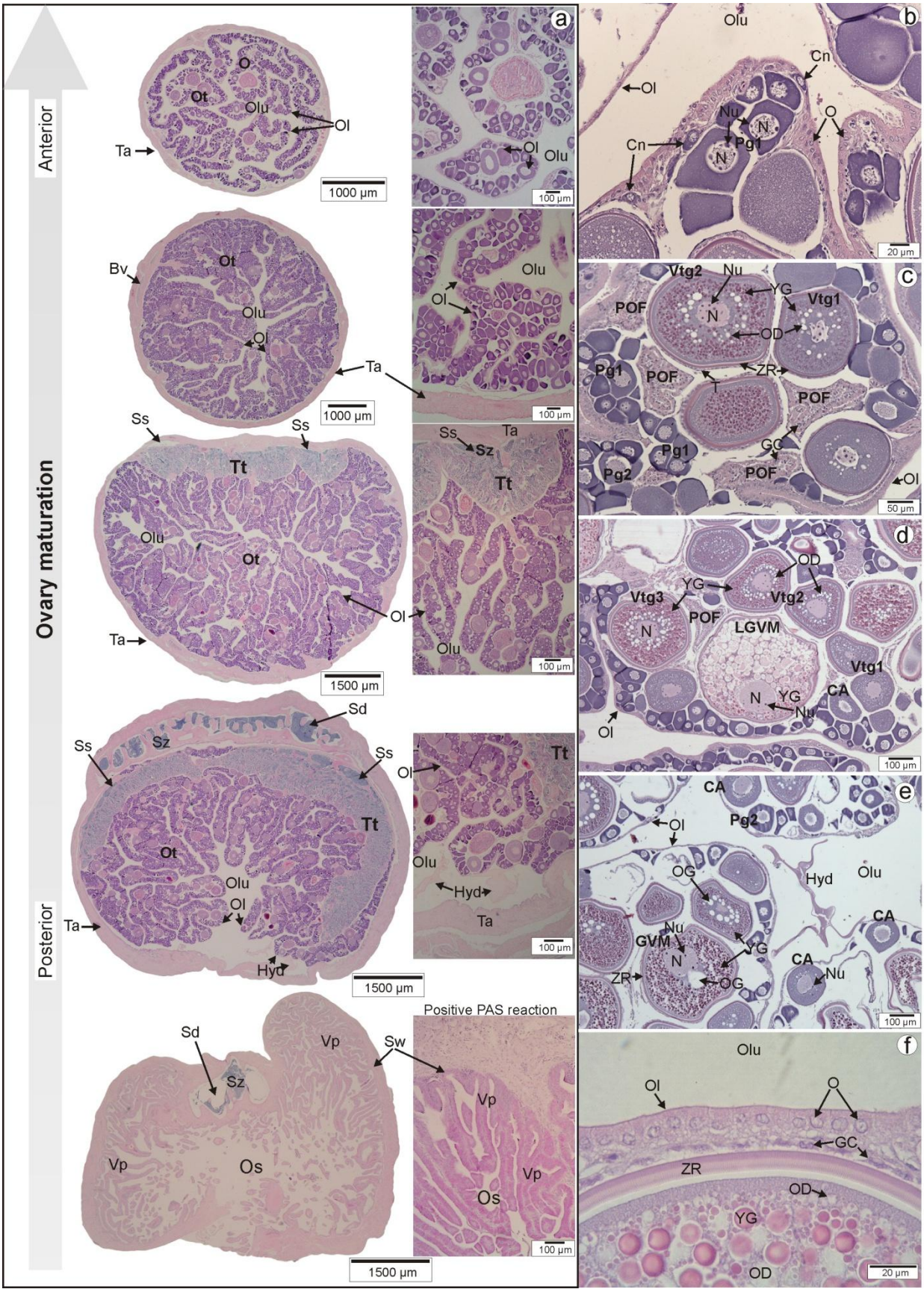

Anterior
Ovary maturation
Posterior
a
b
c
d
e
f
O
Ot
Olu
Ol
Ta
Bv
Tt
Ss
Sz
Sd
Hyd
Vp
Os
Sw
Cn
N
Nu
Pg1
Vtg2
Vtg1
Vtg3
YG
OD
POF
ZR
T
GC
Pg2
LGVM
CA
OG
GVM
1000 µm
1500 µm
100 µm
20 µm
50 µm
Positive PAS reaction

Figure 3. Photomicrographs of the ovotestis of the simultaneous hermaphrodite *Diplectrum radiale*. (a) Different parts of the ovotestis showing the ovarian sinus (Os), accessory reproductive structure; (b) oogonia (O) on the periphery of the ovarian lamellae, oocytes in chromatin nucleolar (Cn) and early primary growth stages (Pg1); (c) spawning capable ovary with post-ovulatory follicle (POF), primary and secondary vitellogenesis stages (Vtg1 and Vtg2); (d) late germinal vesicle migration oocyte (LGVM) with yolk coalescence; (e) specimens in spawning capable with germinal vesicle migration (GVM) and hydrated (Hyd) oocytes; f- zona radiata (ZR) with two different layers. Ot= ovary tissue, Tt= testicular tissue, Ta= tunica albuginea, Os= ovary sinus, Sw= sinus wall, Vp= villae-like projections, Olu= ovarian lumen, Ol= ovarian lamellae, Ss= sperm sinus, Sd= sperm duct, Pg2= late primary growth stage, CA= cortical alveolar stage, Vtg3 tertiary vitellogenic stage, N= nucleus, nu= nucleoli, YG= yolk granules, OG= oil globules, OD= Oil droplets, GC= granulosa cells, T= theca layer, Sz= spermatozoa.

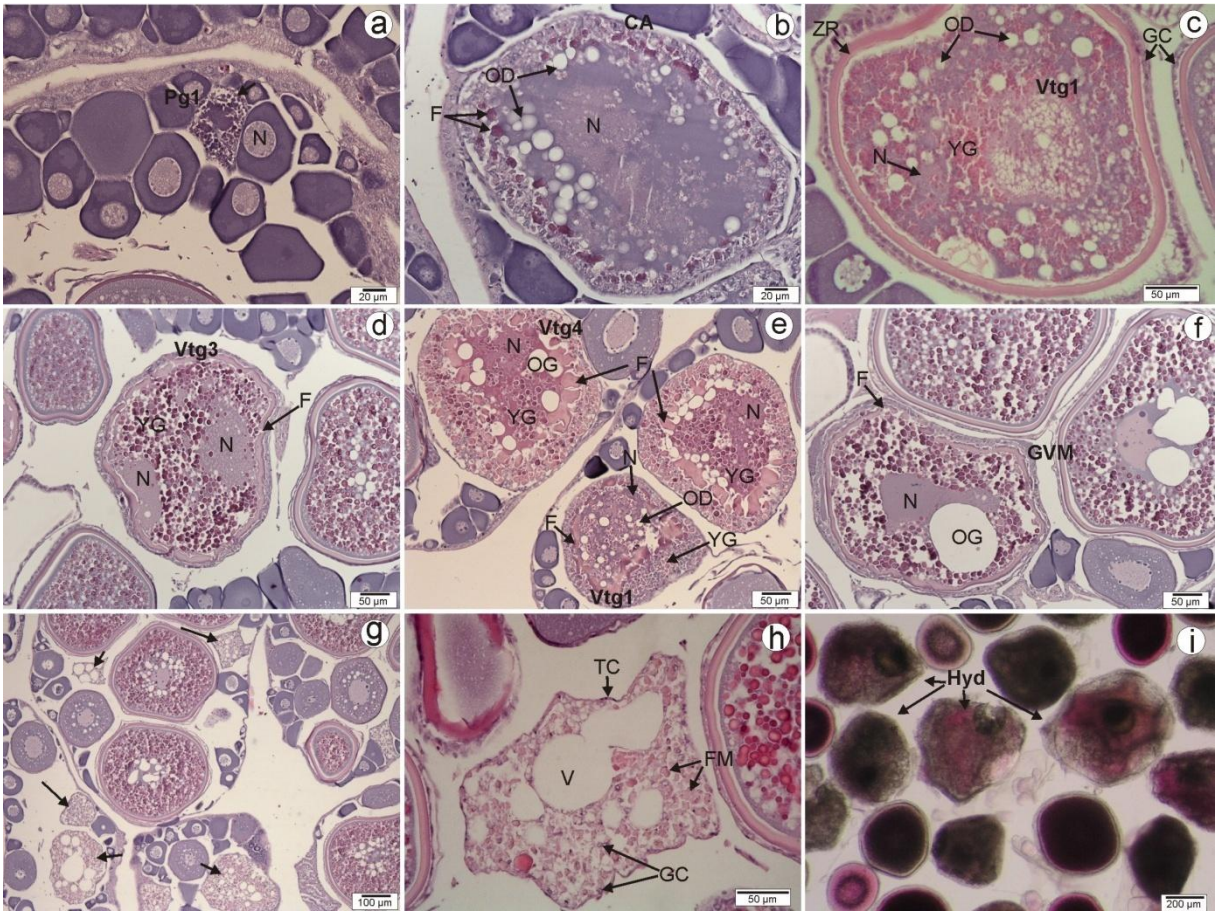


Figure 4. Oocyte atresia in the simultaneous hermaphrodite *Diplectrum radiale*. (a) Early primary growth oocyte (Pg1) with fragmented cytoplasm; (b) disintegration of the nucleus (N) and fragmentation of the zona radiata (F) in cortical alveolar oocyte; (c) primary vitellogenesis oocyte (Vtg1) in early stage of atresia showing the disintegration of the nucleus and uneven diameter of the zona radiata before its fragmentation; (d) atresia of oocyte in tertiary vitellogenesis stage (Vtg3); (e) different atretic oocytes in the same ovarian tissue; (f) germinal vesicle migration oocyte (GVM) with disorganised nucleus and fragmented zona radiata; g- oocytes in late stages of atresia (arrows); (h) yolked oocyte in late stage of atresia with vacuoles (V) and flocculent material (FM); (i) atretic hydrated oocytes (hyd) stained with Rose Bengal. Vtg4 quaternary vitellogenic stage, YG= yolk granules, OG= oil globules, OD= Oil droplets, GC= granulosa cells, ZR= zona radiata.

4.1.2 Post-ovulatory follicle degeneration

In general, 87% of the specimens sampled in Ubatuba and 51.9% in Cananéia were with post-ovulatory follicles (POF). Thus, three POF stages were identified based on their histomorphological characteristics, as follow bellow:

– *Stage I: POF- 6h-old*. Convoluted shape with no sign of degeneration and a large lumen. The theca cells are clearly visible bounding the health and enlarged granulosa cells. Some pycnotic nucleus and the presence of eosinophilic granules of unknown origin in the lumen can also be observed. This POF had the largest cross sectional area measured (Figure 5a-b).

– *Stage II: POF- 12h-old*. Convoluted shape, but the lumen becomes smaller compared to the stage I, as well as the POF cross sectional area. The granulosa cells are smaller with pycnotic nucleus. Some theca cells can also be visualised adhering to granulosa (Figure 5c).

– *Stage III: POF-24h-old*. The lumen cannot be visualised anymore. The follicles have the smallest size, many vacuoles and few number of granulosa cells with pycnotic nucleus dispersed in a compact structure (Figure 5d).

Different POF stages were observed simultaneously, being found up to three stages in the same ovotestis (Figure 5e-f). These follicles were present along with other more developed oocyte stages such as germinal vesicle migration, late germinal vesicle migration and hydrated oocytes. On the other hand, the allometric coefficient (*b*) from the relationship between the diameter and area of the POFs revealed an isometric relationship ($b = 2.067$), meaning that the POF size diminish at the same rate as POF area. The linear discriminant analysis was also statistically significant (Wilks' Lambda = 0.194, $F = 107.3$, $p<0.01$), indicating that the area, circularity and aspect ratio of the POF were important predictors separating the three groups of POFs (Figure 6). The confusion matrix for the cross-validation results indicated that the accuracy for the POF classification, i.e. into POF-6h-old, POF-14h-old and POF-24h-old were 82.8%, 72.1% and 89.3%, respectively.

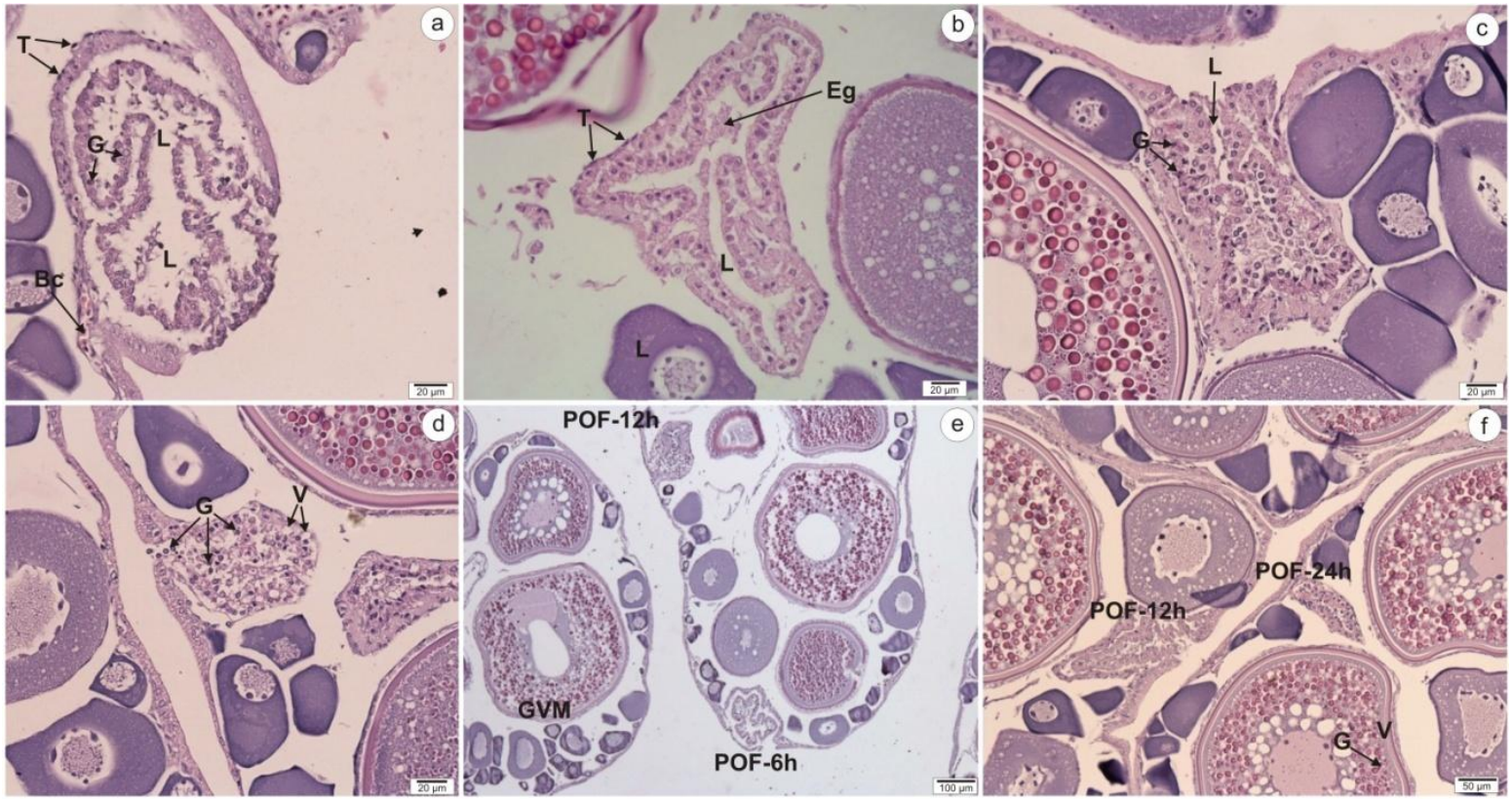


Figure 5. Degeneration of post-ovulatory follicles (POF) of *Diplectrum radiale*. (a-b) 6-h-old POF showing large lumen (L), the theca cells (T), health and enlarged granulosa cells (G); (c) 12-h-old POF after spawning with signs of degeneration indicated by the size reduction of the lumen and free granulosa cells with pycnotic nucleus (Black points); (d) 24-h-old POF showing a compact follicle with many vacuoles (V) and a few number of granulosa cells; (e-f) Different POF stages in the same ovarian tissue. Bc= blood cells, Eg= eosinophilic granules, GVM= Germinal vesicle migration.

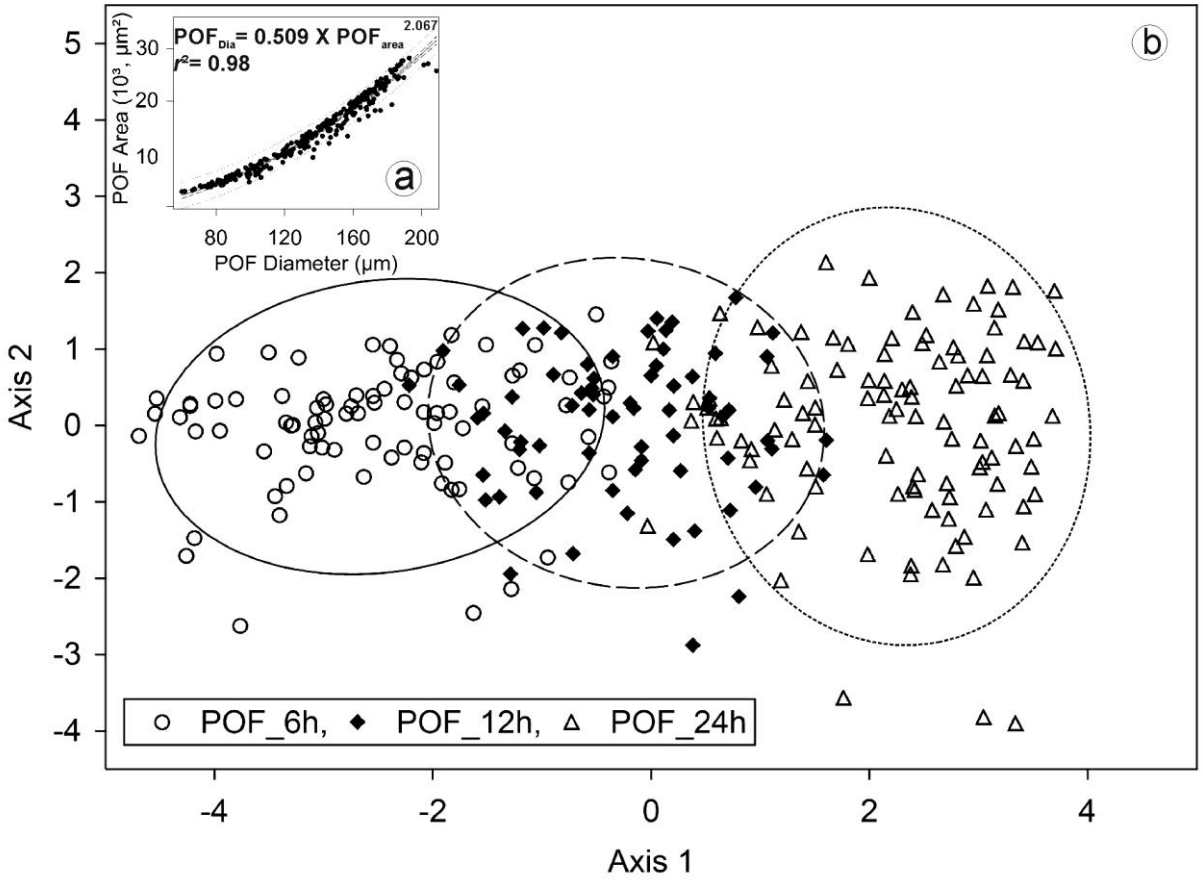


Figure 6. Validation of the pos-ovulatory follicle (POF) classification. (a) Relationship between the diameter and area of the POF, (b) linear discriminant biplot with POFs grouped by age (h). 95% confidence (---) and 95% prediction intervals (···) are given for figure a.

4.4.1.2 Testicular tissues

The testis of *D. radiale* was composed of several seminiferous lobules which were separated from each other by connective tissues bounded by the tunica albuginea. These lobules consisted by cysts containing germ cells and a lumen. Each cyst was bounded by connective tissues containing germ cells in the same development stage (Figure 7). Thus, four development stages were observed in the histological preparations, as follow:

– *Stage I: Spermatogonia*. The spermatogonia are the largest germ cells, lightly stained with Haematoxylin, observed singly or in nest at the periphery of the cysts. These cells are globular with prominent nucleolus.

– *Stage II: Spermatocytes*. These cells are oval with condensed chromatin material stained with Haematoxylin. The spermatocytes are smaller than spermatogonia, although they also have a large size.

– *Stage III: Spermatids*. The spermatids are spherical and strongly basophilic. These cells are smaller than spermatocytes and they can be confused with clusters of spermatozoa.

– *Stage IV: Spermatozoa*. The spermatozoa are the smallest type of germ cells in the testis, and after maturation inside the cysts, they are detached from the basal lamina into the lumen. These cell move from the lobule lumen toward the sperm sinus and sperm duct. Thus, the spermatozoa were always present inside the lobule lumen, sperm sinus and sperm duct in all specimens examined (Figure 7).

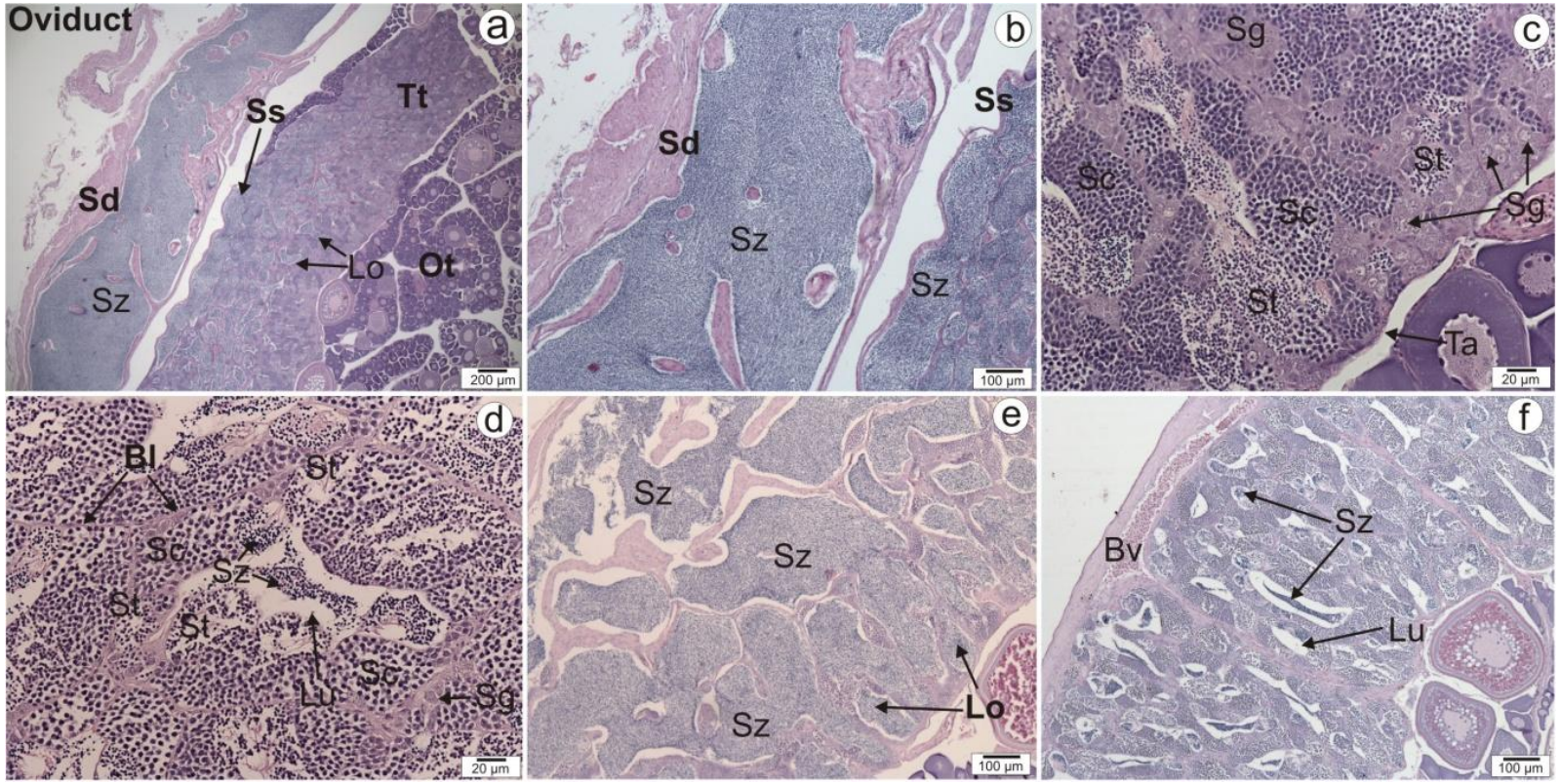


Figure 7. Photomicrographs of the testicular area of ovotestes of the simultaneous hermaphrodite *Diplectrum radiale*. (a) Testicular tissue (Tt) structures showing the lobules (Lo), sperm sinus (Ss) and sperm duct (sd); (b) sperm sinus and sperm duct filled with spermatozoa (SZ); (c) distal region of the testis showing the spermatogonia (Sg), spermatocytes (Sc) and spermatids (St); (d) lobule containing different stages of germ cell within the cysts, and detach of clusters of spermatozoa from the basal lamina (Bl) into the lumen (Lu); (e) male in spawning capable phase with the lobules filled with spermatozoa; (f) male in regressing phase showing the remnant spermatozoa in the lumen.

4.4.3 Oocyte size-frequency distribution

The oocyte size-frequency distribution revealed a similar pattern for specimens captured in both regions. The pre-vitellogenic and vitellogenic oocytes were grouped as a single mode and separated by a gap from those in hydration. In general, the oocytes of the former mode ranged from 104.4 to 532.7 µm (306.9 ± 80.7) and the latter, from 673.1 to 892.6 µm (752 ± 156.8). The hydrated oocytes from specimens captured in the coastal area (769.2 ± 30.6) were larger than those captured in the estuary (742 ± 26.3) ($p < 0.01$) (Figure 8).

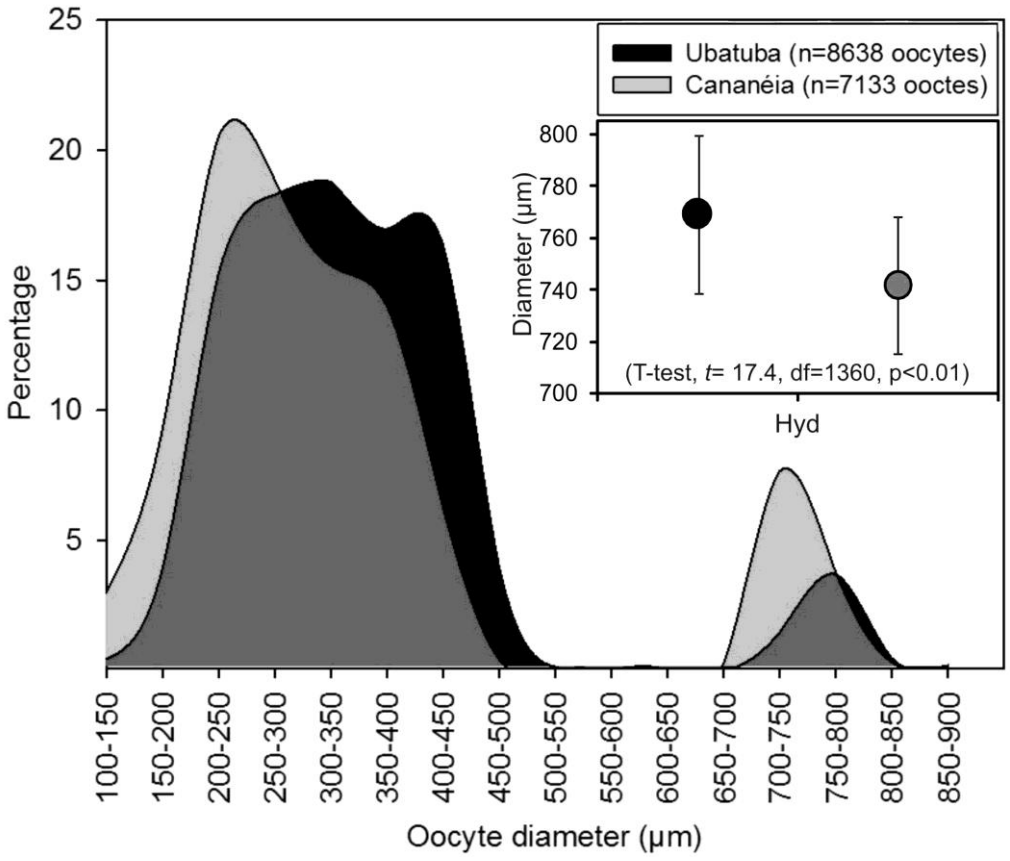


Figure 8. Oocyte size-frequency distribution in the ovotestis of the simultaneous hermaphrodite *Diplectrum radiale*. The mean and ± standard deviation of the hydrated oocytes (Hyd) diameter are indicated by the circles and the bars, respectively.

4.4.4 Relative batch fecundity

The relative batch fecundity (RBF) was estimated for 33 specimens captured in Ubatuba and 6 in the estuary of Cananéia; their total length ranged from 147 to 226 mm (176.1 ± 17) and 142 to 176 mm (155.5 ± 11.9), respectively.

The RBF ranged from 20 to 82 oocytes g^{-1} (51 ± 16) for specimens from Ubatuba, and from 67 to 113 oocytes g^{-1} (90 ± 19) for those from Cananéia. Due to small number of ovotestes with hydrated oocytes sampled in the estuary, the relationship between the RBF and the variables total length (TL), total weight (TW), gonadossomatic index (GSI), stomach index (SI), condition factor (*K*) and hepatossomatic index (HIS) was possible only for specimens from Ubatuba. Thus, the Pearson's correlation coefficient revealed that the RBF was negatively correlated with all variables, but statistically significant with the TL, TW, GSI and K (Figure 9).

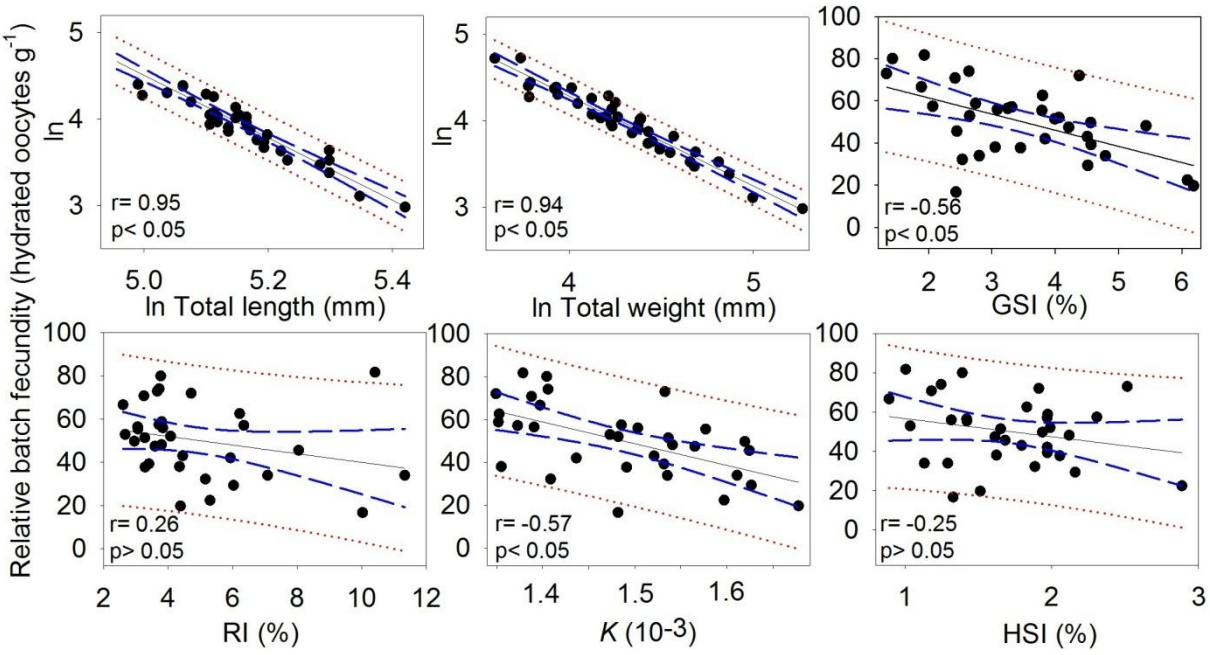


Figure 9. Relationship between the relative batch fecundity (RBF) and the factors total length, total weight, gonadosomatic index (GSI), stomach repletion index (RI), condition factor (*K*) and hepatosomatic index (HSI) for 33 specimens of *Diplectrum radiale* captured in the inner shelf of Ubatuba, southeastern Brazil. 95% confidence interval (---), 95% prediction interval (···), significance of probability (p) and Pearson's correlation coefficient (*r*) are also given. The variables which were transformed to natural logarithms are indicated as ln.

4.5 Discussion

The general ovotestis morphology of *D. radiale* follows the same pattern reported by Bortone (1971, 1977a, 1977b) for species of the same genus. The external aspects of the *Diplectrum* sp. ovotestis such as the position of the ovarian tissue, testicular tissues and the ovarian sinus are very important because such morphological features may be used to discern the phylogenetic relationship among the species of this genus (BORTONE, 1977b). However, in the present study, the testicular tissue was positioned surrounding the ovarian sinus and not between the ovarian sinus and the ovarian tissue, as reported by Bortone (1977b).

The specimens sampled in both regions revealed the presence of hydrated oocytes in the ovarian sinus and a positive PAS reaction of the villae-like projections. The function of the ovarian sinus is complex. Bortone (1977a) has hypothesised that the ovarian sinus is for storage of the hydrated oocytes and for resorption of unshed oocytes. This same author and Touart and Bortone (1980), have also reported a positive PAS reaction of the “villae” in the ovarian sinus of *D. pacificum* and *D bivittatum* identifying a presence of a mucus substance. The villi structures are composed of secretory epithelium and the mucus substance creates the condition for storage of the hydrated oocytes (BUBLEY; PASHUK, 2010); moreover, this substance could also facilitate the movement of the hydrated oocytes through the sinus and oviduct, the adhesion of the spermatozoa in the external environment and hold the hydrated oocytes together after spawning (TOUART; BORTONE, 1980).

The simultaneous occurrence of post-ovulatory follicles at different stages and the ovarian sinus filled only with hydrated oocytes registered in the present study corroborates the theory of the oocyte storage, indicating that the hydrated oocytes were stored in the accessory reproductive structure of *D. radiale* for at least 24h. To date no atretic oocyte has been recorded in the ovarian sinus of *Diplectrum* spp., contradicting the theory of the oocyte resorption (TOUART; BORTONE, 1980; BUBLEY; PASHUK, 2010). However, in the present study was found atretic hydrated oocytes within the ovarian sinus, revealing that the villae-like projections may also reabsorb the unshed oocytes, as stated previously by Bortone (1977a).

In general, the development of the oocytes of *D. radiale* follows the same pattern described for other teleosts, such as: primary growth, cortical alveolar, vitellogenesis and final maturation (WALLACE; SELMAN, 1981; WEST, 1990; TYLER; SUMPTER, 1996; MURUA; SABORIDO-REY, 2003). However, the cortical alveoli vesicles were negative for PAS reaction. According to De Vlaming (1983) and Tyler and Sumpter (1996), lipid bodies often accumulate in the cytoplasm of the oocytes along with cortical alveoli, particularly in marine fish species. For this reason, the oocytes with negative PAS reaction were considered as CA stage supported by the presence of lipid bodies, visible follicle structures (i.e. zona radiata) and absent of yolk granules, as reported by Ganias et al. (2004), McMillan (2011) and Zudaire et al. (2013a). Moreover, the oocyte degeneration occurred in pre-vitellogenic, vitellogenic and hydrated oocytes. The degeneration of pre-vitellogenic oocytes is usually associated with the sex change in protogynous hermaphrodite (HASTING, 1981; SADOVY; SHAPIRO, 1987; ASOH, 2005). Therefore, the results of this work indicate that the atresia in *D. radiale* plays an important role regulating the final reproductive output during the spawning season.

The classification of the teleosts testis is based on the place where the final maturation of spermatozoa occurs within the lobule and the distribution of the spermatogonia along of its length (GRIER et al., 1980; GRIER, 1981; NAGAHAMA, 1986). Thus, the histological analysis of the *D. radiale* testes revealed a cystic spermatogenesis, i.e. the entire spermatogenesis occurs within the cysts releasing mature spermatozoa into the lobule lumen. Moreover, the spermatogonia were found throughout the testicular tissues; according to Grier (1981) this distribution is typical of species with unrestricted spermatogonial testicular type. In general, this type of spermatogenesis and testes organisation occur in the most teleosts, and they have been also reported by several authors for other hermaphrodite fishes (GÁRCIA-DÍAZ et al., 2002; TUSET et al., 2005; ALONSO-FERNÁNDEZ et al., 2011).

Although the oocytes of *D. radiale* showed a heterogeneous distribution along the ovotestis, the presence of oocytes in several development stages without dominant population in spawning capable specimens, reveals a continuous type of oogenesis with an asynchronous ovarian organisation. This type of ovarian organisation is typical of multiple spawning species with a very protracted spawning

season, commonly found in species from low latitudes (KJESBU, 2009, PAVLOV et al., 2009). Similar results have been reported for other hermaphrodite species such as *D. formosum* by Obando and León (1989), *Serranus scriba* by Tuset et al. (2005) and *S. atricauda* by Neves et al. (2014).

The simultaneous occurrence of spermatozoa, hydrated oocytes and post-ovulatory follicles indicates that the both gametes are clearly functional in *D. radiale*, i.e. both matured gametes are ready for fertilisation. However, the testicular and ovarian tissues were separated from each other by different ducts, indicating a little possibility of internal self-fertilisation, but the external self-fertilisation may occur, as observed in the ovotestes of *D. formosum* by Obando and León (1989), and Bubley and Pashuk (2010).

Studies on the oocyte size-frequency distribution to verify the presence or absence of a gap between the pre-vitellogenic and vitellogenic oocytes modes has been widely applied for investigating the fecundity type (determinate or indeterminate) of many fishes (GRANDE et al. 2012; GANIAS, 2013, ZUDAIRE et al., 2013a). Thus, fishes with indeterminate fecundity do not show this gap because the standing stock of pre-vitellogenic oocytes is continuous recruited into yolked oocyte stock (HUNTER; GOLDBERG, 1980; KJESBU, 2009). On the other hand, in those fishes with determinate fecundity, the stocks of pre-vitellogenic and vitellogenic oocytes are clearly separated from each other by a gap, indicating that the potential fecundity is fixed prior to the onset of spawning (MURUA; SABORIDO-REY, 2003; KJESBU, 2009). Thus, the oocyte size-frequency distribution of *D. radiale* revealed an indeterminate fecundity. However, the presence of a mode with pre-vitellogenic and vitellogenic oocytes together, differs from the pattern reported for the most teleosts with indeterminate fecundity, which shows several modes of oocytes in different development stages (MURUA; SABORIDO-REY, 2003). This evidence indicates that only a small number of oocytes are matured and recruited to form the final batch that will be stored in the ovarian sinus up to the next spawning event. The occurrence of vitellogenic oocytes in several stages a long with germinal vesicle migration oocytes, hydrated and post-ovulatory follicles in different stages, corroborate this idea. Moreover, this type of oocyte recruitment may also be a

strategy to regulate the final number of hydrated oocytes and may be related to its spawning frequency.

Multiple spawning fishes with indeterminate fecundity usually produce large batches of hydrated oocytes (GANIAS et al., 2004; MURUA et al., 2006; GRANDE et al., 2014; GARCÍA-SEOANE et al., 2014). Although there was sampled a small number of hydrated ovotestes in Cananéia, the relative batch fecundity estimated for specimens from both regions showed a low production of hydrated oocytes per gram of gonad-free body weight. This relative low production might be related to the spawning frequency of this species that must be high to compensate the low number of hydrated oocytes produced. However, the values of RBF found in this study were close to those reported by Alós et al (2013) and Neves et al. (2014) for the simultaneous hermaphrodites *S. scriba* (mean of 83 hydrated oocytes g^{-1}) and *S. atricauda* (mean of 22 hydrated oocytes g^{-1}), respectively.

Fecundity is also affected by the fish size, weight and condition, being the key parameters to assess fecundity at the population level (KJESBU et al., 1991; MURUA et al., 2003). Thus, the RBF was negatively affected by all factors measured, revealing that the smaller specimens with a better condition produce more hydrated oocytes per gram of body. These results may also indicate that the larger specimens had already spawned at least one batch, explaining the poor condition of these specimens.

The specimens inhabiting different environments revealed significant differences in the size of the hydrated oocytes. The oocyte size is an important factor in fish reproductive strategies and its variation is related with the environmental factors and food availability (TANASICHUK; WARE, 1987; BEACHAM; MURRAY, 1993; LEAL et al., 2009). This results shows that environment may play an important role regulating the oocyte size in *D. radiale*. However, more investigation is needed.

In conclusion, this study reveals that the simultaneous hermaphrodite *Diplectrum radiale* has an oocyte development and fecundity regulation strategy similar to the most teleosts inhabiting tropical and subtropical regions with indeterminate fecundity and asynchronous ovarian organisation. The testis is unrestricted type with spermatogenesis occurring within cysts. Oocyte and testis

maturation occur simultaneously, but with a little possibility of internal self-fertilisation. This study also confirms the theory that the ovarian sinus in *Diplectrum* spp. has the function to store and reabsorb hydrated oocytes. Thus, the hydrated oocytes are stored in this accessory reproductive structure for at least 24h up to the next event of spawning. Smaller specimens with a better condition produce more hydrated oocytes per gram of body.

Chapter III

Reproductive strategy and fecundity of the keystone species *Paralonchurus brasiliensis* (Teleostei, Sciaenidae): An image processing techniques application

5.1 Abstract

This study investigates, for the first time, the reproductive strategy of the keystone species *Paralonchurus brasiliensis* using histology and advanced image processing techniques to estimate the standing stock of the most advanced yolked oocytes (NDO) and the batch fecundity of this species. The reproductive strategy of *P. brasiliensis* was studied in relation to the following aspects: (1) ovary organisation and oocyte development, (2) oocyte size-frequency distribution, (3) oocyte diameter variation and (4) occurrence of the ovary phases during ovarian maturation. A total of 135 mature females were captured during the *P. brasiliensis* spawning season in the inner shelf of Ubatuba, southeastern Brazil. Images from the histological sections and formalin preserved oocytes were taken and analysed using image analysis software. The results indicated the presence of the oocyte stages in primary growth (Pg), cortical alveolar (CA), early vitellogenic (Vtg1) and later vitellogenic (Vtg2) in all ovarian phases. Moreover, no gap between pre-vitellogenic and vitellogenic oocytes was observed. However, a significant increase in the oocyte sizes along with the ovary development indicated constant oocyte recruitment during the spawning season. A significant decrease in the mean oocyte diameter through the study period was also recorded. These findings also revealed an asynchronous ovarian development and an indeterminate fecundity, indicating that on average, a female in the developing phase contains approximately three potential spawning batches ranging from 4,839 to 36,544 hydrated oocytes.

Key words: Oocyte development. Oocyte recruitment. Stereology. Batch fecundity. Spawning season.

5.2 Introduction

Knowledge of oocyte development and fecundity type (or oocyte recruitment process) is essential to understand the reproductive strategy of any fish species (HUNTER et al., 1992; RICHARD; KESTENMONT 2003; MURUA; SABORIDO-REY, 2003; MURUA; MOTOS, 2006). This strategy has been assessed based mainly on the knowledge of the stage-specific oocyte size frequency distribution during the reproductive cycle, evolution of the number of advanced yolked oocytes in the ovary, seasonal variation in the mean diameter of the advanced vitellogenic oocytes and incidence of atretic oocytes through the spawning season (WALKER et al., 1994; MURUA; SABORIDO-REY, 2003). Reproductive strategy among fish species can acclimate to climate and/or anthropogenic changes (STEARNS, 1992). Thus, the results of these studies are of great importance and widely applied in fisheries science because, to some extent, the fecundity studies, including the description of the reproductive strategy, determine the resilience of fish populations to fishing (MORGAN, 2008). Moreover, the studies on reproductive strategies of fishes determine the type of method chosen for estimating the individual fecundity and egg production method for the assessment of the spawning stock biomass (ARMSTRONG; WITTHAMES, 2012; GANIAS, 2013), as well as for understanding fish phenology in a world of changing climate (SORIA et al., 2008; MIRANDA et al., 2009; PANKHURST; KING, 2010; STRÜSSMANN et al., 2010). Fecundity can also be used as an indicator of population's reproductive potential, being a reference point for the management and sustainable fisheries (HUNTER et al., 1992). Nevertheless, there are many more data available on other life history parameters of fish than on the fecundity (TOMKIEWICZ et al., 2003). Fecundity comes from a relatively small number of species, the majority of which are of commercial importance (TYLER; SUMPTER, 1996).

Paralonchurus brasiliensis (Steindachner, 1996), commonly known as the Banded Croaker, is widely distributed along the Atlantic Coast from Central to South America (MENEZES; FIGUEIREDO, 1980). This species is the most frequently and abundant sciaenid captured as by-catch by shrimp fishery trawls in southeastern Brazil (NONATO et al., 1983; BRAGA et al., 1985; BRAGA, 1990; HAIMOVICI et al., 1996; SOUZA et al., 2008). It can reach 300 mm in total length (common to 250 mm),

feeding mainly on crustaceans and polychaetes (MENEZES; FIGUEIREDO, 1980; BRANCO et al., 2005). Moreover, Banded Croaker is considered a keystone species associated with shallow waters (Rossi-WONGTSCHOSKI et al., 2008; COSTA et al., 2012), playing an important role in the trophic structure of the ecosystem (SOARES; VAZZOLER, 2001; SOARES et al., 2008). However, little is known about the fish's reproductive strategy and fecundity.

The use of histological images and stereological methods has been recently and successfully applied to assessing the reproductive parameters for many fish species around the world (MEDINA et al., 2002; COWARD; BROMAGE, 2002; KJESBU, 2009; KJESBU et al., 2010). Thus, the aim of this study was to investigate, for the first time, the reproductive strategy of *P. brasiliensis* using histology and advanced image processing techniques to estimate the standing stock of the most advanced yolked oocytes and batch fecundity of this species. The reproductive strategy of *P. brasiliensis* was studied in relation to the following aspects: (1) ovary organisation and oocyte development, (2) oocyte size-frequency distribution, (3) oocyte diameter variation and (4) occurrence of the ovary phases during ovarian maturation.

5.3 Materials and Methods

5.3.1 Area of study and sampling strategy

The area of study is located in a transition region between the tropical and subtropical zones on the inner shelf of Ubatuba, on the northern coast of São Paulo state, southeastern Brazil (Figure 1). The specimens of *P. brasiliensis* were captured bimonthly during research cruises, from June (2012) to April (2013) using an otter trawl net. To capture females in spawning condition, the samples were taken twice a day (between 08:00–09:30 am and 13:30–17:00 pm) on three consecutive days of each sampled month. Each station was trawled over for 15 min at depths that ranged from 7.2 to 20.6 m. After trawling, the specimens were immediately taken to the laboratory to measure their total length (to the nearest millimetre) and weight (total weight to the nearest gram, 0.1 g), identify their maturity phase by macroscopic examination, and remove their ovaries. Then, the ovaries were weighed and fixed in 10% neutral formalin buffered with sodium phosphate monobasic ($NaH_2PO_4 \bullet H_2O$)

and dibasic ($Na_2H_2PO_4$) to ensure adequate preservation of the oocytes (HUNTER, 1985).

Homogeneity of the distribution of oocytes in the most advanced stage within the ovary was tested for five females in different phases. Thus, the number of oocytes was estimated as the weight mean density of the six subsamples (right and left ovaries — anterior, middle and posterior portion) multiplied by the ovary's total weight.

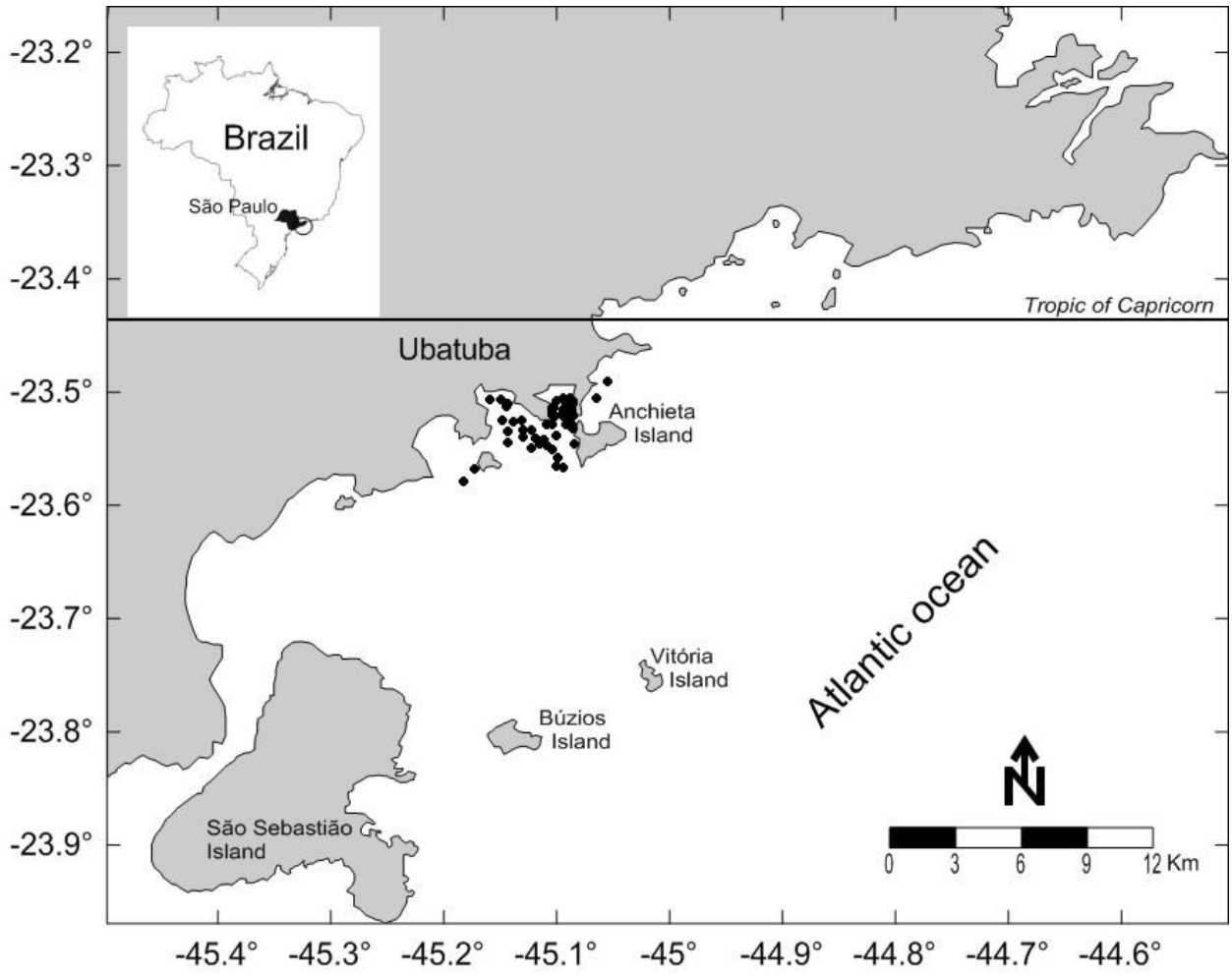


Figure 1. Area of study showing the sampled stations (black circles).

5.3.2 Histological analysis

A total of 189 females were captured during the sampling period. From this total, 135 females were mature and their ovaries were examined histologically. Sampled tissues were dehydrated, cleared in xylol, embedded in paraffin blocks,

sectioned at 5 µm and stained with Harris' Hematoxylin and Eosin. Periodic acid-Schiff's Hematoxylin, counterstained with metanil yellow (PAS-MY) was also used to correctly identify the cortical alveolus (GARCÍA-SEOANE et al., 2014).

The spawning season of *P. brasiliensis* was defined based on the occurrence of spawning capable females during the sampled period, i.e., the presence of females capable of spawning within the current reproductive cycle due to advanced oocyte development (BROWN-PETERSON et al., 2011).

5.3.3 Oocyte and ovarian classification

The analyses of the histological preparations were performed using a light microscope. Thus, the oocyte stages were classified based on the histological criteria established by Wallace and Selman (1981). The ovarian phases were classified according to Brown-Peterson et al. (2011). Atresia stages were classified following a modified Hunter and Macewicz (1985) classification: α and β-atresia were subdivided into early and late α;β-atresia based primarily on the zona radiata fragmentation, disintegration of the yolk granules and appearance of vacuoles in the cell.

5.3.4 Stereology

The number of developing oocytes (NDO), defined as the standing stock of advanced yolked oocytes (late vitellogenesis, Vtg2) present in the ovaries at any time (MURUA; SABORIDO-REY, 2003) without considering atresia loses, was estimated stereometrically in 24 ovaries. Thus, the NDO was estimated by applying the model-based methodology described in Emerson et al. (1990) and Murua et al. (2003) according to the equation proposed by Weibel et al. (1966): $\mathrm{NDO} = Ov \times \frac{K}{\beta} \times \frac{Na^{1/2}}{Vi^{1/2}}$ where Ov is the ovary volume; K is the size distribution coefficient; β is the shape coefficient; N_a is the number of Vtg2 oocytes transected per unit area; and V_i is the volume fraction occupied by Vtg2 in the histological section.

The ovary volume was calculated for 62 females applying the methodology proposed by Scherle (1970). *K* was calculated by the following equation:

$K = \left[\frac{M_3}{M_1}\right]^{3/2}$ where M_1 is the mean oocyte diameter, i.e., $M_1 = (D_1+D_2+D_n)/n$, and M_3 is the third moment for the mean of the oocyte distribution, i.e., $M_3 = \left[\frac{(D_1)^3+(D_2)^3+(D_n)^3}{n}\right]^{1/3}$; D is the individual oocyte diameter calculated by the arithmetic mean of the longest (L) and shortest (S) diameter measured in the histological section (KORTA et al., 2010b) and n is the number of Vtg2 oocytes counted. A total of 53 oocytes sectioned through the nucleus, from three specimens in spawning capable phase, were measured to estimate K as suggested by Emerson et al. (1990). However, β was calculated by the ratio between the longest and shortest axis of the oocytes transected. A Weibel grid (WEIBEL et al., 1966) with 256 test points and an area of 0.050 cm² was utilised to calculate N_a and V_i (Figure 2). Three to seven images from the non-overlapped area in the same histological section were taken with a magnification of 40X. Then, a routine developed by Haslob et al. (2013) to overlay histological images with a grid was applied using the free image analysis software ImageJ version 1.46r (Figure 2) (RASBAND, 1997–2009).

5.3.5 Oocyte size-frequency distribution and fecundity

In the histological preparations, 51 to 66 oocytes in pre-vitellogenic and vitellogenic growth sectioned through the nucleus, from the anterior, middle and posterior portion of the ovary of three to five individuals, were selected to measure the individual oocyte diameter. However, a total of 400 oocytes in germinal vesicle migration (GVM) and 611 hydrated oocytes were measured (whole mounts) from the formalin-preserved ovaries due to shrinkage caused by the histological procedures in these stages, following the methodology proposed by Ganias et al. (2010). All sub-samples were taken from the anterior, middle and posterior part of the right ovary. Then, the oocyte diameters were calculated and grouped into classes of 50 µm to analyse the size frequency distribution by ovary phase, identifying a presence or absence of a gap (hiatus) between the size distribution of pre-vitellogenic and vitellogenic oocytes. All measures also were performed using the software ImageJ (RASBAND, 1997–2009).

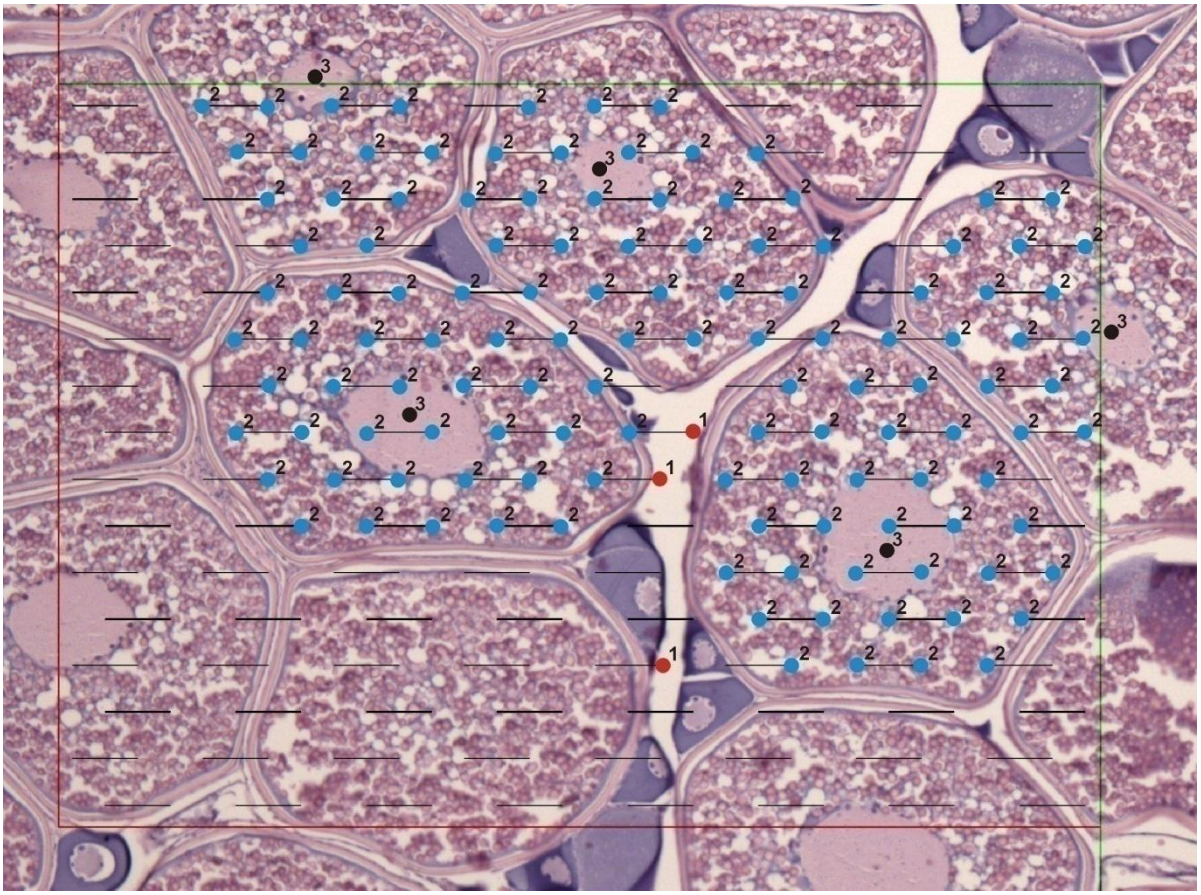


Figure 2. Illustrative image after ImageJ application showing a Weibel grid overlaid on a histological section with test points. There are 128 bars in this grid and the end of each bar represents a test point (*n*= 256 points). N_a was calculated by dividing the number of oocytes/profiles in the Vtg2 stage (3) by the frame area, i.e., N_a = 5 /0.050 = 100 profiles/ cm², whereas V_i was calculated by dividing the number of points laid on Vtg2 (2) by the total points subtracted from the negative grid (1), i.e., V_i = 124/ (256-3)= 0.047. Only oocytes overlapping the green borders of the grid were included in the analyses.

The NDO was estimated stereometrically for 24 females as previously described. BF, defined as the number of hydrated oocytes present in the ovary (HUNTER et al., 1985), was estimated on 18 hydrated ovaries with no sign of spawning (post-ovulatory follicle). Three subsamples of ≈140 mg each from the anterior, middle and posterior position of the right ovary were taken and weighed to the nearest 0.0001 g. To separate the hydrated oocytes, each sub-sample was sieved (using a 500–300 µm mesh sieve) and sprayed with high-pressure water using a pipette filled with distilled water. Then, the oocytes were stained with Rose

Bengal, placed in a Petri dish and photographed with a digital camera. All hydrated oocytes present in each image were counted and measured using the automated method proposed by Ganias et al. (2010). The BF was estimated as the weighted mean density of the three subsamples multiplied by the ovary's total weight. In addition, the relative number of advanced yolked oocytes (RNDO) and relative batch fecundity (RBF) also was calculated by dividing the absolute NDO and BF values by the female ovary-free weight.

The number of potential batches present in the ovaries was calculated by the ratio of NDO to BF, and refers to the number of batches of Vtg2 oocytes which are recruited to form the stock of germinal migration/hydrated oocytes, no considering atresia losses, CA and Vtg1oocytes.

5.3.6 Data analysis

Prior to applying any statistical test, each numeric variable was tested for normality and homogeneity of variances using Kolmogorov-Smirnov and Levene's test, respectively (ZAR, 1999). A *t*-test for the independent variable was performed to test the differences among the means of the total length (TL) and total weight (TW) of the females utilised to estimate the NDO and BF. The differences among oocyte diameters by stage and month were analysed applying a one-way analysis of variance (ANOVA) with Bonferroni correction followed by a Tukey post-hoc test. A two-way ANOVA was applied to verify the homogeneity in the distribution of the number of oocytes in the ovaries, considering the left and right ovary and the anterior, middle and posterior position as factors (GOTELLI; ELLISON, 2008). The coefficient of determination (r^2) was used to measure the linear relationship (goodness of fit of the model) between ovary weight (Ow) and ovary volume (Ov). Linear regressions among oocyte stages and their diameters in relation to ovary phases and the sample period were also performed (ZUUR et al., 2010). However, the correlation between the oocyte quantification variables (NDO and batch fecundity) and female parameters (Ow, TL and TW) was evaluated by applying the Spearman's Rank Correlation test (r_s). In the results, mean ± standard deviations are given between brackets. All tests were performed at a level of 0.01 or 0.05 when appropriate.

5.4 Results

5.4.1 Oocyte development and ovary organisation

The histological analyses of the *P. brasiliensis* ovaries showed the presence of the following oocyte stages: perinucleolar (Pg); cortical alveolar (CA); early vitellogenic (Vtg1); later vitellogenic (Vtg2); germinal vesicle migration (GVM); hydration (Hyd); early and late α-atresia; and early and late β-atresia (Figures 3–4). The differences among the oocyte stages are described in Table 1.

The analysis of the oocyte development stages and the presence of the post-ovulatory follicles (POF) during the peak of spawning suggested four ovary development phases: spawning capable, spawning capable subphase with GVM, spawning subphase with hydrated oocytes (hydration) and spawning capable with POFs (Figure 3). The oocyte stages Pg, CA, Vtg1 and Vtg2 were observed in all ovarian phases. Thus, females in spawning capable and spawning capable subphase with POFs were not ready to spawn and showed Vtg2 as the most advanced oocyte stage; however, the presence of the POFs was the main characteristic of the later phase which indicated that the females had already spawned in the current reproductive cycle. In the spawning capable subphase with GVM, the specimens were developmentally ready to spawn shortly in the current cycle as indicated by the presence of the oocytes in GVM; whereas in hydration, the presence of hydrated oocytes indicated that the females were close to spawn the next batch of oocytes.

5.4.2 Oocyte size-frequency distribution

The distribution of the oocyte diameters of *P. brasiliensis* showed a progressive increase in size from the oocytes in primary growth to the most advanced oocyte stages in all ovarian phases. On the one hand, no gap between pre-vitellogenic (Pg and CA) and vitellogenic oocytes diameters could be observed, which indicates asynchronous oocyte developing in *P. brasiliensis* where all types of oocytes appeared without dominant populations. On the other hand, a clear gap between later vitellogenic and hydrated oocytes was recorded in hydrated females. The most advanced mode (Hyd) indicated a new group of oocytes that will be

spawned in the subsequent batch, with the remaining oocytes forming the standing stock of oocytes from where the next batch will be developed (Figure 5).

Table 1. Histological description of the oocyte development stages in *Paralonchurus brasiliensis.* The mean oocyte diameter ± the standard deviation and the total number of oocytes measured (*n*) are provided between brackets.

Oocyte development stages	Histological description
Pre-vitellogenic oocyte growth	
Primary growth (Pg)	Unyolked oocytes with a basophilic cytoplasm stained with Haematoxylin and nucleus enlarged showing several nucleoli arranged at the periphery (112.2 µm ± 30.5, n=238).
Cortical alveolar (CA)	Small oil droplets and cortical alveolus begin to accumulate and disperse in the cytoplasm. The zona radiata, theca and granulosa cells become visible at this stage; however, yolk granules are still absent (207.1 µm ± 45.3, n=211).
Vitellogenic oocyte growth	
Early vitellogenic (Vtg1)	The oocyte begins to fill with protein yolk granules. The eosinophilic granules are arranged as a ring in the cytoplasm and the oil droplets start to increase in number and size. Cortical alveolus are visibly arranged at the periphery of the oocyte (280.5 µm ± 52.1, n=232)
Late vitellogenic (Vtg2)	Yolk granules increase in number and fill the cytoplasm. The oil droplets increase in size and distribute around the nucleus (402.8 µm ± 73.7, n=231).
Oocyte maturation	
Germinal vesicle migration (GVM)	The nucleus (germinal vesicle) starts to migrate toward the animal pole at the periphery of the oocyte, and yolk granules and oil droplets start to coalesce and rapidly increase in size. An oil globule larger than others is apparent in this stage (507.2 µm ± 24.7, n=488).
Hydration (Hyd)	The oocyte enlarges rapidly due to the intake of water and becomes translucent. The cytoplasm becomes homogeneous, which makes it difficult to identify the cell structures. Due to the histological process, the oocytes appear misshapen and stained lightly with eosin (693.9 µm ± 27.6, n=611).
Atresia	
Early α- Atresia	Yolk granules and oil droplets are clearly visible as in a healthy yolked oocyte; however, this stage is characterised by the fragmentation of the zona radiata and disintegration of the nucleus.
Late α- Atresia	The ZR is highly fragmented invading the cytoplasm. Disintegration of the nucleus is still visible, yolk granules are fused in the cytoplasm and oil droplets are fused as oil globules.
Early β- Atresia	The nucleus and yolk granules have already been absolved, some fragments of the ZR are visible, the granulosa cells are located inside the cell and numerous vacuoles start to appear.
Late β- Atresia	The cell has a small and irregular shape. The granulosa cells are almost absent inside the cell, the ZR has already been completely absolved and the vacuoles are larger and more numerous than the previous stage.

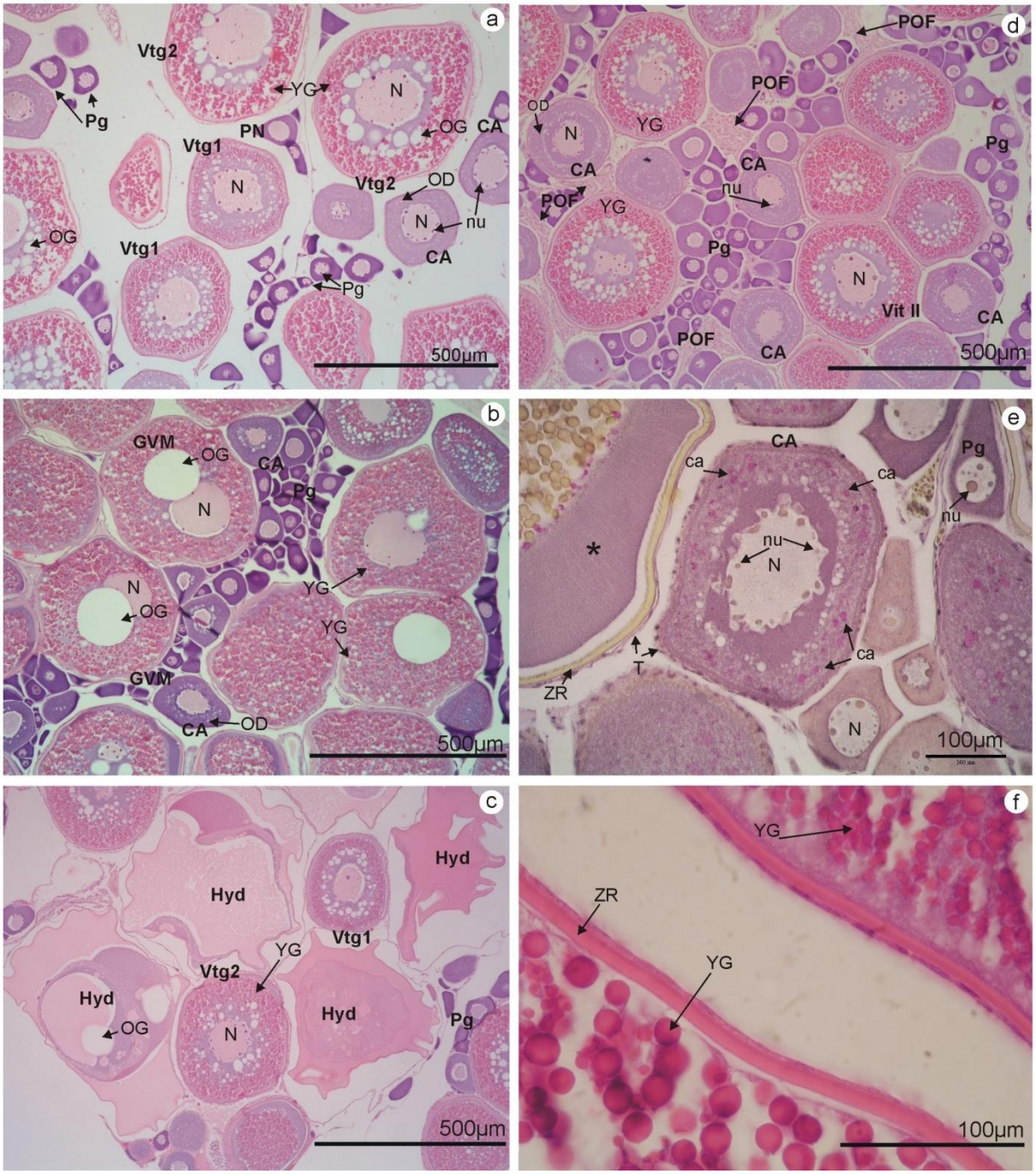


Figure 3. Photomicrographs of *Paralonchurus brasiliensis* ovaries. Ovarian development phases showing different oocyte stages. (a) spawning capable (Pg= primary growth; CA= cortical alveolar; Vtg1= early vitellogenic and Vtg2= later vitellogenic stage); (b) Spawning capable subphase with GVM; (c) Spawning capable subphase with hydrated oocytes (hydration); (d) Spawning capable with post-ovulatory follicle (POFs); (e) CA stage showing cortical alveolus; (f) Follicle layer (ZR= zona radiata; T= theca layer). N= nucleus, nu= perinucleolar nucleoli; ca= cortical alveolar; YG = yolk granules; OG= oil globules; OD= Oil droplets). * artefacts

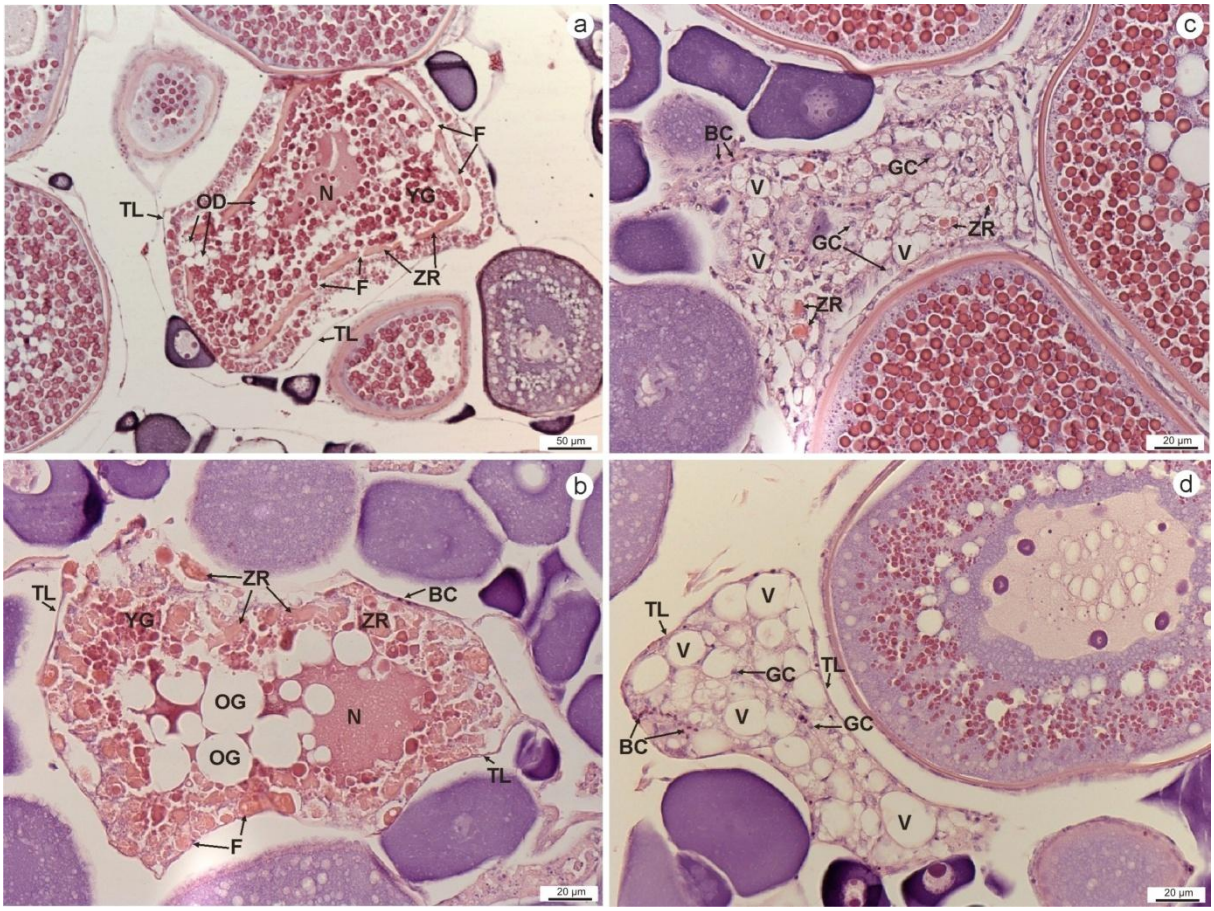


Figure 4. Histological sections of *Paralonchurus brasiliensis* ovaries showing different stages of atresia. (a) Early α-atresia; (b) late α-atresia, (c) early β-atresia, (d) late β-atresia. N= nucleus, ZR= zona radiata, F= spaces indicating fragmentation of the zona radiata, TL= theca layer, YG= yolk granules, OG= oil globules, OD= Oil droplets, BC= blood cell, GC= granulosa cell, V=vacuoles.

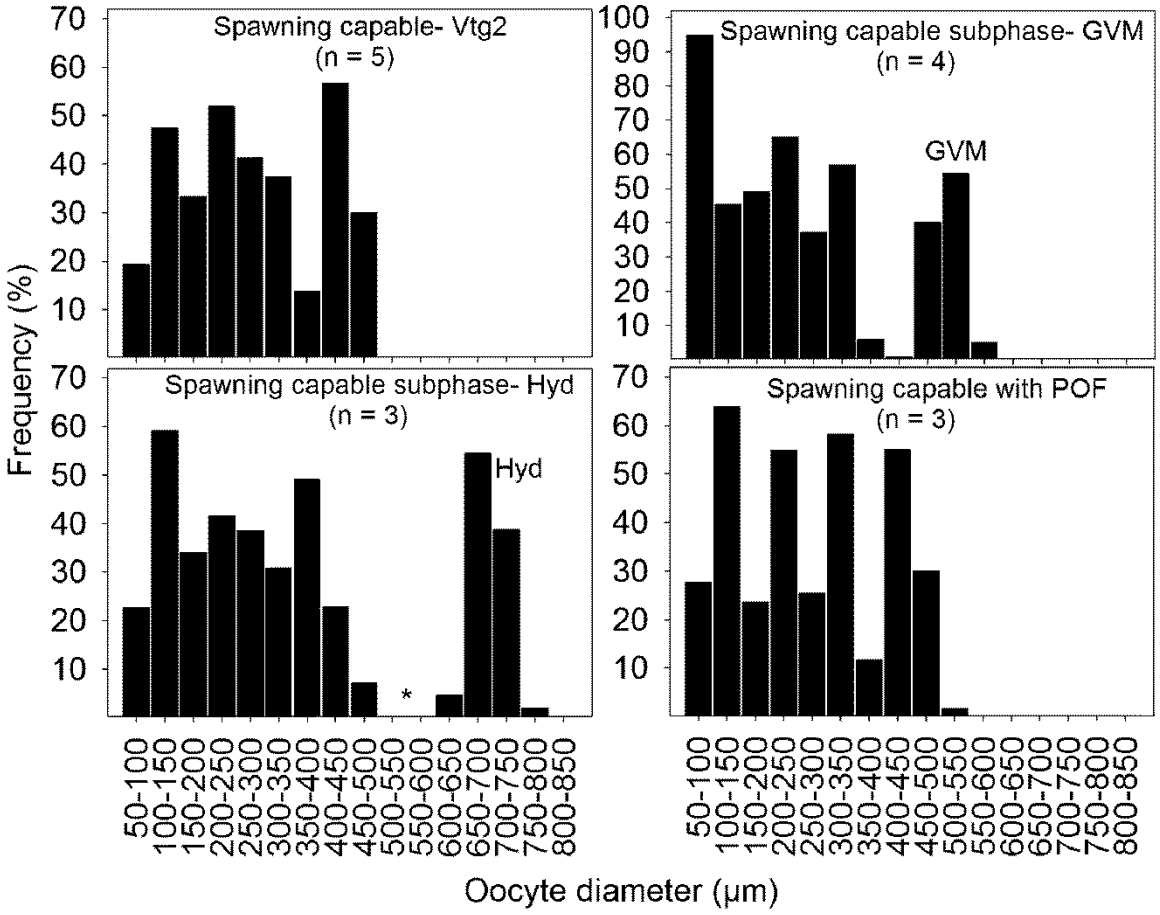


Figure 5. Oocyte size-frequency distribution in relation to ovarian development phases in *Paralonchurus brasiliensis*. GVM= germinal vesicle migration and Hyd= hydrated oocytes. The asterisk indicates a gap between the vitellogenic and hydrated oocytes and the number of ovaries analysed is given between brackets.

5.4.3 Oocyte diameter variation

In general, the oocyte diameters ranged from 57 µm in the Pg stage to 807 µm in the hydrated oocytes. The one-way ANOVA showed significant differences among the diameters of all oocyte stages independent of the ovarian phase ($p < 0.01$). Descriptive statistics of the oocyte diameters for the most advanced oocyte stage for each ovarian phase are summarised in Table 2.

A linear regression ($p<0.01$) between the diameter of the pre-vitellogenic (Pg, CA) and vitellogenic oocytes (Vtg1 and Vtg2) and their respective stages revealed a

significant increase in the oocyte sizes in relation to the oocytes development stages in each ovarian phase (Figure 6). Moreover, these oocytes showed significant variation when compared by phases, indicating that their sizes in the spawning capable subphase (GVM) are smaller than in the hydration, spawning capable with POF females and spawning capable phases (Table 3).

The seasonal analyses through linear regressions of the oocyte diameters by stage indicated a significant decrease in size from August (2012) to February (2013) in all oocyte stages analysed (Pg, CA, Vtg1 and Vtg2). Moreover, the mean oocyte diameters by stage also varied significantly by month (two-way ANOVA, $p < 0.01$, Supplementary Table S1). However, the Tukey post-hoc test indicated that the mean diameter of the most advanced stage (Vtg2) differed significantly from one month to the other (Figure 7; Supplementary Table S2).

Table 2. Mean, standard deviation (sd), number of oocytes measured (n) and minimum and maximum values (min-max) of the most advanced oocyte stage diameter with regards to the ovary development phase in *Paralonchurus brasiliensis.* The number of ovaries examined is given between brackets

Phases	Most advanced oocyte stage	Oocyte diameter (µm)	n	Min-max (µm)
Spawning capable	Late vitellogenic (Vtg2)	473.5 ± 49.9	62	282.1–541.4
Spawning capable subphase	Germinal vesicle migration (GVM)	507.2 ± 24.7	400	400.0–587.0
Spawning capable subphase	Hydrated (Hyd)	693.9 ± 27.6	611	543.8–807.5
Spawning capable with POF	Late vitellogenic (Vtg2)	435 ± 33.5	60	339.6–503.7

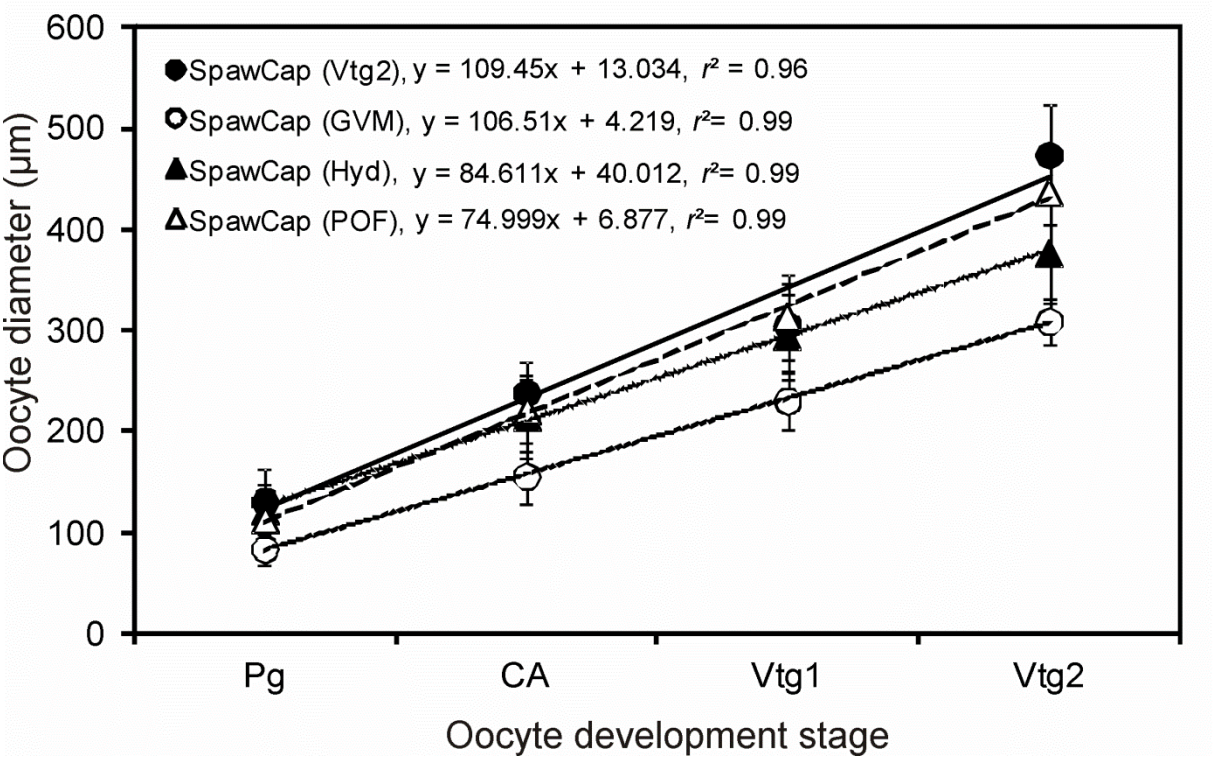


Figure 6. Variation in mean oocyte diameters of *Paralonchurus brasiliensis* in relation to oocyte stages (Pg= primary growth, CA= cortical alveolar, Vtg1= early vitellogenic, Vtg2= late vitellogenic). The linear regression by ovarian phases (spawning capable= SpawCap (Vtg2), spawning capable subphase with germinal vesicle migration= SpawCap (GVM), spawning capable subphase with hydrated oocytes= SpawCap (Hyd) and spawning capable with post-ovulatory follicles= SpawCap (POF)) are also shown. Bars represent ± standard deviations.

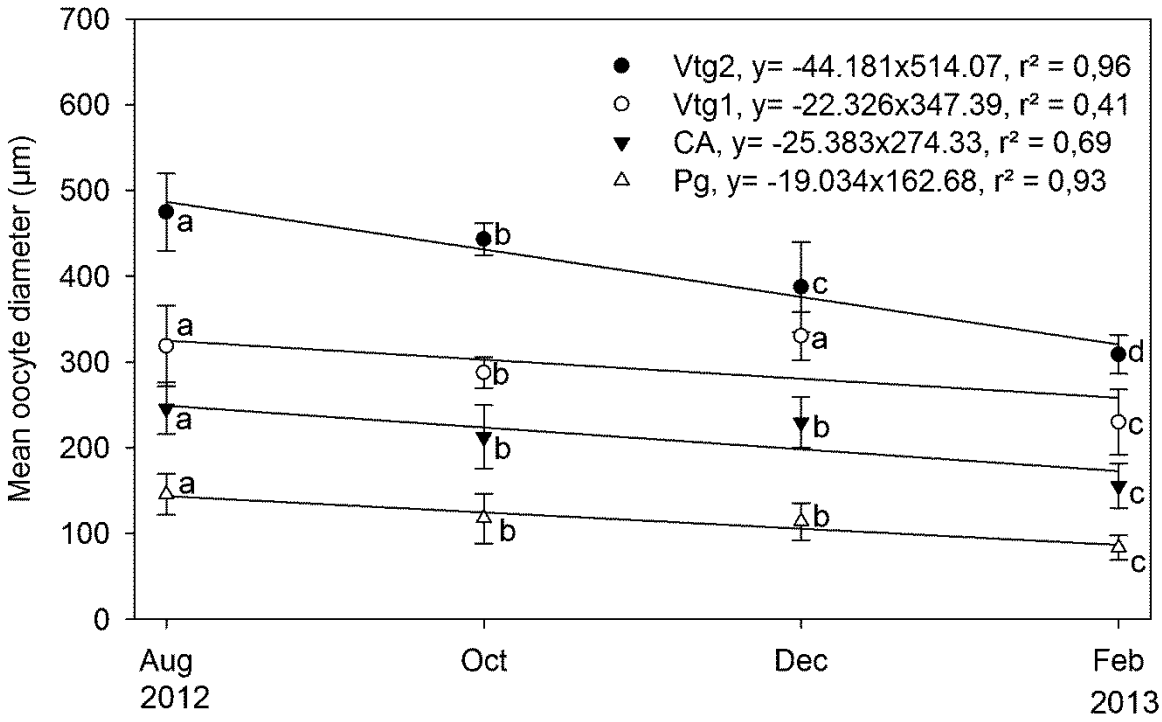


Figure 7. Monthly mean diameter of pre-vitellogenic and vitellogenic oocytes of *Paralonchurus brasiliensis* in ovaries in spawning capable phase, spawning capable subphase with germinal vesicle migration, spawning capable subphase with hydrated oocytes and spawning capable with post-ovulatory follicles. The linear regressions from the period Aug (2012) to Feb (2013) are also shown. Bars indicate the standard deviation. Different letters indicate significant differences at a level of 1% (Tukey post-hoc test) among months. Pg = primary growth, CA= cortical alveolar, Vtg1= early vitellogenic, Vtg2= late vitellogenic.

Table 3. Descriptive analyses results and comparisons of the *Paralonchurus brasiliensis* oocyte diameters among ovarian phases by stages: primary growth (Pg), cortical alveolar (CA), early vitellogenic (Vtg1) and late vitellogenic (Vtg2). GVM= germinal vesicle migration, Hyd= hydrated oocytes, POF= post-ovulatory follicle, *n* = number of oocytes measured, *sd* = standard deviation, *min-max* = minimum and maximum values, *df* = degrees of freedom. The number of ovaries examined is given between brackets. Different letters indicate significant differences at a level of 1% (Tukey post-hoc test).

Stages	Spawning capable (Vtg2)			Spawning capable subphase (GVM)			Spawning capable subphase (Hyd)			Spawning capable (POF)		
	n	Mean ± sd	Min - max	n	Mean ± sd	Min - max	n	Mean ± sd	Min - max	n	Mean ± sd	Min - max
Pg	57	130.8 ± 33.2 [a]	73.2–193.5	57	83.4 ± 14.5 [c]	57.0–153.2	66	120.9 ± 28.0 [ad]	63.7–198.6	58	112.2 ± 20.7 [b]	77.6–163.3
CA	54	238.0 ± 31.8 [a]	161.4–303.6	53	155.5 ± 25.7 [b]	108.4–254.5	53	214.5 ± 41.2 [c]	123.7–339.6	51	220.3 ± 31.8 [c]	157.9–288.6
Vtg1	51	304.4 ± 43.9 [a]	199.3–396.7	74	229.9 ± 28.2 [a]	166.9–303.4	52	294.4 ± 41.8 [a]	229.4–372.4	55	313.4 ± 42.9 [a]	205.9–405.1
Vtg2	62	473.5 ± 48.9 [ad]	282.1–541.4	51	308.6 ± 22.5 [c]	262.8–374.8	58	376.3 ± 49.5 [ab]	249.1–468.5	60	435.5 ± 33.5 [d]	339.6–503.7

One-way ANOVA $_{Pg}$; df = 3, F = 36.86, P < 0.01
One-way ANOVA $_{CA}$; df = 3, F = 47.31, P < 0.01
One-way ANOVA $_{Vtg1}$; df = 3, F = 32.63, P < 0.01
One-way ANOVA $_{Vtg2}$; df = 3, F = 23.51, P < 0.01

5.4.4 Occurrence of the ovary phases during the spawning season

The distribution of ovarian phases of *P. brasiliensis* by month revealed that the spawning capable and spawning capable with POF females were present in all sampled months. The occurrence of females in hydration was confirmed from August to December by the presence of spawning capable with hydrated oocytes. Thus, 35.6% of the all females sampled in October were hydrated. Females in the spawning capable subphase (GVM) were captured in August, October, February and April with a maximum in February (45.4%), which indicates that the oocytes in the GVM stage would develop and spawn in the next days, indicating a long spawning season (Figure 8) (see Supplementary Material Table S3 for details about the time of spawning).

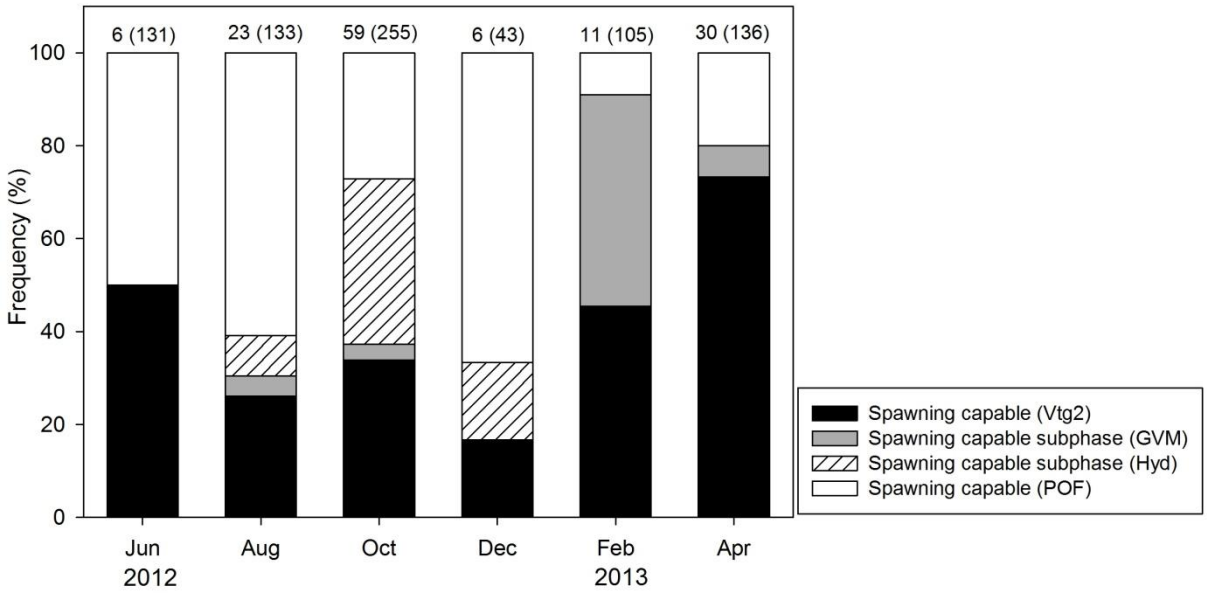


Figure 8. Variation of the ovarian development phases in *Paralonchurus brasiliensis* during the sample period. The number of females analysed histologically and the total number of species captured are provided in bold and between brackets, respectively. Vtg2= late vitellogenic oocyte stage, GVM= germinal vesicle migration, Hyd= hydrated oocytes, POF= post-ovulatory follicles.

5.4.5 Fecundity

The analyses applied to verify homogeneity in the oocyte distribution indicated that the oocytes were equally distributed in the ovaries (two-way ANOVA; F= 0.12; df= 2; p> 0.05) and sub-samples collected from the right lobe represented the entire ovary. Thus, from a total of 135 females analysed histologically, twenty-four ovaries from females between 162 and 223 mm were utilised to estimate the NDO, whereas eighteen females measuring between 157 and 240 mm were utilised for batch fecundity. The rest of the ovaries were excluded due to the presence of post-ovulatory follicles. No significant differences were found between the total length (t = -0.39; df = 40; p>0.05) and weight (t = -1.10; df = 40; p>0.05) of the females utilised to estimate fecundity.

The linear regression model adequately described the relationship between the ovary weight and ovary volume estimated using Scherle's method (Ov = 0.969 (0.006, se) x Ow, r^2 = 0.99, p<0.01). The stereological parameters K and β were estimated to be 0.97 and 1.16, respectively. The NDO ranged from 13,326 to 108,311 (48810 ± 22117) oocytes and the relative RNDO ranged from 224 to 1,789 (902 ± 368.9) oocyte g^{-1} fish. The values of BF ranged from 4,839 to 36,544 (18925 ± 9521) oocytes and the RBF ranged from 144 to 622 (321 ± 129.2) oocyte g^{-1} fish. In general, the average NDO (48810 oocytes) was 2.6 times higher than the average BF (18925 oocytes), which indicates on average a female in the developing phase contains approximately 3 potential spawning batches. Moreover, the values of the NDO and BF fecundity showed a positive and significant relationship with the ovary weight, total length and total weight (Figure 9).

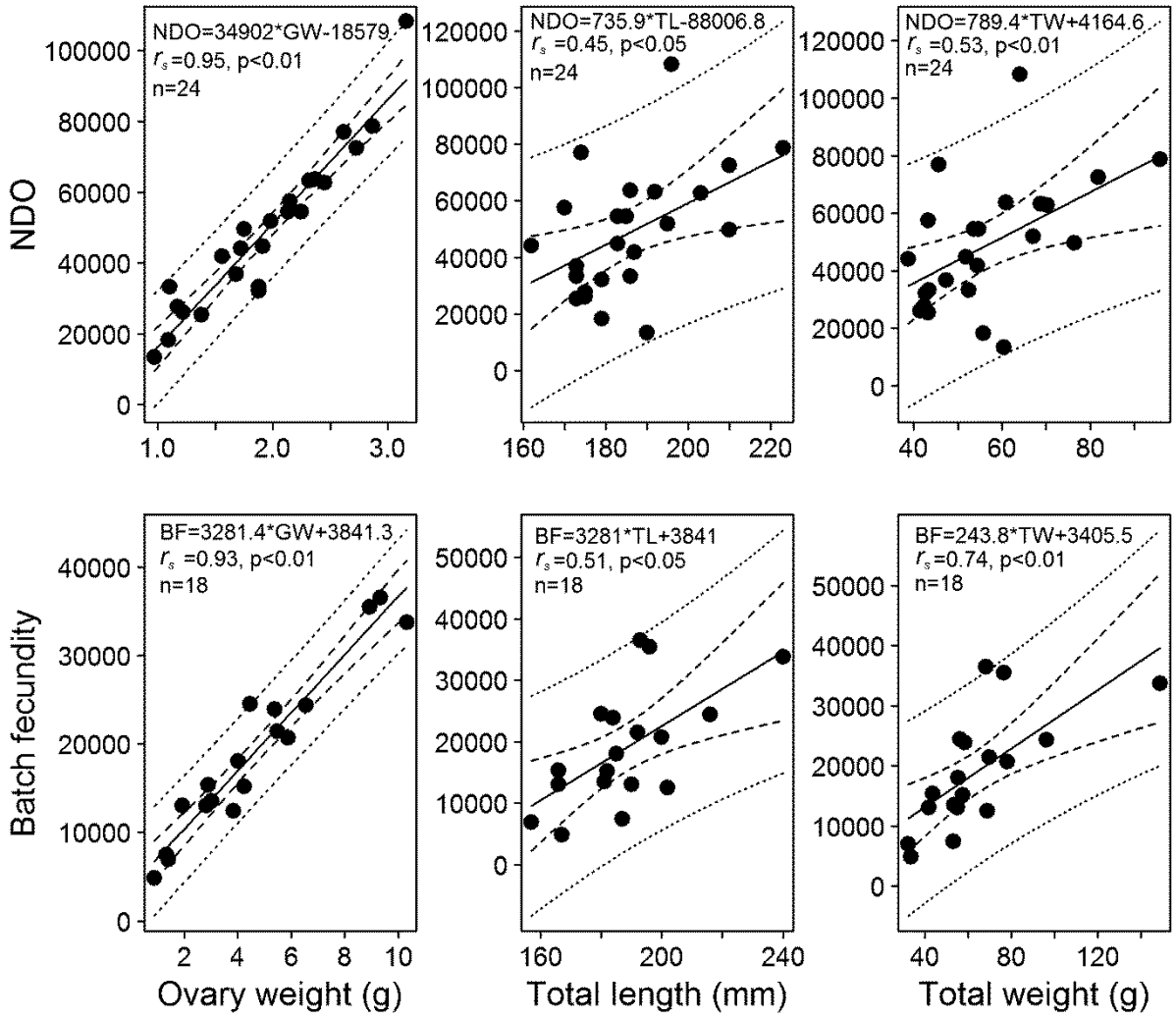


Figure 9. Relationship between fecundity (number of developing oocytes – NDO and Batch fecundity) and parameters of females of *Paralonchurus brasiliensis* (ovary weight, total length and total weight). A 95% confidence interval (---), 95% prediction interval (···), p-value, number of ovaries sampled (n) and Spearman's Rank Correlation (ρ) are also given.

5.5 Discussion

5.5.1 Oocyte development and ovary organisation

An accurate evaluation of ovarian development is essential to describe the maturation process, the period of spawning and oocyte recruitment (LOWERRE-BARBIERI et al., 2011). In general, the ovary of the teleost fish species follows the same pattern of oocyte development stages, such as primary growth, cortical

alveolar, vitellogenesis and maturation (WALLACE; SELMAN, 1981; WEST, 1990; TYLER; SUMPTER, 1996; MURUA; SABORIDO-REY, 2003).

In general, the atresia process in *P. brasiliensis* showed similar degeneration patterns as described by Hunter and Macewicz (1985) for *Engraulis mordax* (Engraulidae), with *α*-atresia characterised by the total resorption of the yolk granules and *β*-atresia by the major degeneration of the granulosa and theca cells. However, the absence of other advanced stages, such as *gamma* and *delta* that are characterised by the increased pigmentation, can indicate that the duration of the most advanced atresia stages in *P. brasiliensis* are very short or the follicles are completely resorbed during the *beta* stage, following the second pattern of β-atresia described by Hunter and Macewicz (1985). Another hypothesis could be that the time that is needed for atretic follicles to be fully degenerated and reach the gamma and delta stages over-exceeds the lifetime of the studied population (GANIAS et al., 2008).

The simultaneous occurrence of pre-vitellogenic (Pg and CA) and vitellogenic oocytes (Vtg1 and Vtg2) in all ovarian phases also was observed in *P. brasiliensis*. The presence of all oocyte developmental stages mixing at the simultaneously time without a dominant population can be found only in ovaries with asynchronous ovarian development (TYLER; SUMPTER, 1996; MURUA, SABORIDO-REY, 2003). Thus, these findings suggest an asynchronous ovary organisation for *P. brasiliensis* as registered for several fishes such as the sciaenid *Micropogonia undulatus* in Chesapeake Bay (BARBIERI et al., 1994), *Pollachius pollachius* in the Galician coast (ALONSO-FERNANDEZ et al., 2013), the macrourid fish (*Nezumia aequalis* and *Coelorinchus mediterraneus*) in the Mediterranean continental margin (FERNANDEZ-ACAYA et al., 2013) and *Argyrosomus regius* in the Balearic Islands (GIL et al., 2013).

5.5.2 Oocyte size-frequency distribution

Oocyte size-frequency distribution has been widely applied for investigating the fecundity type (determinate or indeterminate) and oocyte recruitment of many fishes (GRANDE et al., 2012; GANIAS, 2013, ZUDAIRE et al., 2013a). On the one hand, in fishes with indeterminate fecundity, the standing stock of pre-vitellogenic

oocytes can develop and be recruited into yolked oocyte stock at any time and is expected to be a more common pattern in tropical and sub-tropical fishes that show a long spawning period (HUNTER; GOLDBERG, 1980; KJESBU, 2009). On the other hand, in fishes with determinate fecundity, the potential fecundity is fixed prior to the onset of spawning and usually reflects a short spawning period (MURUA; SABORIDO-REY, 2003; KJESBU, 2009). Thus, in this study, the oocyte size-frequency distribution of *P. brasiliensis* indicated continuous growth from the developing phase up to the spawned females showing no gap (hiatus) between pre-vitellogenic and vitellogenic oocytes. According to Ganias (2013), a lack of gap between pre-vitellogenic and vitellogenic oocyte modes in the oocyte size-frequency distribution does not necessarily imply indeterminacy for a fish species, which can lead to mistakes in the choice of method for estimating fecundity and stock biomass. This lack of gap was also observed for fishes with determinate fecundity by Walker et al. (1994), Tyler et al. (1994) and Alonso-Fernández et al. (2008). However, this evidence is not an unequivocal sign of indeterminate fecundity (ZUDAIRE et al., 2013a), but additional criteria, such as variation in the diameter of the most advanced stage and levels of atresia during the spawning season, have also been applied (HUNTER at al., 1992; GORDO et al., 2008; KJESBU, 2009; GRANDE et al., 2012; ZUDAIRE et al., 2013a).

5.5.3 Oocyte diameter variation

The significant increase in mean oocyte diameter along with the oocyte development among ovarian phases also indicates continuous oocyte recruitment during the sample period. This is corroborated by the analysis of the oocyte size-frequency distribution, which showed continuous modes from primary growth through the most advanced stage in each ovarian phase. The relationship between the mean oocyte diameter and the volume-based oocyte diameter by stage in studies of oocyte packing density theory (OPD) applied to indeterminate species show a similar pattern (KURITA; KJESBU, 2009; KORTA et al., 2010b; SCHIMENOU et al., 2012). Differences in the diameter of pre-vitellogenic and vitellogenic oocytes among ovarian phases must be related with the rate at which the oocytes are recruited to form the next cohort in each ovarian phase, showing smaller sizes in spawned fishes

(spawning capable with POFs) and those females with oocytes in maturation (GVM and Hyd).

The mean oocyte diameter can change during the spawning season and is related to the fecundity type of the species (GRANDE et al., 2012). In accordance with Hunter et al. (1992) and Murua and Saborido-Rey (2003), for fishes with determinate fecundity, a seasonal increase in the mean diameter of the advanced vitellogenic oocytes is expected due to a lack of oocytes to replace those that have been spawned. However, a significant decrease in oocyte size from August (2012) to February (2013) in all oocyte stages analysed was observed in this study, suggesting the replenishment of newly formed oocytes to the standing stock of the advanced vitellogenic oocytes as described by Hunter et al. (1992). This supports the idea that *P. brasiliensis* shows an indeterminate fecundity indicating that new yolked oocytes are recruited into the new yolk class. A similar pattern using the advanced vitellogenic stage during the spawning season was observed by Murua and Motos (2006) for *Merluccius merluccius* and Zudaire et al. (2013a) for *Thunnus albacares*.

5.4.4 Occurrence of the ovary phases during the spawning season

Previous studies using the gonadosomatic index (GSI) have shown that the spawning season of *P. brasiliensis* in the inner Brazilian shelf ranged from July to December for the south-eastern population (PAIVA-FILHO; ZANI-TEIXEIRA, 1980; PAIVA-FILHO; ROSSI, 1980; VAZZOLER et al., 1999) and August to March for the southern population (LEWIS; FONTOURA, 2005). The occurrence of mature females in different phases for all months sampled was registered in this study. Although the samples of *P. brasiliensis* have not covered the whole year, the histological examination of those ovaries suggest that the spawning season is longer than previously reported, showing peaks of spawning from August to December indicated by the presence of hydrated females. This longer spawning season could be related with a reproductive strategy that is more common in fishes with indeterminate fecundity (KJESBU, 2009).

5.5.5 Fecundity

In the present study, the NDO estimated using stereological methods ranged from 13,326 to 108,311 oocytes. Paiva-Filho and Rossi (1980), in a study conducted in the south-eastern region of Brazil, estimated the number of oocytes in the later vitellogenic stage to be between 12,110 and 151,320 oocytes (through the application of the volumetric method, but without a prior histological examination of the ovaries). Although Paiva-Filho and Rossi (1980) may have counted oocytes in early and later vitellogenesis as the most advanced stages, the NDO estimated stereologically in the present study was similar to those estimated by these authors. Moreover, fecundity is affected by several factors such as the year, environmental conditions, fish condition, fish size and when the samples were collected (HUNTER et al., 1985; RIJNSDORP, 1991; WITTHAMES et al., 1995; KJESBU et al., 1998).

The values of batch fecundity (hydrated oocytes being the most advanced stage) estimated for *P. brasiliensis* in the Ubatuba region were similar to those estimated using the gravimetric method for the population in the Buenos Aires coastal zone. In this study the BF ranged from 4,839 to 36,544 with an average of 18,925 oocytes, whereas in the Argentine waters it ranged from 5,560 to 32,400 with a mean of 18,683 oocytes (MITITELLI et al., 2013). However, the relative BF was higher for the Ubatuba population than for the Argentine, with a mean of 199 oocyte g^{-1} fish (ovary-free weight). This difference can be attributed to a narrow size range of females sampled by Militelli et al. (2013; from 19 to 21 cm in total length) when compared with those of this study.

Fecundity is usually proportional to fish size, weight and condition, those being the key parameters to assess fecundity at the population level (KJESBU, 1991; MURUA et al., 2003). In general, *P brasiliensis* showed a significant relationship between fecundity (NDO and BF) and the female's parameters (ovary weight, total length and total weight). However, the gonad weight explained 93% of the fecundity variability, which indicates a larger explanatory power than female size and weight for quantifying the reproductive condition of *P. brasiliensis*. A similar pattern was observed by Fernadez-Arcaya et al. (2012) for *Trachyrincus scabrous* and by *Fernadez-Arcaya* et al. (2013) for *Nezuma aequalis*.

The histological and stereological techniques provided a complete analysis of the reproductive status of *P. brasiliensis*, including the correct identification and classification of the oocytes, spawning status of the females through the analysis of post-ovulatory follicles, fecundity and oocyte size. Another advantage of these methods is that the images of the ovaries can be kept for a long time and be used in futures studies.

5.6 Conclusions

In conclusion, the results showed, for the first time, that *P. brasiliensis* has an oocyte development similar to that of other teleosts inhabiting tropical and subtropical waters with asynchronous ovarian organisation. The oocytes are recruited in all ovary phases during the spawning season, and their sizes decrease with the elapsed spawning season, thus showing that the fecundity of *P. brasiliensis* could be of an indeterminate type strategy where batch fecundity and spawning fraction should be measured for fecundity studies. The species has a long spawning season, and the batch fecundity ranges from 4,839 to 36,544 oocytes per female measuring between 157 and 240 mm.

The knowledge obtained from this study can help to better understand the reproductive strategy of *P. brasiliensis* inhabiting shrimp fishery grounds. Despite their ecological importance, this species is often captured as by-catch by shrimp fishery trawls in the inner shelf of São Paulo state, Brazil. Thus, the by-catch pressure on the population may result in the need to assess the stock and these reproductive parameters are essential to an assessment.

6.6 Supplementary Material

For: **Reproductive strategy and fecundity of the keystone species *Paralonchurus brasiliensis* (Teleostei, Sciaenidae): An image processing techniques application.**

Appendix A. Supplementary tables.

Table S1. Two-way ANOVA to analyse the seasonal variation in oocyte size by development stage. SS= sum of squares, MS= mean square, df= degrees of freedom, Significant p-values are highlighted in red.

Effect	SS	*df*	MS	*F*	p
Month	1.19E+06	3	3.98E+05	347	0.000
Oocyte stages	9.36E+06	3	3.12E+06	2720	0.000
Month*Oocyte stages	2.40E+05	9	2.67E+04	23	0.000

Table S2. One-way ANOVA and post-hoc test applied to analyse the seasonal variation in oocyte size by development stage. SS= sum of squares, df= degrees of freedom, p= alpha level of 0.008 (Bonferroni Correction). Significant p-values are highlighted in red.

Primary growth (Pg)				
One-way ANOVA				
Effect	SS	*z*	*F*	p
Month	83800	3	47.74	0.000
Tukey HSD post-hoc test				
	Aug_2012	Oct_2012	Feb-2013	Dec_2012
Aug_2012		0.000008	0.000008	0.000008
Oct_2012	0.000008		0.000008	0.816733
Feb_2013	0.000008	0.000008		0.000008
Dec_2012	0.000008	0.816733	0.000008	
Cortical alveolar (CA)				
One-way ANOVA				
Effect	SS	*df*	*F*	p
Month	219000	3	71.35	0.000
Tukey HSD post-hoc test				
	Aug_2012	Oct_2012	Feb-2013	Dec_2012
Aug_2012		0.000008	0.000008	0.133957
Oct_2012	0.000008		0.000008	0.041297
Feb_2013	0.000008	0.000008		0.000008
Dec_2012	0.133957	0.041297	0.000008	
Early vitellogenic (Vtg1)				
One-way ANOVA				
Effect	SS	*df*	*F*	p
Month	326000	3	82.56	0.000
Tukey HSD post-hoc test				
	Aug_2012	Oct_2012	Feb-2013	Dec_2012
Aug_2012		0.000012	0.000008	0.592744
Oct_2012	0.000012		0.000008	0.000016

Continued

Feb_2013	0.000008	0.000008		0.000008
Dec_2012	0.592744	0.000016	0.000008	

Late vitellogenic (Vtg2)

One-way ANOVA

Effect	SS	*df*	*F*	p
Month	876000	3	176.9	0.000

Tukey HSD post-hoc test

	Aug_2012	Oct_2012	Feb-2013	Dec_2012
Aug_2012		0.000008	0.000008	0.000533
Oct_2012	0.000008		0.000008	0.000008
Feb_2013	0.000008	0.000008		0.000008
Dec_2012	0.000533	0.000008	0.000008	

Table S3*.* Sampling date, time, season and the environmental variables, bottom temperature (BT) and bottom salinity (BS), and depth measured during the period of study in the inner shelf of Ubatuba, São Paulo State, Brazil. GVM= germinal vesicle migration and Hyd= hydrated females.

Sampling date (yyyy-mm-dd)	Sampling time (hh:mm)	GVM/ Hyd	Season	BT (°C)	BS	Sampling depth (m)
2012-06-25	16:23		Autumn	22.6	35.0	20.60
2012-06-25	9:00		Autumn	22.2	34.0	14.80
2012-06-25	15:46		Autumn	22.3	36.0	13.10
2012-06-27	9:30		Autumn	22.2	34.0	16.00
2012-06-27	14:46		Autumn	22.0	35.0	15.50
2012-06-28	8:43		Autumn	22.4	35.0	15.50
2012-08-21	16:05		Winter	21.0	36.6	15.60
2012-08-21	16:05		Winter	21.0		15.60
2012-08-23	8:41	GVM	Winter	19.8	37.2	15.00
2012-08-23	15:49		Winter	22.1	36.5	9.80
2012-08-24	9:40	Hyd	Winter	20.9	36.4	10.50
2012-08-24	15:22		Winter	21.5	36.0	7.70
2012-10-24	9:03	Hyd+POF	Spring	21.3	36.0	12.00
2012-10-24	15:52	Hyd	Spring	21.2	36.0	11.00
2012-10-25	8:58		Spring	21.5	36.0	8.00
2012-10-25	15:52		Spring	20.6	36.0	10.30
2012-10-26	8:49	GVM	Spring	21.5	36.0	10.80
2012-10-26	13:50	GVM, Hyd	Spring	21.1	36.0	10.00
2012-12-11	8:52		Spring	23.0	35.0	9.60
2012-12-11	15:56	Hyd	Spring	17.6	36.0	12.60
2012-12-12	8:37		Spring	17.2	36.0	13.70
2012-12-12	14:33		Spring	21.5	36.0	12.10
2012-12-13	8:43		Spring	25.3	36.0	10.00
2012-12-13	15:34		Spring	23.3	36.0	10.30
2013-02-23	8:42		Summer	28.4	36.0	7.20
2013-02-23	14:49		Summer	27.9	36.0	9.70
2013-02-24	8:48		Summer	27.6	36.0	9.70
2013-02-24	15:24		Summer	28.8	36.0	10.30
2013-02-25	8:56		Summer	28.5	36.0	9.30
2013-02-25	15:41	GVM	Summer	28.3	37.0	10.40
2013-04-26	15:38		Autumn	24.0	36.0	9.90
2013-04-29	9:08	GVM	Autumn	24.0	36.0	8.00
2013-04-29	15:58		Autumn	24.3	36.0	8.40
2013-04-30	8:30		Autumn	24.5	36.5	9.60
2013-04-30	15:24	GVM	Autumn	24.5	35.8	8.60

Chapter IV

Do congeneric fish species inhabiting different ecosystems exhibit the same oocyte production and recruitment pattern?

6.1 Abstract

Information on oocyte production and recruitment in phylogenetically related species can help to understand the evolution in reproductive life history traits in multiple spawning fishes. The present study compared, for the first time, the oocyte production and recruitment patterns between two congeneric species *Stellifer brasiliensis* and *S. rastrifer* (Perciformes, Sciaenidae) in southwestern Atlantic, Brazil. The specimens of *S. brasiliensis* and *S. rastrifer* were sampled in the inner shelf of Ubatuba and in the estuarine system of Cananéia, respectively. The ovaries were investigated using histology and model-based stereology. The results revealed that total number of oocytes per individual (*N*) and stage-specific oocyte packing density did not differ significantly between *S. brasiliensis* and *S. rastrifer*. The females' total weight, total length and ovary weight were positively correlated with *N* of pre-vitellogenic and vitellogenic oocytes in both species. The analysis of the oocyte recruitment across their development stage showed that 13.1% of the standing stock oocytes larger than 50 µm in *S. brasiliensis* and 8.8% in *S. rastrifer* will develop to form the next batches up to the final maturation, generating 22–29 batches of oocytes in different development stages. The similarities between *S. brasiliensis* and *S. rastrifer* could be attributed to their phylogenetic relationship, exhibiting the same oocyte production and recruitment pattern.

Key words: *Stellifer brasiliensis*, *S. rastrifer*. Sciaenidae. Ovarian maturation. Histology. Model-based stereology.

6.2 Introduction

Comparing the reproductive traits among congeneric species from different environments is very important for understanding the evolution in fish life history (PARTRIDGE; HARVEY, 1988; WIEDMANN et al., 2014). This kind of information can also be used to assess the sensitivity and vulnerability of marine ecosystems under exploitation and environmental change (WIEDMANN et al., 2014). Furthermore, a simple measure of the life history of marine fishes can provide a useful tool for predicting rates of recovery from low population abundance (DENNEY et al., 2002).

The congeneric species *Stellifer brasiliensis* (Schultz, 1945) and *S. rastrifer* (Jordan, 1889) (Perciformes, Sciaenidae) are usually found in coastal and estuarine areas over sand or muddy bottoms, as the other species of this genus; however, the former species is restricted to the Brazilian coast from Bahia to Santa Catarina, whereas the late occurs in the western and southwest Atlantic Ocean (MENEZES; FIGUEIREDO, 1980b; MENEZES et al., 2003; PINA; CHAVES, 2009). These fishes feed mainly on crustaceans, sharing the same habitat during some part of their life cycle, e.g. larvae and juveniles of both species have been recorded in coastal and estuarine waters in the southeastern region, Brazil (SOUZA et al., 2008; POMBO et al., 2012; SCHMIDT; DIAS, 2012; PORCARO et al., 2014). However, the adults of *S. brasiliensis* can reach 180 mm in total length and they are associated with shallow coastal waters, showing peaks of reproductive activity in the spring (MENEZES; FIGUEIREDO, 1980b; RODRIGUES-FILHO et al., 2011). On the other hand, *S. rastrifer* can reach 212 mm in total length, being abundant in estuaries where the spawning takes place during the spring and winter (CHAVES; VENDEL, 1997b; CAMARGO; ISAAC, 2005; PINA; CHAVES, 2009).

Estimating and understanding variation in the oocytes production in fish is challenging because of the interaction between environmental, demographic and genetic influences on the reproductive traits (reproductive potential) (WRIGHT, 2013). Several studies have showed divergences in the final oocyte production among specimens of the same species or among closely related species, and attributed such differences to these factors (LAMBERT et al., 2003; NISSLING; DAHLMAN, 2010; McELROY et al., 2013; McBRIDE et al., 2013; NISSLING et al.

2014). Therefore, using oocytes in advanced development stages for understanding the evolution of reproductive life history traits in fishes must be biased. Thus, underlying production of oocyte and recruitment are still poorly understood, mainly due to the difficult for quantifying the pre-vitellogenic oocytes using traditional methods (KJESBU, 2009; KORTA et al., 2010b; GANIAS et al., 2015).

Information on oocyte production and recruitment in phylogenetically related species can help to understand the evolution in reproductive life history traits in multiple spawning fishes (WIEDMANN et al., 2014). Thus, the present study compared, for the first time, the oocyte production (pre-vitellogenic and vitellogenic oocytes) and its recruitment patterns between two congeneric species, *Stellifer brasiliensis* and *S. rastrifer*, sampled in two different ecosystems using histological and stereological techniques.

6.3 Materials and Methods

6.3.1 Areas of study

The coastal system of Ubatuba and the estuary of Cananéia are located in the south-west Atlantic Ocean, São Paulo State, Brazil (Figure 1). Both areas were established as MPA (Marine Protected Area) by the Brazilian Ministry of the Environment for their sustainable use, preservation and conservation of the marine living resources (Proclamation No. 53525 and 53527, on 8 October 2008).

6.3.1.1 Coastal of Ubatuba

This area is located in the northern coast of the São Paulo state, and it is strongly influenced by three water masses: Tropical Waters (TW) with high temperature and salinity (T > 20 °C and S > 36); the South Atlantic Central Water (SACW) with low temperature and salinity (T < 20 °C and S < 36), and the Coastal Water (CW) with high temperature and low salinity (T > 20 °C and S < 36) (CASTRO-FILHO; MIRANDA, 1998). However, the seasonal presence of the SACW during the summer and spring months is the main factor influencing the environmental conditions in this area (PIRES-VANIN; MATSUURA, 1993). A strong thermocline is established during the SACW intrusion in isobaths from 10 to 50 m (PIRES-VANIN; MATSUURA, 1993). On the other hand, during the autumn and winter, the SACW

retreats toward the margin of the continental shelf and is replaced by the CW (CASTRO-FILHO; MIRANDA, 1998). Thus, these water masses affect the nourishment condition and the reproduction of species inhabiting in this ecosystem (MATSUURA, 1990; MATSUURA et al., 1992; KATSURAGAWA et al., 1993; MYASHITA et al., 2011)

6.3.1.2 Estuary of Cananéia

The Cananéia estuarine system is located in the southern coast of the São Paulo State, near the latitudinal limit for mangroves (25° S). This area is shaped mainly by the Cardoso, Cananéia and Comprida islands. These islands are separated by channels and rivers with a maximum depth of 12 m that flow into the ocean (SCHAEFFER-NOVELLI; CINTRÓN-MOLERO, 1990). The tides are semidiurnal (with diurnal inequality) with mean amplitude of 0.82 m, and 1.25 m for spring tides (MESQUITA; HARARI, 1983). The environment is controlled mainly by the intrusion of the tides, freshwater runoff and pluvial input. Thus, the salinity and temperature ranges from approximately 16 to 34 and 19 to 28 °C, respectively. The cumulative monthly rainfall is never less than 80 mm showing peaks during the summer and minima during the winter (SILVA, 1989; SCHAEFFER-NOVELLI; CINTRÓN-MOLERO, 1990).

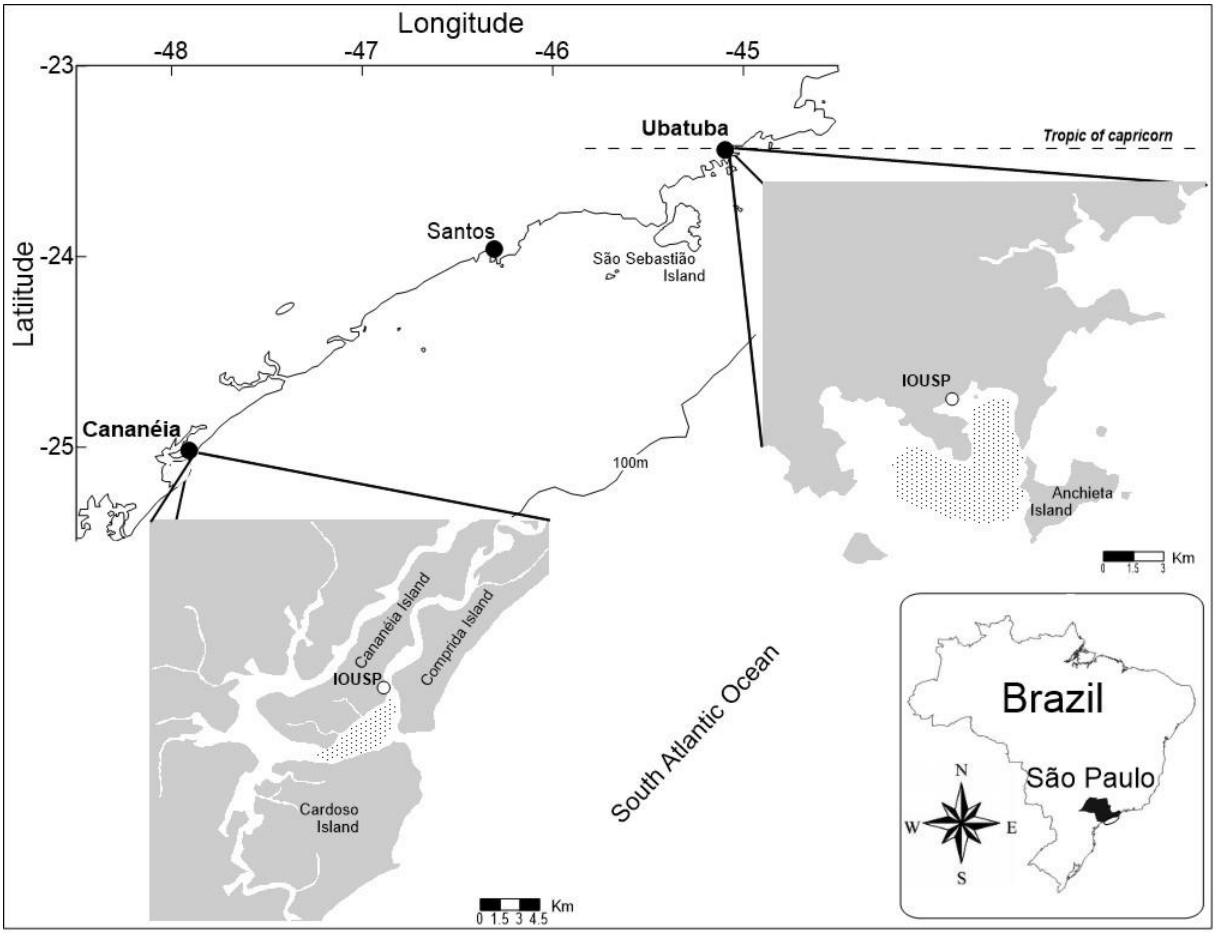


Figure 1. Coastal of Ubatuba and the estuary of Cananéia showing the sampled areas. IOUSP: research base of the Instituto Oceanográfico da Universidade de São Paulo, Brazil.

6.3.2 Sampling strategy

The fishes were captured between June (2012) and May (2013), during prospecting research cruises using an otter trawl. The trawls were carried out twice per day (between 08:00-10:30 am and 13:30-17:00 pm) on three consecutive days in each sampled month. Each station was trawled over 15 min in depths that ranged from 1.9 m in the estuary to 20.6 m in the coastal of Ubatuba. After trawling, the specimens were immediately taken to the laboratory to measure the total length (to the nearest millimetre), weigh total weight (to the nearest gram, 0.01 g), identify the maturity phase by macroscopic examination, and remove the ovaries. Then, the ovaries were weighed and fixed in 10% formalin buffered with sodium phosphate

monobasic ($NaH_2PO_4 \cdot H_2O$) and dibasic ($Na_2H_2PO_4$) to ensure the adequate preservation of the oocytes (HUNTER, 1985).

Ten ovaries by species were sampled for histological analysis, taking only ovaries from females in spawning capable phase, i.e. females capable of spawning within the current spawning cycle (BROWN-PETERSON et al., 2011). Thus, the specimens of *S. brasiliensis* were larger than *S. rastrifer* ($U=25$, $df=9$, $p<0.05$). On the other hand, the total weight and ovary weight did not differ significantly between species (Table 1) (Table 1 in appendix A, Supplementary material).

Table 1. Mean ± standard deviation (sd), minimum (Min) and maximum (Max) for biological variables measured in ten specimens of *Stellifer brasiliensis* and *S. rastrifer*.

Biological variables	*Stellifer brasiliensis*		*S. rastrifer*	
	Mean ± sd	Min–max	Mean ± sd	Min–max
Total length (mm)	153.8 ± 16.6	126–174	133 ± 11.2	111–149
Total weight (g)	43.1 ± 14.1	20.8–64.2	30.5 ± 9.1	16.4–44.6
Ovary weight (g)	1.93 ± 1.31	0.3–4.5	1.36 ± 0.8	0.44–2.7

6.3.3 Histological analysis

All histological preparations were carried out with three to four sub-samples from the right ovary (anterior, middle and posterior portion), containing the whole thickness of the ovarian wall. For doing histology, the tissue were dehydrated, cleared in xylol, embedded in paraffin blocks, sectioned at 5 µm and stained with Harris' Hematoxylin and Eosin. Periodic Acid-Schiff's, counterstained with Hematoxylin and metanil yellow (PAS-MY), was also performed for correct identification of cortical alveolus (GARCIA-SEOANE et. al, 2014).

6.3.4 Oocyte classification

The oocyte stages were classified based on the histological criteria established by Wallace and Selman (1981) and Tyler and Sumpter (1996). However, oocytes in primary growth were classified as early and late primary growth (Pg1 and Pg2) and those oocytes in vitellogenic growth were divided into three development

stages (Vtg1, Vtg2 and Vtg3). The ovaries containing the most development stages such as germinal vesicle migration and hydrated oocytes were not analysed in this study due to their shrinkage in the histological sections, which could affect the final result overestimating the stereological variables, e.g. the volume fraction, as observed by Schismenou et al. (2012) and Saber et al. (2014). The descriptions of each oocyte stage are detailed in Table 2 (Figure 1 in appendix B, Supplementary material).

6.3.5 Oocyte size measurements and distribution

At least 50 oocytes sectioned through the nucleus, per development stage, were selected to measure the individual oocyte diameter (OD_{ind}) and to estimate the volume-based mean oocyte diameter (OD_{vi}) (KORTA et al., 2010b). The number of oocytes measured was based on the work published by Aragón et al. (2010), which observed that the oocyte size distribution became stable after 40 measurements. Thus, OD_{ind} was calculated by the arithmetic mean of the longest (L) and shortest (S) diameter measured in the histological section (OD_{ind}= (L+S)/2), and OD_{vi} as follows: $\mathrm{OD_{vi}} = \left[\sum_{j=1}^{n_i} \frac{(\mathrm{OD_{(ind)i,j}})}{n_i}\right]^{1/3}$. All oocytes were measured using the software ImageJ (RASBAND, 1997-2009). The oocyte diameters were grouped in classes of 40 µm and plotted to analyse the size frequency distribution.

Table 2. General histological description of the oocyte development stages for *Stellifer rastrifer* and *S. brasiliensis*.

Development stages	Histological description
Pre-vitellogenesis	
Early primary growth (Pg1)	Unshaped and unyolked oocytes with a basophilic cytoplasm stained with hematoxylin and nucleus enlarged showing nucleolus arranged at the periphery with at least one nucleoli larger than others.
Late primary growth (Pg2)	Regular shape in comparison with Pg1, unyolked oocytes with a basophilic cytoplasm stained with hematoxylin and nucleus showing nucleolus arranged at the periphery.
Cortical alveolar (CA)	Small oil droplets begin to accumulate dispersed in the cytoplasm. The zona radiata, theca and granulosa cells become visible from this stage. Cortical alveoli show PAS positive reaction and yolk granules are still absent.
Vitellogenesis	
Primary vitellogenesis (Vtg1)	The eosinophilic protein granules start to fill the cytoplasm. Small oil droplets are more abundant and cortical alveoli may be visible arranged at the periphery of the oocyte.
Secondary vitellogenesis (Vtg2)	The protein granules are arranged as a ring in the cytoplasm and oil droplets start to arrange around the nucleus.
Tertiary vitellogenesis (Vtg3)	Yolk granules increase in number and fill the cytoplasm. The oil droplets increase in size and are distributed around the nucleus.

6.3.6 Stereology and oocyte production

The density of *i* oocyte stage per unit of volume (N_v) was estimated stereometrically for 20 ovaries showing no sign of spawning (post-ovulatory follicles) and atresia (oocyte degeneration). Thus, N_v was estimated by applying model-based methodology described in Emerson et al. (1990) and Murua et al. (2003) following the equation proposed by Weibel et al. (1966):

$$N_v = \frac{\mathrm{K}}{\beta} \times \frac{N_a^{\frac{1}{3}}}{V_i^{\frac{1}{2}}}$$

where K= size distribution coefficient; β= is a shape coefficient; N_a = number of *i* oocyte stage transected per unit area and V_i = volume fraction occupied by i oocyte in the histological section. Then, the total number of oocytes within the ovary (N) was estimated extrapolating N_v to the total volume of the ovary (Ov) (N= Nv×Ov). Thus, Ov was estimated by following the methodology proposed by Scherle (1970).

The coefficients K and β were calculated as follow:

$$\mathrm{K} = \left(\frac{M_3}{M_1}\right)^{3/2}$$

where M_1 is the mean oocyte diameter, i.e. $M_1 = [(OD_1 + OD_2 + OD_{\mathrm{n}})/\mathrm{n}]$ and M_3 is the third moment about the mean of oocyte distribution, i.e.

$$M_3 = \left[\frac{(D_1)^3 + (D_2)^3 + (D_{\mathrm{n}})^3}{\mathrm{n}}\right]^{\frac{1}{3}}$$

; D = OD_{ind} and n is the number of oocytes counted. However, β was calculated by the ratio between the longest and shortest axis of the oocytes transected. At least 60 oocytes were measured to estimate K and β as suggested by Emerson et al. (1990).

The stereological variables N_a and V_i were estimated using a Weibel grid (WEIBEL et al. 1966) with 3,200 test points and an area of 0.05 cm². Four to ten images from the non-overlapped area in the same histological section were taken with a magnification of 40X (resolution of 2560x1920 pixels). All images were analysed also using the ImageJ (RASBAND, 1997-2009).

The relative number of oocyte per gram of female (*RN*) was calculated dividing *N* by the ovary-free body weight, and the stage-specific oocyte packing density (OPD_i) dividing *N* of i oocyte stage by the ovary weight. The oocyte packing density (OPD), i.e. the total number of oocyte per gram of ovary, was the sum of OPD_i of all stage i oocyte in the ovary.

The approximation number of batches (NB) that will be recruited to the next development stage were estimated dividing the mean *N* of i oocyte stage by the mean *N* of the next stage, e.g. Pg1/Pg2, Pg2/CA, and so on.

6.3.7 Data analysis

The tests of Kolmogorov-Smirnov and Levene were applied prior any statistical test to verify the normality distribution and homogeneity of variances, respectively (ZAR, 1999). Thus, linear regressions were performed to analyse the relationship between the ovary volume (Ov) and ovary weight (Ow). The correlation between *N* (number of pre-vitellogenic and vitellogenic oocytes) and the female parameters (TL, TW and Ow) was evaluated by application of the Spearman's rank correlation test (r_s). Moreover, polynomial regressions were also fitted to describe the relationships OD_{vi}–V_i and OD_{vi}–OPD_i between species, using the coefficient of determination (r^2) as a measure of goodness of fit.

The Mann-Whitney test compared N_a, V_i, *N*, *RN*, OPD_i and OPD between species. However, the analysis of covariance (ANCOVA) was applied to compare two regressions lines by testing the effect of a categorical variable (species- *S. brasiliensis* and *S. rastrifer*) on a dependent variable (Ov or *N*), taking into account the effect of a continuous co-variable (TL, TW or Ow). All analyses were performed at the significance level of 0.01 and 0.05.

6.4 Results

6.4.1 Ovary volume and coefficients for model-based stereology

The linear regressions with the intercept forced through the origin described adequately the relationship between the ovary weight (Ow) and ovary volume (Ov) in *S. brasiliensis* (Ow=1.0695×Ov, r^2=0.99, p<0.01) and *S. rastrifer* (Ow=1.0848×Ov, r^2=0.99, p<0.05). However, the analysis of covariance indicated that the slopes of the regressions differed significantly between species (F=12,281; p<0.05) (**Figure 2**).

The coefficients of oocyte size distribution (K) and shape (β) showed low variability (less than 7%) among oocyte development stages. Thus, K ranged from 1.00 to 1.08 in *S. brasiliensis* and from 0.99 to 1.06 in *S. rastrifer*, whereas β ranged from 1.20 to 1.40 in the former species and from 1.16 to 1.35 in the latter (**Table 1**).

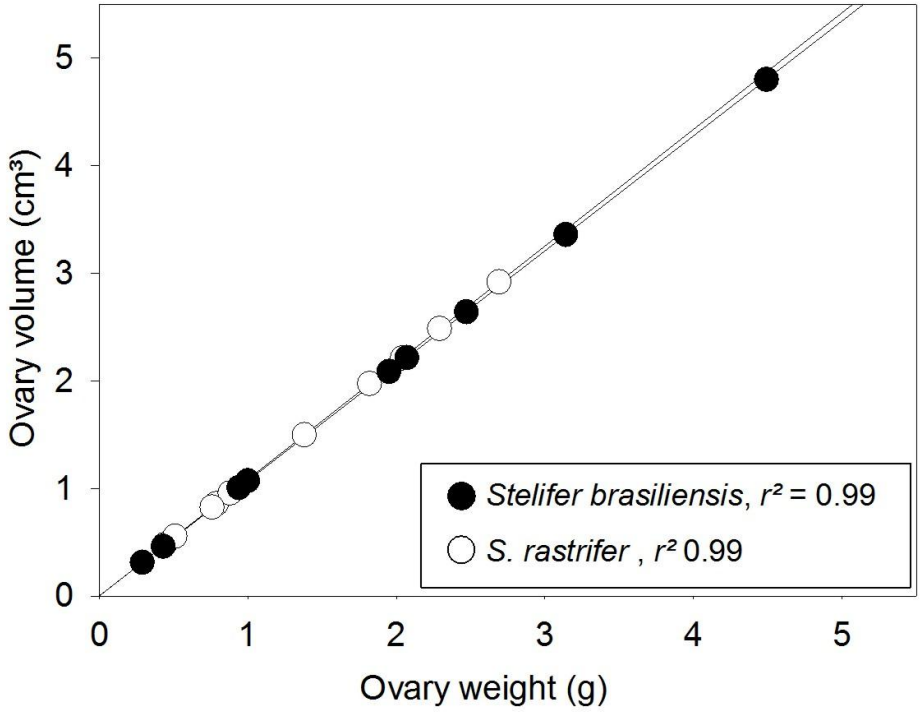


Figure 2. Linear relationship between the ovary weight (Ow) and ovary volume (Ov) for *Stellifer brasiliensis* and *S. rastrifer*.

Table 3. Coefficients of the oocyte size distribution (K) and shape (*β*) estimated for each oocyte development stage in *Stellifer brasiliensis* and *S. rastrifer*.

Oocyte development stages	*Stellifer brasiliensis*		*S. rastrifer*	
	K	*β*	K	*β*
Early primary growth (Pg1)	1.12	1.40	1.02	1.35
Late primary growth (Pg2)	1.00	1.30	1.02	1.32
Cortical alveolar (CA)	1.08	1.29	1.06	1.23
Primary vitellogenesis (Vtg1)	1.02	1.25	1.00	1.18
Secondary vitellogenesis (Vtg2)	1.05	1.21	1.04	1.16
Tertiary vitellogenesis (Vtg3)	1.05	1.20	0.99	1.16
Coefficient of variation (%)	4.1	5.8	2.5	6.8

6.4.2 The oocyte production estimation

The total number of pre-vitellogenic oocytes ranged from 1,016,621 to 10,841,313 in *S. brasiliensis* and from 1,392,838 to 5,917,151 in *S. rastrifer*. On the other hand, the number of vitellogenic oocytes ranged from 7,681 to 272,048 in the former species, and from 24, 569 to 116,118 in the latter. However, the stereological variables (N_a and V_i), total number of oocytes per individual (N) and the stage-specific oocyte packing density (OPD_i) did not differ significantly between *S. brasiliensis* and *S. rastrifer* (Mann-Whitney test, $p>0.05$) (Table 1).

The females' total weight, total length and ovary weight were positively correlated with N of pre-vitellogenic and vitellogenic oocytes. The analysis of covariance (ANCOVA) also showed that these biological variables affected significantly the number of oocytes within the ovaries, but this effect was similar between species (Figure 3).

Table 4. The stereological variables (N_a= number of oocyte transected per area and V_i= volume fraction), pre-vitellogenic (Pg1, Pg2 and CA) and vitellogenic oocytes (Vtg) quantification, and stage-specific oocyte packing density (OPD_i) in *Stellifer brasiliensis* and *S. rastrifer*. The values are indicated as mean ± sd, *N*= oocytes per ovary, *RN*= oocytes $^{-g}$ ovary-free body weight. The Mann-Whitney test was applied to test differences between the species.

	Stellifer brasiliensis	*Stellifer rastrifer*
Early primary growth (Pg1)		
N_a (oocytes per cm²)	752.4 ± 274.4	732.9 ± 365.1
V_i (%)	3.96 ± 1.90	3.99 ± 1.90
N	4,181,022 ± 2,851,354	3,515,791 ± 2,048,065
RN	93,549 ± 46,468	110,994 ± 53,036
OPD_i	2,134,046 ± 795,417	2,346,506 ± 921,764
Late primary growth (Pg2)		
N_a (oocytes per cm²)	108.3 ± 72.9	75 ± 57.5
V_i (%)	1.23 ± 0.77	0.98 ± 0.77
N	304,982 ± 294,388	164,909 ± 109,123
RN	6,845 ± 6,189	5,279 ± 2,958
OPD_i	145,160 ± 121,630	114,047 ± 60,556
Cortical Alveolar (CA)		
N_a (oocytes per cm²)	62.9 ± 48.8	39.0 ± 34.9
V_i (%)	1.41 ± 1.12	0.89 ± 1.12
N	118,941 ± 115,187	65,348 ± 54,296
RN	2,3687 ± 2,523	2,090 ± 1,865
OPD_i	53,029 ± 33,470	49,756 ± 72,642
Primary vitellogenesis (Vtg1)		
N_a (oocytes per cm²)	52.1 ± 36.4	37.4 ± 28.9
V_i (%)	2.10 ± 1.53	1.41 ± 1.53
N	52,667 ± 45,784	31,607 ± 19,468
RN	1,176 ± 1,026	979 ± 581
OPD_i	22,228 ± 15,143	20,100 ± 10,362
Secondary vitellogenesis (Vtg2)		
N_a (oocytes per cm²)	54.2 ± 35.1	35.6 ± 30.7
V_i (%)	4.12 ± 2.55	2.98 ± 2.55
N	28,870 ± 28,889	16,002 ± 16,488
RN	652 ± 586	482 ± 545
OPD_i	13,055 ± 8,539	10,054 ± 10,530
Tertiary vitellogenesis (Vtg3)		
N_a (oocytes per cm²)	150.6 ± 47.6	159.2 ± 43.6
V_i (%)	20.1 ± 6.43	20.9 ± 6.43
N	41,431 ± 30,472	31,417 ± 18,793
RN	946 ± 644	954 ± 449
OPD_i	18,036 ± 6,700	19,255 ± 6,129

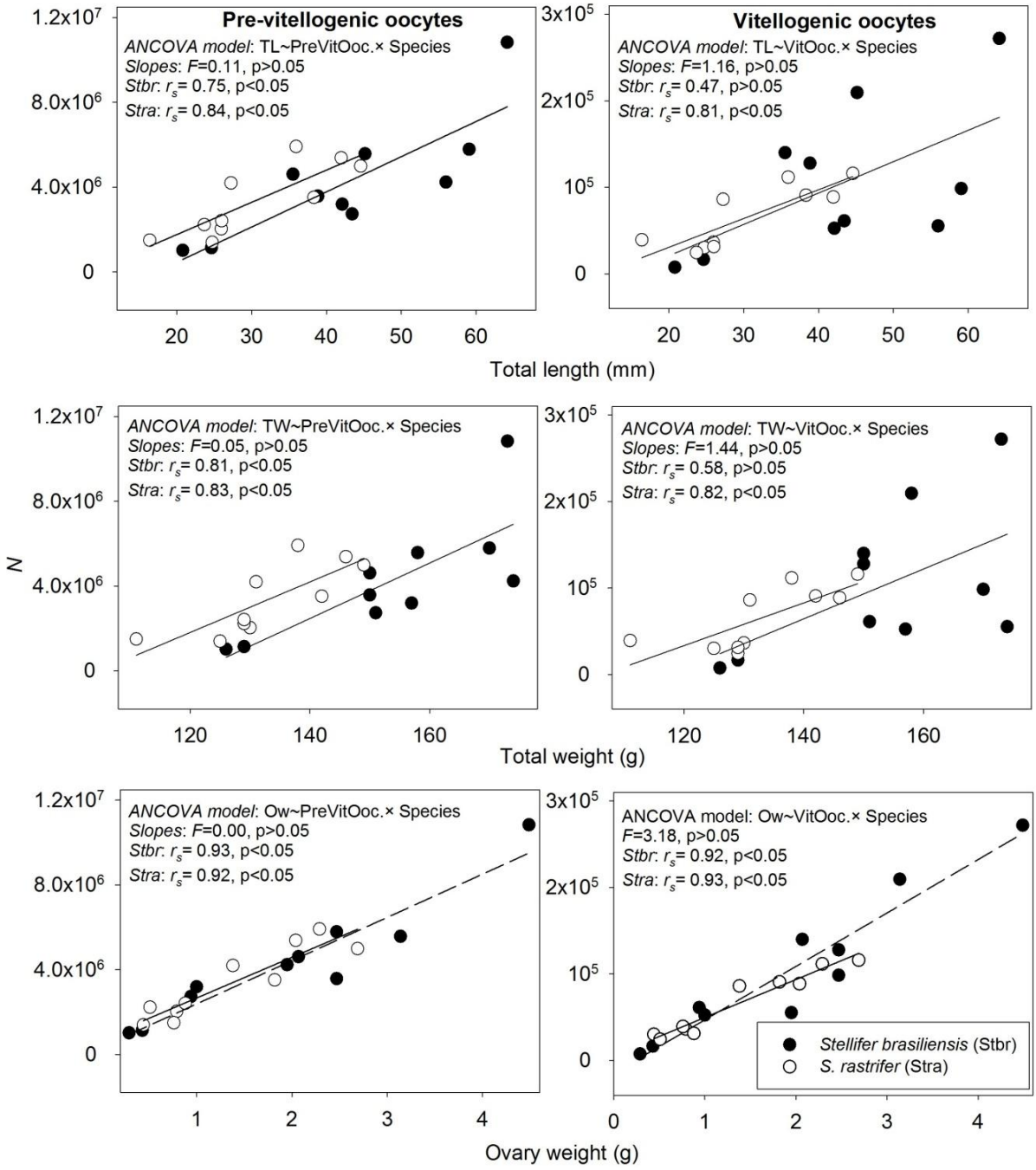


Figure 3. Relationship between the biological variables (total weight–TW, total length –TL, ovary weight –Ow) and the total number of oocytes (*N*) in *Stellifer brasiliensis* and *S. rastrifer*. The results of the analysis of covariance (ANCOVA) and the Spearman rank correlation test (r_s) are also given. PreVitOoc= pre-vitellogenic oocytes, VitOoc= vitellogenic oocytes.

6.4.2.1 The volume fraction (V_i)

The maximum volume fraction estimated in the ovaries of *S. brasiliensis* and *S. rastrifer* were 20.2% and 20.9%, respectively. The cubic polynomial function was the best fit to describe the relationship between V_i and the volume-based mean oocyte diameter (OD_{vi}) in both species (Figure 4). Thus, V_i decreased from a maximum of 7.8% to a minimum of 0.3% in *S. brasiliensis*, and from a maximum of 6.2% to a minimum of 0.4% in *S. rastrifer*. Decreasing in V_i values was observed when OD_{vi} increased from 85.9 to 137.6 µm in the former species and from 91.2 to 134.9 µm in the latter, corresponding to those oocytes in pre-vitellogenesis, i.e. Pg1, Pg2 and CA stages. On the other hand, V_i increased with increasing OD_{vi} of vitellogenic oocytes, Vtg1 (OD_{vi}: *S. brasiliensis* 184.0 µm and *S. rastrifer* 182.6 µm), Vtg2 (OD_{vi}: *S. brasiliensis* 253.7 µm and *S. rastrifer* 243.0 µm) and Vtg3 stages (OD_{vi}: *S. brasiliensis* 333.2 µm and *S. rastrifer* 351.3 µm) (Figure 4).

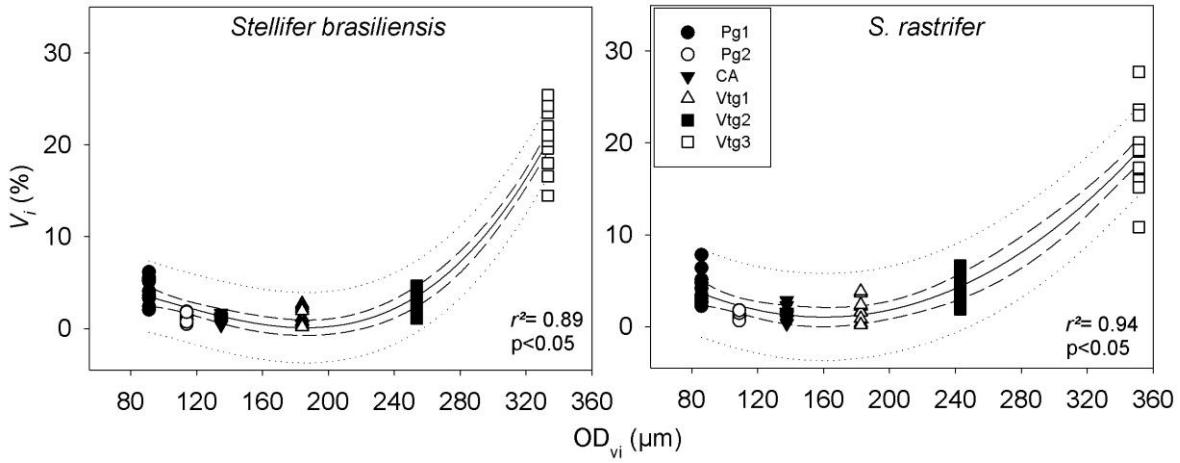


Figure 4. Relationship between the volume-based mean oocyte diameter (OD_{vi}) and oocyte volume fraction (V_i) across different oocyte development stages. The main line is fitted by cubic polynomial regression. 95% confidence interval (---), 95% prediction interval (···), p-value and coefficient of determination (r^2) are also given. Abbreviations of the oocyte development stages are defined in Table 2.

6.4.2.2 Oocyte packing density (OPD)

In general, the estimated OPD_i decreased with increasing OD_{vi}. The pre-vitellogenic oocytes decreased sharply from about 3×10^6 (Pg1) to 4×10^4 oocytes g^{-1} of ovary (CA) when OD_{vi} increased from ≈ 80 to 135 µm. OPD_i of vitellogenic oocytes

(Vtg1, Vtg2 and Vtg3) also decreased when OD_{vi} increased from ≈ 180 to 350 µm. Thus, the maximum OPD_i observed in vitellogenic oocytes was 28,329 in *S. brasiliensis* and 35,456 in *S. rastrifer* with minimum of 3,129 and 4,687, respectively. Moreover, the 3rd-order polynomial regression was highly significant describing adequately the relationship between $log(OD_{vi})$ and $log(OPD_i)$ (*S. brasiliensis*: r^2=0.94, p<0.05; *S. rastrifer*: r^2=0.95, p<0.05), in agreement with the findings above (Figure 5).

The total number of oocytes per gram of ovary (OPD) in *S. brasiliensis* was similar to that in *S. rastrifer* (*U*=48, p>0.05); the OPD ranged from 1,595,432 to 3,848,673 g^{-1} (2,657,757 ± 662,727.1) in the former species, and from 1,899,923 to 3,893,245 g^{-1} (2,683,099 ± 625,192.4) in the latter.

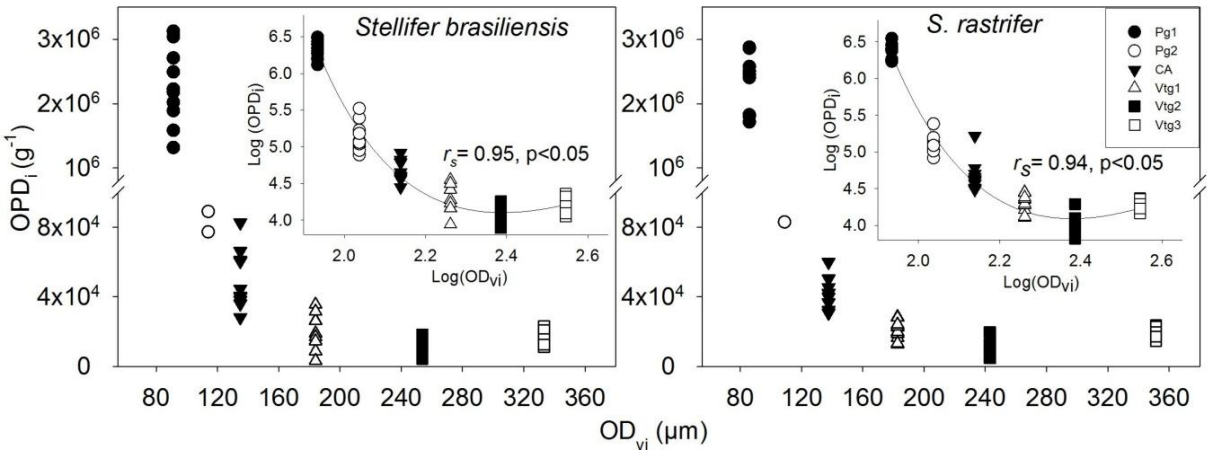


Figure 5. Relationship between volume-based mean oocyte diameter (OD_{vi}) and stage-specific oocyte per gram of ovary (OPD_i) across different oocyte development stages. Relationship between $log(OD_{vi})$ and $log(OPD_i)$ and the Spearman rank correlation test (r_s) are also given. The main line is fitted by 3rd-order polynomial regression. Abbreviations of the oocyte development stages are defined in Table 2.

6.4.3 Oocyte recruitment

The distribution of the oocyte size by development stage revealed no gap between the pre-vitellogenic and vitellogenic oocytes in spawning capable females of *S. brasiliensis* and *S. rastrifer*.

The analysis of the oocyte recruitment across their development stage revealed that approximately 13.1% of the standing stock oocytes larger than 50 µm, i.e. Pg1 stage, in *S. brasiliensis* and 8.8% in *S. rastrifer* will develop to form the next

batches. Thus, approximately 39% of the Pg2 standing stock will be recruited to form the CA batch; 42% from CA to Vtg1; 53% from Vtg1 to Vtg2 and all oocytes from Vtg2 to Vtg3. Both species exhibited the same oocyte recruitment pattern. Furthermore, the standing stock of Pg1 oocytes will generates approximately 22–29 batches, including the next development stages up to Vtg3. In general, the number of batches that will reach the next development stage decreased with increasing OD_{vi}, as well as the oocytes progression in maturation (Figure 6).

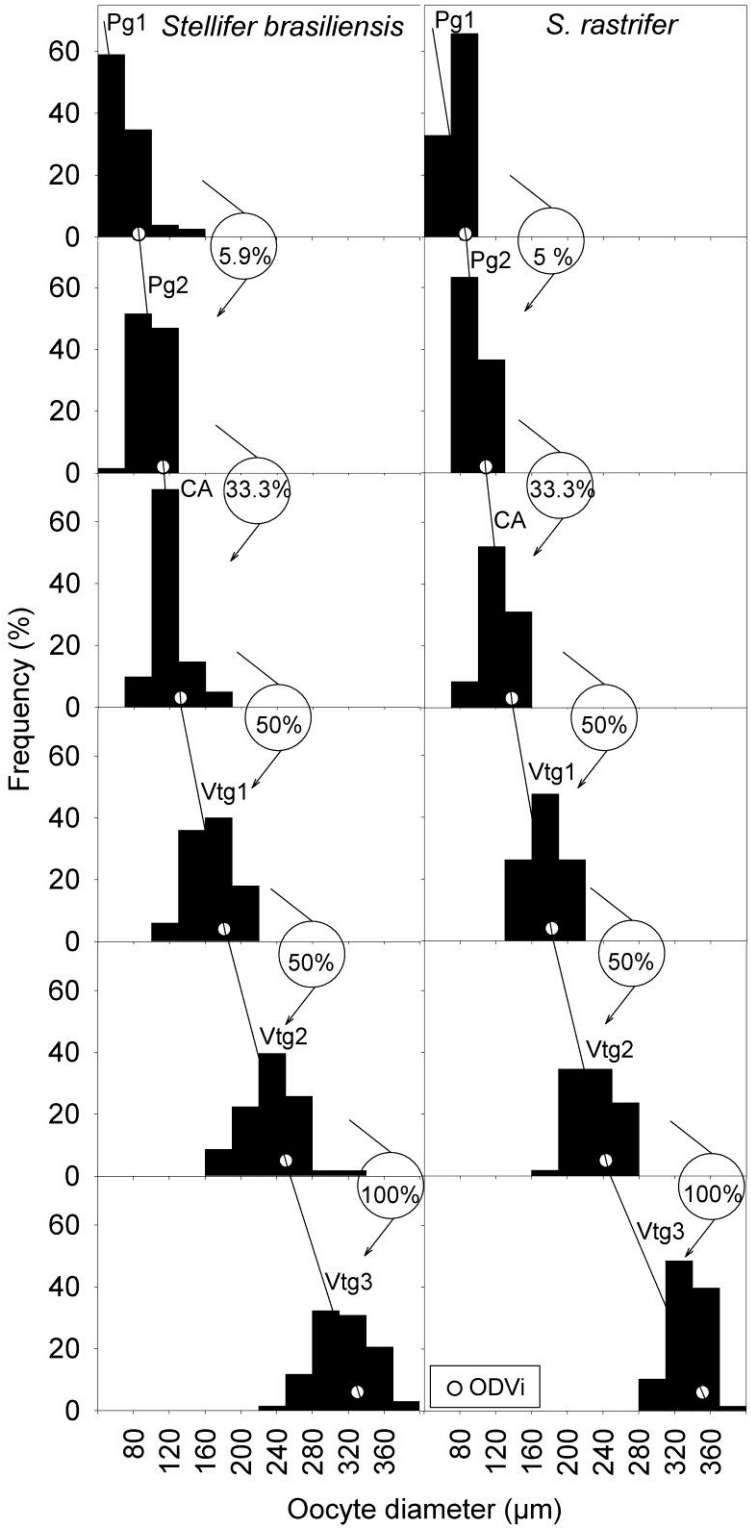


Figure 6. Oocyte size-frequency distribution and recruitment in *Stellifer brasiliensis* and *S. rastrifer*. OD_{vi} is the volume-based mean oocyte diameter. The circles and arrows indicate the percentage of oocytes that will be recruited to the next development stage. The abbreviations of the oocyte development stages are defined in Table 2.

6.5 Discussion

The stereological method is a powerful tool for assessing the reproductive strategy in fishes; it has mainly been applied for estimating the number of developing oocytes (potential fecundity) in many species (MURUA et al., 2003; GOZÁLEZ-RUFINO et al., 2013; HASLOB et al., 2013; GÁRCIA-SEOANE et al., 2014). Furthermore, studies which take into account the oocyte production by development stage for understanding the oocyte dynamics are scarce. Thus, this study reports, for the first time, the oocyte production and recruitment of two congeneric species using histology and mode-based stereology.

The coefficients for correction of the oocyte size distribution (K) and shape (β) are very important to reduce the bias in model-based stereology, although they may vary among species and among oocyte development stages (EMERSON et al., 1990). In general, the K estimated for *S. brasiliensis* and *S. rastrifer* were in agreement with those reported for *Tilapia zilli* (Cichlidae), *Thunnus thynnus* (Scombridae) and *Sprattus sprattus balticus* (Clupeidae) by Coward and Bromage (2002), Aragón et al. (2010) and Haslob et al. (2013), respectively. However, the low values of β (1.16–1.40) reveal that the shrinkage rate of the oocytes in the histological sections of both species was less than those reported by Emerson et al. (1990), Medina et al. (2002) and Aragón et al. (2010) (β, 1.42–1.56). Therefore, the coefficient of variation less than 7% indicates that K and β were adequately estimated in the present study.

Substantial changes among species and individuals of the same species due to their different environments might be expected (LAW, 2000). Thus, fishing activities, climate changes and the environment are the main factors driving changes in fish reproductive life history, affecting oocyte maturation, size-age at the first maturity, spawning frequency, longevity, fecundity, egg size and offspring size (WAGGY et al., 2006; MARTIN et al., 2009; PANKHURST; MUNDAY, 2011; WIEDMANN et al., 2014). However, the oocyte production in *S. brasiliensis* (*N*, *RN,* OPD_i and OPD) was similar to the *S. rastrifer*, although they were sampled in different environments. Wiedmann (2014), comparing the life history of several fishes in two distinct environments in the Barents Sea, observed that species phylogenetically related exhibited similar reproductive traits. On the other hand,

Waggy et al. (2006) have reported a convergence evolution of distinct fishes of the family Sciaenidae in the Gulf of Mexico and Caribbean Sea. These authors separated different species into three groups with the same reproductive strategy based on their life span, maximum size, size-age of maturity, duration of the spawning season, spawning frequency and fecundity. Thus, biological variables such as size, weight and ovary weight of females affect significantly the number and size of oocytes produced by a female (BELL, 1980; KORTA et al., 2010a; BUCHOLTZ et al., 2013; HASLOB et al., 2013). Therefore, similarities between *S. brasiliensis* and *S. rastrifer* might be attributed to their phylogenetic relationship, once the effect of the biological variables on the oocyte production was similar between these species.

The volume fraction (V_i) estimated also revealed that the stage-specific oocyte V_i occupied a similar volume in the histological sections, when compared *S. brasiliensis* and *S. rastrifer*. According to Ganias (2013), V_i of the standing stock of vitellogenic oocytes should be high than those in pre-vitellogenesis. In general, this pattern was observed in this study, also corroborating the results found by Saber et al. (2014) in *Thunnus alalunga*. These authors have reported values of V_i (pre-vitellogenic and vitellogenic oocytes) less than 50% and described the relationship between OD_{vi} and V_i by a polynomial function, as recorded in both species here.

The production of pre-vitellogenic oocytes was greater than those in vitellogenesis, revealing also that the recruitment of vitellogenic oocytes from the standing stock of pre-vitellogenic occurs continuously in both species. Korta et al. (2010b) and Saber et al. (2014) have reported similar result for fishes with indeterminate fecundity. Therefore, the asynchronous oogenesis with no gap between the pre-vitellogenic and vitellogenic modes in the oocyte size-frequency distribution are associated with multiple spawning fishes, and this represents a strategy for investment of large numbers of oocytes when resources are patchy (WINEMILLER; ROSE, 1993; MURUA; SABORIDO-REY, 2003). This mode of spawning is typical of marine species of low latitudes if the environmental conditions are comparatively stable over a prolonged period of the year (PAVLOV et al., 2009).

According to Pavlov et al. (2009), in an asynchronous ovary organisation, two or three groups of vitellogenic oocytes are separated in the ovary during the vitellogenesis, and then they are released in the same number of batches. Murua

and Saborido-Rey (2003) have reported that different species exhibit different oocyte-size frequency distributions. However, both species exhibited the same oocyte development pattern with similar number of oocytes recruited from a stage-specific oocyte group to form the subsequent batch, supporting the idea of the phylogenetic relationship between this species. Although the number of batches that will be recruited from Vtg3 to hydration was not estimated here, these results indicate that the oocyte recruitment occurs in pulses. According to Schismenou et al (2012), the recruitment of oocytes from one to another pulse is activated by hydration of the spawning batch in fishes with indeterminate fecundity. Thus, both spawning capable females of *S. brasiliensis* and *S. rastrifer* have one potential batch (Vtg3) within the ovaries to supply the subsequent pulse/batch (hydration) after each event of spawning. However, only a part of this standing stock of advanced vitellogenic oocytes will be hydrated and then released to the environment.

In conclusion, the combinations of histological and stereology techniques, applied for comparing the oocyte production and the course of batch generations in *S. brasiliensis* and *S. rastrifer*, allow us to conclude that the similarities reported here must be attributed to their phylogenetic relationship with both species exhibiting, practically, the same oocyte production and oocyte recruitment patterns.

6.6 Supplementary Material

For: **Do congeneric fishes inhabiting different subtropical environments exhibit the same oocyte production and recruitment patterns?**

Appendix A. Supplementary table.

Table 1. Sampling date, time, season and biological variables for each specimen of *Stellifer brasiliensis* (Stbr) and *S. ratrifer* (Stra) sampled in the inner shelf of Ubatuba and the estuarine system of Cananéia, southeastern Brazil. TL= total length, SL= standard length, TW= total weight, Ow= ovary weight.

Region	Specimen	Sampling Date (yyyy-mm-dd)	Sampling Time (hh:mm)	Season	TL (mm)	SL (mm)	TW (g)	Ow (g)
Ubatuba	Stbr_1	2012-10-26	13:50	Spring	170	130	59.07	2.47
Ubatuba	Stbr_2	2012-01-26	13:50	Spring	150	113	38.85	2.47
Ubatuba	Stbr_3	2012-12-13	15:34	Spring	173	133	64.16	4.49
Ubatuba	Stbr_4	2012-12-13	15:34	Spring	174	131	55.97	1.95
Ubatuba	Stbr_5	2012-12-13	15:34	Spring	158	118	45.15	3.14
Ubatuba	Stbr_6	2012-12-13	15:34	Spring	150	112	35.52	2.07
Ubatuba	Stbr_7	2013-04-26	15:30	Autumn	129	95	24.64	0.43
Ubatuba	Stbr_8	2013-04-30	08:30	Autumn	157	123	42.11	1.00
Ubatuba	Stbr_9	2013-04-30	15:24	Autumn	151	116	43.43	0.94
Ubatuba	Stbr_10	2013-04-30	15:24	Autumn	126	95	20.80	0.29
Cananéia	Stra_1	2013-03-26	08:15	Summer	146	112	41.97	2.04
Cananéia	Stra _2	2013-03-26	08:15	Summer	138	105	35.94	2.29
Cananéia	Stra _3	2013-03-26	08:15	Summer	142	110	38.31	1.82
Cananéia	Stra _4	2013-03-26	08:15	Summer	149	110	44.58	2.69
Cananéia	Stra _5	2013-03-26	08:15	Summer	131	100	27.24	1.38
Cananéia	Stra _6	2013-03-26	08:15	Summer	125	94	24.74	0.44
Cananéia	Stra _7	2013-03-26	08:15	Summer	130	102	25.94	0.79
Cananéia	Stra _8	2013-03-27	08:15	Summer	111	87	16.38	0.76
Cananéia	Stra _9	2013-03-28	08:24	Summer	129	96	23.68	0.51
Cananéia	Stbr_10	2013-03-28	08:24	Summer	129	99	26.00	0.88

Appendix B. Supplementary figure.

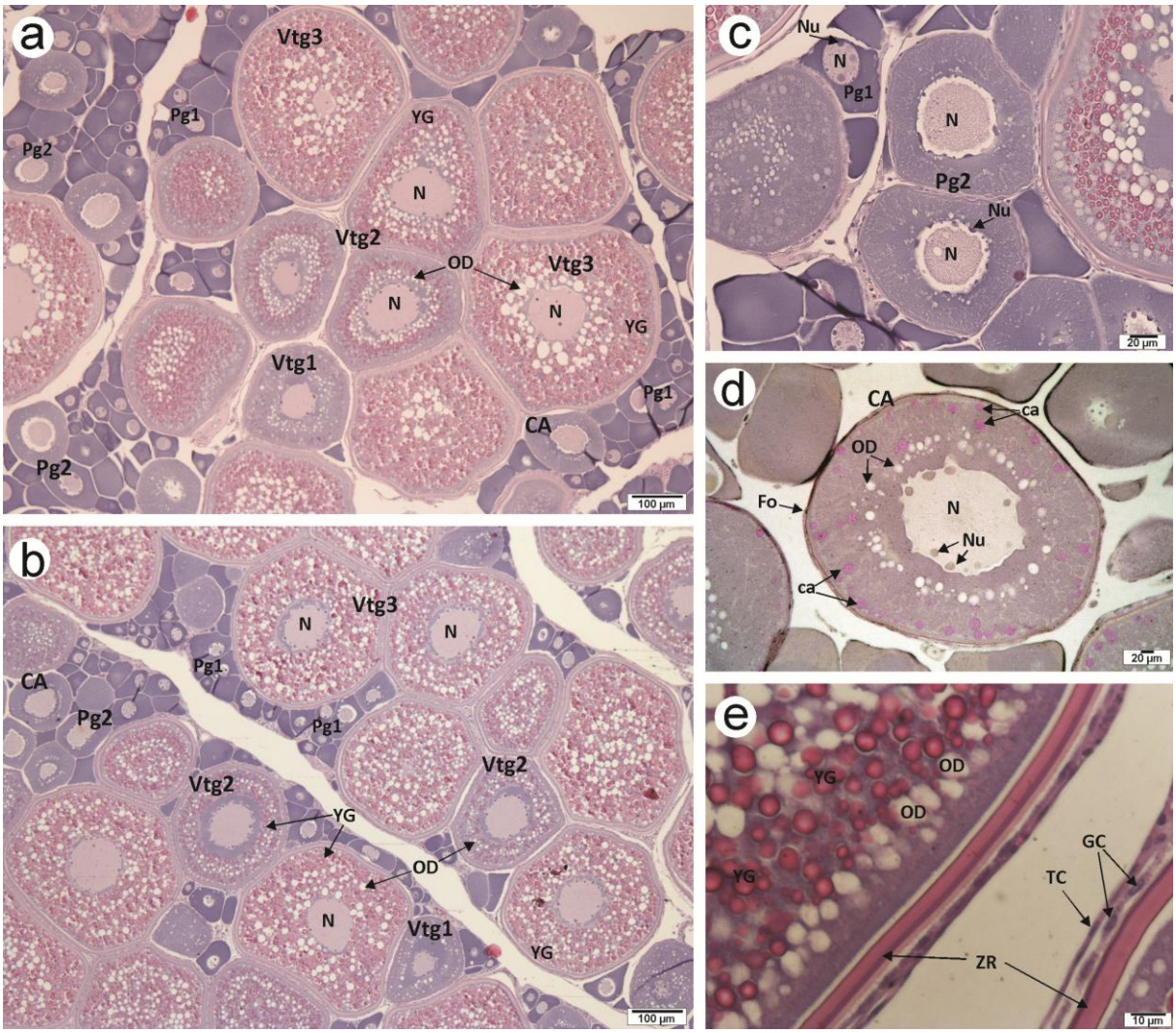


Figure 1. Photomicrography of the histological sections of females in spawning capable phase showing different oocyte development stages in *Stellifer brasiliensis* (a) and *S. rastrifer* (b); (c) oocytes in early (Pg1) and late primary growth (Pg1); (d) cortical alveolar stage (CA) showing the PAS-positive reaction of the cortical alveolus (ca); (f) zona radiata (ZR), theca (TC) and granulosa cells (GC). Vtg1-Vtg2-Vtg3= early, secondary and tertiary vitellogenic stages. N = nucleus, nu = nucleoli, YG = yolk granules, OD = Oil droplets.

GENERAL DISCUSSION

7. GENERAL DISCUSSION

This thesis presents, for the first time, data on reproductive strategy and fecundity for the fish species *Anchoa filifera*, *Cetengraulis edentulus*, *Citharichthys spilopterus*, *Stellifer brasiliensis*, *S. rastrifer*, *Menticirrhus americanus*, *Paralonchurus brasiliensis* and the simultaneous hermaphrodite *Diplectrum radiale* from two different ecosystems located in the southwestern Atlantic Ocean, São Paulo State, Brazil. This study contributes to the knowledge of the reproductive strategy of these species inhabiting coastal and estuarine ecosystems.

7.1 Oocyte development and ovary organisation

The correct evaluation of the ovary is crucial to describe the maturation process, the time of reproduction and the oocyte recruitment (ALONSO-FERNÁNDEZ, 2011). In general, oogenesis is similar for most teleosts with the presence of oocytes in primary growth, cortical alveolar, vitellogenesis and maturation (WALLACE; SELMAN, 1981; WEST 1990; TYLER; SUMPTER, 1996; MURUA; SABORIDO-REY, 2003). However, species-specific histological criteria may be used to develop more specific divisions of oocyte stages for comparative purposes (LOWERRE-BRABIERI et al., 2011a). Thus, the oocytes of all species in spawning capable phase in the **Chapters I, II, and III** were separated into two groups: early developing oocytes, which encompassed early primary growth, late primary growth and cortical alveolar, and vitellogenic oocytes such as primary, secondary, tertiary and quaternary vitellogenesis. The CA stage was not observed in the ovaries of *A. filifera* and *C. edentulus*, differing from the other species **(Chapter I)**. The main function of the cortical alveoli is the prevention of the polyspermy and its formation may vary among teleost species (KOBAYASHI, 1985; GUARAYA, 1986; OHTA et. al., 1990). The absence of CA in the histological sections may indicates that this stage have a short period of time followed by a rapid yolk accumulation in these species or the abundance of these oocytes in spawning capable phase ovaries is low, which became difficult its visualisation in the histological sections. Furthermore, the presence of germinal vesicle migration and hydrated oocytes, as well as post-ovulatory follicles, indicated that all species studied here spawn in the

sampled areas, with *D. radiale* spawning in both coastal and estuarine environments (**Chapter II**).

Oocytes in quaternary vitellogenesis stage, i.e. the last stage before the nucleus migration, were recorded only in *C. spilopterus*, *S. brasiliensis* and *D. radiale* **(Chapters I and II)**. According to Yoda and Yoneda (2009), the transition between Vtg4 and germinal vesicle migration takes a short period of time and occurs during a specific time of the day, which could explain the absence of this stage in the histological sections of the ovaries of *A. filifera*, *C. edentulus*, *S. rastrifer*, *M. americanus* and *P. brasiliensis* (**Chapters I and III)**. Thus, all species exhibited the same pattern of oocyte development, and the differences recorded here must be attributed to rate of oocyte maturation and the sampling time to capture specimens in spawning capable with Vtg4 stages. Therefore, the simultaneous presence of oocytes in several development stages in these species revealed a continuous type of oogenesis with an asynchronous ovarian organisation (TYLER; SUMPTER, 1996; MURUA; SABORIDO-REY, 2003). This type of ovary organisation is typical of batch spawning species with a very protracted spawning season, usually in those species from tropical regions, and represents a strategy for investment of larger number of smaller eggs when resources are patchy on a relatively large spatial scale (OVEN, 1976; KJESBU, 2009; PAVLOV et al., 2009).

7.2 Oocyte recruitment and fecundity strategy regulation

Assessing the type of fecundity, i.e. indeterminate or determinate, is necessary to select the correct method for fecundity estimation and egg production (MURUA; SABORIDO-REY, 2003; KJESBU, 2009; BERNAL et. al., 2012). The asynchronous ovarian organisation is usually associated with species with indeterminate fecundity, although species with determinate fecundity can also exhibit this type of ovary organisation (TYLER et al., 1994; MURUA; SABORIDO-REY, 2003; ALONSO-FERNÁNDEZ et al., 2008). Both fecundity types refer to the way at which the oocytes are recruited during the ovary maturation. Thus, the analysis of the oocyte size-frequency distribution has been widely applied for investigating the fecundity type of many fishes based on the presence or absence of a gap between the modes of pre-vitellogenic and vitellogenic oocytes (GRANDE et al., 2012; GANIAS, 2013, ZUDAIRE et al., 2013a). Therefore, fishes with indeterminate

fecundity do not show this gap because the standing stock of pre-vitellogenic oocytes is continuously recruited into yolked oocyte stock (HUNTER; GOLDBERG, 1980; KJESBU, 2009). On the other hand, in those species with determinate fecundity, the stocks of pre-vitellogenic and vitellogenic oocytes are clearly separated from each other by a gap in diameter, indicating that the potential fecundity is fixed prior to the onset of spawning (MURUA; SABORIDO-REY, 2003; KJESBU, 2009).

Different species show different oocyte size-frequency distributions to the dynamics of early cycle oocyte recruitment that may vary considerably among species (MURUA; SABORIDO-REY, 2003; KORTA et al., 2010b). However, the oocyte size-frequency distribution in the **Chapters I, III** and **IV** showed several modes with a clear overlap between pre-vitellogenic and vitellogenic oocytes, revealing an indeterminate fecundity for these species.

On the other hand, the simultaneous hermaphrodite *D. radiale* revealed a different pattern in this distribution showing a single mode with pre-vitellogenic and vitellogenic oocytes **(Chapter II)**. It differs from the general pattern reported for the most teleosts with indeterminate fecundity, which shows several modes of oocytes in different development stages (MURUA; SABORIDO-REY, 2003). This evidence indicates that only a small number of oocytes are matured and recruited to form each batch that will be stored in the ovarian sinus up to the next spawning event. The occurrence of pre-vitellogenic and vitellogenic oocytes in several stages a long with oocytes in germinal vesicle migration, hydration and post-ovulatory follicles may indicate continuous oocyte recruitment, revealing an indeterminate fecundity type for *D. radiale* **(Chapter II)**. This fecundity type is typical of tropical and some subtropical fishes, which exhibit continuous oocyte recruitment repeatedly recruiting oocytes from pre-vitellogenic to vitellogenic growth (LOWERRE-BARBIERI et al., 2011b).

The oocyte size-frequency distribution does not necessarily imply indeterminacy for a fish species because a lack of the gap between pre-vitellogenic and vitellogenic modes has also been reported for fishes with determinate fecundity (GREER WALKER et al., 1994; TYLER et al., 1994; ALONSO-FERNÁNDEZ et al., 2008). Moreover, the presence or absence of the gap is not an unequivocal sign of indeterminate fecundity (GANIAS, 2013; ZUDAIRE et al., 2013a), but additional criteria, such as variation in the diameter of the most advanced stage, seasonal

evolution of the number of advanced vitellogenic oocytes in the ovary and levels of atresia during the spawning season, have also been applied (HUNTER at al., 1992; GORDO et al., 2008; KJESBU, 2009; GRANDE et al., 2012; ZUDAIRE et al., 2013a). Thus, the variation of the oocyte diameter of *P. brasiliensis* in relation to the sampling period corroborates this idea confirming the indeterminate fecundity for this species **(Chapter III)**. In addition, the differences in the size at which the oocytes are recruited to vitellogenesis (ORS) among ovarian phases, in each species, may be considered as evidence for indeterminate fecundity, indicating that the size at which the oocytes begin the vitellogenesis and are recruited to form the new development group is ovary-phase dependent, and it is related with the time of spawning in these species **(Chapter II)**. This idea is supported by the tendency of females in spawning capable phase show the largest ORS-values, i.e. females that have not been spawned in the current reproductive cycle yet, while those in spawning capable phase with GVM and POFs have lower values of ORS indicating that recruitment of pre-vitellogenic oocytes is occurring. Therefore, ORS could be used as additional criteria for investigating the fecundity regulation strategy in teleosts.

7.3 Fecundity

Stereological methods have been successful applied in fishery science for estimation of the number of pre-vitellogenic and vitellogenic oocytes, so-called developing oocytes (NDO), in fishes with indeterminate and determinate fecundity (COWARD; BROMAGE, 2002; MURUA et al., 2003; DOMÍNUEZ-PETIT, 2007; MEDINA et al., 2007; ARAGÓN et al., 2010; DOMÍNUEZ-PETIT; SABORIDO-REY, 2010; HASLOB et al., 2013; HERNÁNDEZ-PORTOCARRERO et al., 2014; GARCÍA-SEOANE et al., 2014; KNAPP et al., 2014; SABER et al., 2014). Differences in the final output among species often reflect different reproductive strategies, although the final oocyte production of a given species may vary in response to changeable environment and biological factors (WOOTON, 1984; WITTHAMES et al., 1995) HELFMAN et al., 1997; MURUA; SABORIDO-REY, 2003).

Concerning environmental conditions, several studies have shown relationships between fish fecundity and environmental factors, both biological and physical or hydrographical, such as food abundance and availability, temperature, and density dependence (LAMBERT et al., 2003). These studies have been focused

on species with economical importance, as haddock, herring, winter flounder and anchovy. However, as the environmental factors act simultaneously, the understanding of the responses is quite complex, because of differences in patterns of energy allocation to growth and reproduction.

On the other hand, this study showed that the NDO estimated for the species in the **Chapters I and III** was significantly affected by the biological variable ovary weight, total length and total weight. This result could explain the differences in the values of NDO among these species, in agreement with Murua et al. (2003), which have stated that the fecundity, as well as NDO, is usually proportional to the biological measurements of the females, being the key parameters to assess fecundity at the population level. Similar pattern has been reported for other teleosts with indeterminate fecundity such as *Merluccius merluccius*, *Trachyrincus scabrous* and *Nezuma aequalis* by Domínuez-Petit (2007), Fernadez-Arcaya et al. (2012) and Fernadez-Arcaya et al. (2013), respectively.

The relative NDO (RNDO) revealed a similar production of advanced vitellogenic oocytes between the engraulids *A. filifera* and *C. edentulus*, and between the congeneric species *S. brasiliensis* and *S. rastrifer* (**Chapters I and IV)**. Although these species inhabit different ecosystems, the similarities reported here might be attributed to their phylogenetic relationship, in agreement with those results found in **Chapter IV**. Similarities in the reproductive traits of phylogenetically related fishes inhabiting different environments have been reported by Wiedmann (2014). On the other hand, Vtg3 produced (corresponds to Vtg2 in **Chapter III)** in the ovaries of *P. brasiliensis* was approximately two times greater than those estimated in *M. americanus* (**Chapter I).** In addition, the standing stock of Vtg3 oocytes in the former species generates approximately three batches of hydrated oocytes (**Chapter I)**, whereas in the latter only one batch is formed (**Chapter III)**. Therefore, the difference between the numbers of Vtg3 oocytes produced per gram of body weight in the sciaenids *P. brasiliensis* and *M. americanus* must be related to the rate that the oocytes are recruited during the vitellogenesis, the spawning frequency or the spawning period of these species.

The relative batch fecundity (RBF) in *A. filifera* was close to that in *C. edentulus*, in agreement with the RNDO results discussed previously **(Chapters I)**. In

general, there was low variation of RBF values among the sciaenid species, which ranged from 306 to 444 germinal vesicle migration/hydrated oocytes per gram of ovary-free body weight **(Chapters I and III)**. However, this type of results for comparison purposes must be used with caution because the final number of mature oocytes (GVM and Hyd) could be affected by atresia of vitellogenic oocytes during the ovarian maturation. Several studies have showed divergences in the fecundity among specimens of the same species or among closely related species, and attributed such differences to biotic and abiotic factors such as intensity of atresia, oocyte size, water temperature, food availability, feeding condition and geographical distribution (LAMBERT et al., 2003; LEAL et al., 2009; NISSLING; DAHLMAN, 2010; STRÜSSMANN et al., 2010; McELROY et al., 2013; McBRIDE et al., 2013; NISSLING et al. 2014). Therefore, BF fecundity is most related to phenotypic plasticity of species in response to the environmental change or fishing activity than phylogenetic relationships (LEAL et al., 2009; NISSLING; DAHLMAN, 2010; THORSEN et al., 2010).

The RBF estimated for *D. radiale* in both regions showed low production of hydrated oocytes **(**from 20 to 113 g^{-1}, **Chapter II)** compared to the gonochoristic species**,** which ranged from 215 to 444 g^{-1} **(Chapters I and III)**. This relative low production may be related to the short interval between the batches released in *D. radiale* to compensate the low number of oocytes produced, and the capacity of the ovary sinus (the accessory reproductive structure) to store these oocytes. Therefore, the ovarian sinus volume in simultaneous hermaphrodite could play an important role in regulating the number of oocytes recruited during the ovarian maturation, as well as the number of hydrated oocytes stored in this structure prior to spawning. The simultaneous presence of post-ovulatory follicles in different ages, and atretic hydrated oocytes in the ovarian sinus corroborate the idea of oocyte production regulation. Furthermore, the mean values of RBF found in **Chapter II** were close to those reported for the simultaneous hermaphrodites *Serranus scriba* and *S. atricauda* reported by Alós et al (2013) and Neves et al. (2014), respectively.

7.4 Number of potential batches

Oocyte development in fishes with indeterminate fecundity is less temporally constrained, and these species usually show a very lengthy reproductive period,

sometimes with a period of major reproductive activity (RIDEOUT; TOMKIEWICZ, 2011). In these fishes, oocyte recruitment may occur either before the beginning or during the spawning period (KORTA et al., 2010b). Extended spawning in fishes with indeterminate fecundity is linked with the ability to continually recruit oocytes into vitellogenesis (RIDEOUT; TOMKIEWICZ, 2011). In the **Chapters I and III**, the number of batches recruited from the standing stock of advanced yolked oocytes to form the subsequent batch (hydrated oocytes) is probably an evidence for differences in the length of spawning, rate of replenishment and release of oocytes among these species.

The spawning period of *A. filifera*, *C. edentulus* and *M. americanus* with one potential batch is probably much shorter than those with at least two batches in the ovary, i.e*. C. spilopterus*, *S. brasiliensis*, *S. rastrifer* and *P. brasiliensis* **(Chapters I and III)**. Thus, the presence of more than one batch, composing the standing stock of advanced vitellogenic oocytes, is a strategy adopted for these species guaranteeing the replenishment of released oocytes for an extensive spawning season (GANIAS et al., 2015). The registration of three to four well defined groups of vitellogenic oocytes in the ovaries of spawning capable females is also an evidence for a long period of spawning (PAVLOV et al., 2009; BROWN-PETERSON et al., 2011; ZUDAIRE et al., 2013a), although these groups of oocytes were also recorded in *A. filifera*, *C. edentulus* and *M. americanus* **(Chapter I)**. Furthermore, according to Hunter and Leong (1981), the oocyte growth rate of yolked oocytes is much faster than those smaller pre-vitellogenic oocytes in fishes with indeterminate fecundity, as observed in figure 5, **Chapter I,** which shows exponential growth of the oocytes with a rapid vitello accumulation. Therefore, the number of batches is also an evidence of high rate of oocyte replenishment during the vitellogenesis in *C. spilopterus*, *S. brasiliensis*, *S. rastrifer* and *P. brasiliensis* **(Chapters I and III)**. The **Chapter IV** corroborates this idea showing that a high number of oocytes is recruited to form the subsequent batch during the vitellogenesis in *S. brasiliensis* and *S. rastrifer*.

The number of advanced vitellogenic oocyte is probably linked with the spawning interval between batches in gonochoristic species **(Chapters I and III)**. Thus, species with a larger number of advanced vitellogenic batches, ready to be recruited into maturation during the spawning period, would have a short interval

between the successive spawnings. Many studies have reported a tendency of the engraulids exhibit a long and variable spawning intervals when compared to the Sciaenidae species, in agreement with this idea (Table 1). For instance, Hunter and Leong (1981) have reported that *Engraulis mordax* spawns every seven day, and the oocytes take 28 days to grow from primary growth to the most advanced vitellogenic stage. However, studies which assess the ovary dynamics in multiple spawning fishes are scarce, and further investigation is needed to test this hypothesis in other teleosts.

Table 1. Variation in the spawning intervals in species of the families Engraulidae and Sciaenidae.

Family/ species	Spawning interval (days)	Reference
Engraulidae		
Engraulis mordax	7	Hunter and Leong (1981)
Engraulis ringens	6	Alheit et al. (1984)
Engraulis australis	3-7	Dimmlich et al. (2009)
Encrasicholina heteroloba	2-17	Wright (1992)
Sciaenidae		
Micropogonias furnieri	3-4	Waggy et al. (2006)
Pogonias cromis	3-4	Waggy et al. (2006), Militelli et al. (2013)
Bairdiella chrysoura	1-2	Waggy et al. (2006)
Sciaenops ocellatus	2-4	Waggy et al. (2006)
Otolithes ruber	2-3	Waggy et al. (2006)

Therefore, the annual population fecundity for *C. spilopterus*, *S. brasiliensis*, *S. rastrifer* and *P. brasiliensis*, which is the real number of hydrated oocytes that all females spawn during the reproductive period (BAGENAL, 1978; MURUA; SABORIDO-REY, 2003), is probably much higher than that for *A. filifera*, *C. edentulus* and *M. americanus* **(Chapters I and III)**. This reveals that the former species invest more in reproduction than the latter, increasing the number of oocytes spawned per year and, consequently, the probability of the hydrated oocytes to be fertilised in the environment. However, high mortality of the egg and larval stages

occurs in many fish species (McBRIDE, 2013). Therefore, the number of the eggs produced by a species during the spawning period does not imply that the larvae will survive, reach the sexual maturity and increase the adult population (RUSSEL, 1932).

7.5 Implications for ecosystem management

To date, fisheries management has often been ineffective because the models being used were focused on maximisation of the captures of a single target species, ignoring important factors such as habitat, predators, prey of the target species and other ecosystem components and interactions (PITCHER, 2001; PIKITCH et al., 2004). Growing concern over issues such as bycatch species, impacts of gear on habitat and the influence of the climate change on the natural regulation of populations, has led agencies to develop more holistic approaches to the assessment and management of the marine resources (KING; McFARLANE, 2003). Thus, ecosystem-based management for fisheries (EBMF) is proposed as an attempt to implement management practices focused on the entire ecosystem rather than a particular species, which include target and non-target species (PIKITCH et al., 2004, LIMA, 2011). Therefore, basic reproductive information, such as oocyte development, fecundity regulation strategy and fecundity, which are essential for assessment of fish reproductive potential and spawning stock biomass are often lacking for newly exploited species, as well as for those species captured as bycatch (KING; McFARLANE, 2003; KJESBU, 2009; FARLEY, 2013; GANIAS et al., 2014).

The species studied in the **Chapters I, II, III and IV** play an important role in the trophic structure of ecosystems (ROCHA et al., 2008; SOARES et al., 2008). Thus, *Anchoa fifilifera*, *Cetengraulis edentulus*, *Citharichthys spilopterus*, and the sciaenids *Stellifer brasiliensis*, *S. rastrifer*, *Menticirrhus americanus* and *Paralonchurus brasiliensis* are commonly captured as bycatch by shrimp fishery trawl in the southeastern and southern coast of Brazil (VIANNA et al., 2004; VIANNA; ALMEIDA, 2005; BRANCO; VERANI, 2006; GOMES; CHAVES, 2006). These species have little importance as a fishery resource, but participate in the annual Brazilian fishery production, with exception of *C. spilopterus* and the simultaneous hermaphrodite *Diplectrum radiale* (MPA, 2011). Therefore, the reproductive aspects

studied here are essential for understanding the reproductive strategy of these species, and hence develop effective ecosystem management measures.

The reproductive traits of the fishes are linked to population dynamics and response to environmental forcing (KING; McFARLANE, 2003). Thus, changes in the environment caused by pollution alter the oocyte development (detrimental effects on oogenesis), reproductive behaviours, and reduce the number of oocytes spawned (DENSLOW et al., 2007). Furthermore, variations in oocyte size-frequency distribution, oocyte diameter and size at which the oocyte are recruited to vitellogenesis (ORS) could be use to monitor alterations in the ecosystems caused by pollution, climate changes and fishing activities. However, variables such as temperature, salinity, dissolved oxygen, organic matter and other important factors associated to the fishing activities, should be taking into account.

High fecundity species with protracted spawning and short spawning intervals such as *C. spilopterus*, *S. brasiliensis*, *S. rastrifer* and *P. brasiliensis* allow support high predation and rapid recovery from unfavourable conditions, e.g. climate changes, fisheries or perturbation caused by human activities. Therefore, the application of histological and advanced stereological techniques for monitoring reproduction of these fishes becomes important management issues.

At the present stage, there is a lack of information concerning changes in fish fecundity related with global changes. The major difficulties are how reproductive strategies and tactics are adaptive to different environments occupied by the same species, and relate causes and effects of possible patterns found (POTTS; WOOTTON, 1984). As the same species can live under different environmental pressures, experimental studies and modeling of effects of environmental factors on fecundity must be subject of further investigations.

CONCLUSIONS

8. CONCLUSIONS

The differences in the reproductive strategy among species were recorded. Therefore, the hypothesis “different species inhabiting coastal and estuarine ecosystems will exhibit the same reproductive strategies in terms of ovarian development, oocyte recruitment and fecundity” was rejected. Thus, the main conclusions, concerning reproductive strategies of *Anchoa filifera*, *Cetengraulis edentulus*, *Citharichthys spilopterus*, *Stellifer brasiliensis*, *S. rastrifer*, *Menticirrhus americanus*, *Paralonchurus brasiliensis* and *Diplectrum radiale* were:

1. Although all species showed an asynchronous ovary organisation, indicating an indeterminate fecundity strategy, they exhibited different oocyte development patterns.

2. The oocyte size-frequency distribution in the gonochoristic species was similar to that reported for other teleosts, but the oocyte development stages, which composed the modes, varied among species. This could be considered a tactic as a result of the interaction between environmental factors and life history traits.

3. *Diplectrum radiale*, a simultaneous hermaphrodite, presented an unimodal size-frequency distribution of pre-vitellogenic and vitellogenic oocytes, differing from the other teleosts with indeterminate fecundity. It is a strategy imposed by the size of the ovarian sinus, resulting in a small number of oocytes spawned per batch and a high spawning frequency.

4. There is a positive relationship between total number of advanced vitellogenic oocytes and females’ ovary weight, total length and total weight in gonochoristic species, as expected. As the number of potential batches present in the ovaries of specimens in spawning capable phase differed among species, a single correlation cannot be considered as a proxy to access the reproductive status of the species.

5. The batch fecundity is an essential parameter in studies of spawning stock biomass. The results presented in this study, based on ecosystems structuring species, emphasises the importance of understanding patterns in oocyte development, as well as using reproductive performance and fecundity as tools to predict impacts of global climate change on biological systems.

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Glossary

10. GLOSSARY

Definitions of the terms used in the text. See also Hunter et al. (1992), Tyler and Sumpter (1996), Murua and Saborido-Rey (2003), Brown-Peterson et al. (2011), Mcbride et al. (2013), Pickitch et al. (2004) for further details.

Annual fecundity: total number of oocytes spawned by a female per year.

Asynchronous oocyte development: oocytes of all stages of development are present in the ovary as a random mixture.

Atresia: Resorption of oocytes, referring normally to those oocytes containing yolk which affect the annual realized fecundity.

Batch: population of oocytes at the same development stage.

Batch fecundity: number of germinal vesicle migration/ hydrated oocytes that could be released in one spawning event.

Bycatch: species incidentally captured while catching certain target species.

Clutch: in this thesis, refers a group of oocytes in pre-vitellogenesis or vitellogenesis growth.

Determinate fecundity regulation: the standing stock of vitellogenic oocytes is fixed prior to the onset of spawning season. Therefore, the annual or total potential fecundity can be measured at any time.

Ecosystem-based fishery management: is new direction for fishery management, essentially reversing the order of management priorities so that management starts with the ecosystem rather than a target species, which includes specie with no economic importance.

Fecundity: number of oocytes produced by a female.

Follicle: refers to the structure formed by the theca, granulosa/follicular cells and zona radiata.

Haploid: refers a cell (egg or sperm) containing a single set of chromosomes.

Gonochoristic species: species with separate sexes (non-sex changing).

Hydration (Hydrated oocytes): enlarged and transparent oocytes due to rapid uptake of water during the final maturation and prior to the next spawning event.

Indeterminate fecundity regulation: the standing stock of vitellogenic oocytes is not fixed prior to the onset of spawning season because primary oocytes continue develop into vitellogenic oocytes during the spawning season.

Oocyte: a female germ cell in development process.

Oocyte envelope: synonymous to follicle.

Oocyte maturation: duration of oocyte development from pre-vitellogenesis to spawning.

Stage: term commonly used to describe the development of individual gametes.

Oogenesis: the process of transformation of oogonia to oocytes.

Ovary phase: refers to the one part of the ovary development cycle.

Ovary sinus: accessory reproductive structure found in some serranids used to store hydrated oocytes before the next spawning event.

Ovotestis: gonad containing simultaneously testicular and ovarian tissues.

Potential Fecundity: The number of developing oocytes present in the ovary any time.

Potential annual fecundity: total number of advanced vitellogenic oocytes matured per year, uncorrected for atretic losses.

Pre-vitellogenic oocytes: Refers in this thesis to oocytes without the presence of yolk in the cytoplasm.

Relative fecundity: Fecundity dived by female weight.

Resilience: capacity of fish stock to respond to environment perturbation or fishing pressure.

Simultaneous hermaphrodite: individual which produces simultaneously both male and female gametes.

Skipped spawners: omission of annual spawning in a sexually mature female.

Spawning capable phase: Females being capable of spawning within the current reproductive cycle due to advanced gamete development, i.e. the oocytes are capable of receiving hormonal signals for final maturation.

Spawning event: each event of spawning releasing one batch of hydrated oocytes to the environment

Spawning fraction: the proportion of mature females spawning per day (ratio less than one or percentage).

Spawning frequency: average time between successive event of spawnings of females in a population, but presented in frequency terms (once per X days). Also deduced from the inverse of the spawning fraction.

Spawning interval: time between successive spawnings of an individual, normally measured in number of days or hours.

Spawning period: length of spawning of an individual within a season measured in months or days.

Spawning season: time in the year when the population produce egg/ offspring. Commonly set being synonymous to spawning period.

Standing stock oocytes: refers a population of oocytes at the same development stage or groups of pre-vitellogenic or vitellogenic oocytes

Stereology: quantitative descriptions of three-dimensional structures from interpretation of two-dimensional cross sections of materials or tissues.

Synchronous oocyte development: only a single cohort of oocytes progress from pre-vitellogenesis to vitellogenesis and is released during a spawning event.

Total fecundity: standing stock of advanced vitellogenic oocytes at any time in the ovary.

APPENDIX

11. APPENDIX A

11.1 Sampling strategy

The fishes were captured bimonthly, from June (2012) to may (2013), using the research boats Albacora and Veliger II of the Instituto Oceanografico da Universidade de São Paulo equipped with an otter trawl net. The trawls were carried out twice per day (between 08:00-10:30 am and 13:30-17:00 pm) on three consecutive days in each sampled month. Each station was trawled over a 15 min in depths that ranged from 20.6 m in the coastal of Ubatuba to 1.9 m in the estuary of Cananéia. After trawling, the specimens were immediately taken to the laboratory for taxonomical identification, to measure their total length (to the nearest millimetre), to weight (to the nearest gram, 0.01 g), to identify the maturity phase by macroscopic examination, and to remove their ovaries. Then, the ovaries were weighed and fixed in 10% neutral buffered formalin (Figure 1).

Figura 1. The sampling strategy adopted to capture the fishes. (a) Research boats Albacora and Veliger II; (b) the otter trawl; (c) fishes sampled; (d) processing the biological material; (e) specimen of *Stellifer rastrifer* showing the hydrated ovary before its fixation; (f) sampled ovaries fixed in 10% buffered formalin solution.

11.2 Estimation of the ovary volume

The volume of the ovaries was estimated according to the method proposed by Scherle (1970)[5]. This method is based on the principle of Archimedes, which states that a body submerged in a liquid will lose weight quantitatively equal to the weight of the liquid displaced by the body. However, the specific gravity (density) of the liquid must be known. Thus, only intact ovaries were taken for this purpose, and their whole volumes (Ov) were estimated according to the equation $Ov = m/ \rho$, where m is the buoyancy of the ovary (g) and ρ the density of the liquid. The liquid used was 10% neutral buffered formalin which has an approximate density of 1.029 g/cm³. Two different balances were used according to the weight of the ovary. A beaker filled with formalin solution was placed on the balance and adjusted to zero. Then, each ovary was completely immersed in the formalin solution avoiding its direct contact with the bottom and the walls of the beaker (Figure 2). Finally, a linear regression between the volume and weight of the ovaries, forced to pass through the origin, was performed to validate the results.

[5] SCHERLE, W.A. simple method for volumetry of organs in quantitative stereology. *Mikroskopie*, v. 26, p. 57-60, 1970.

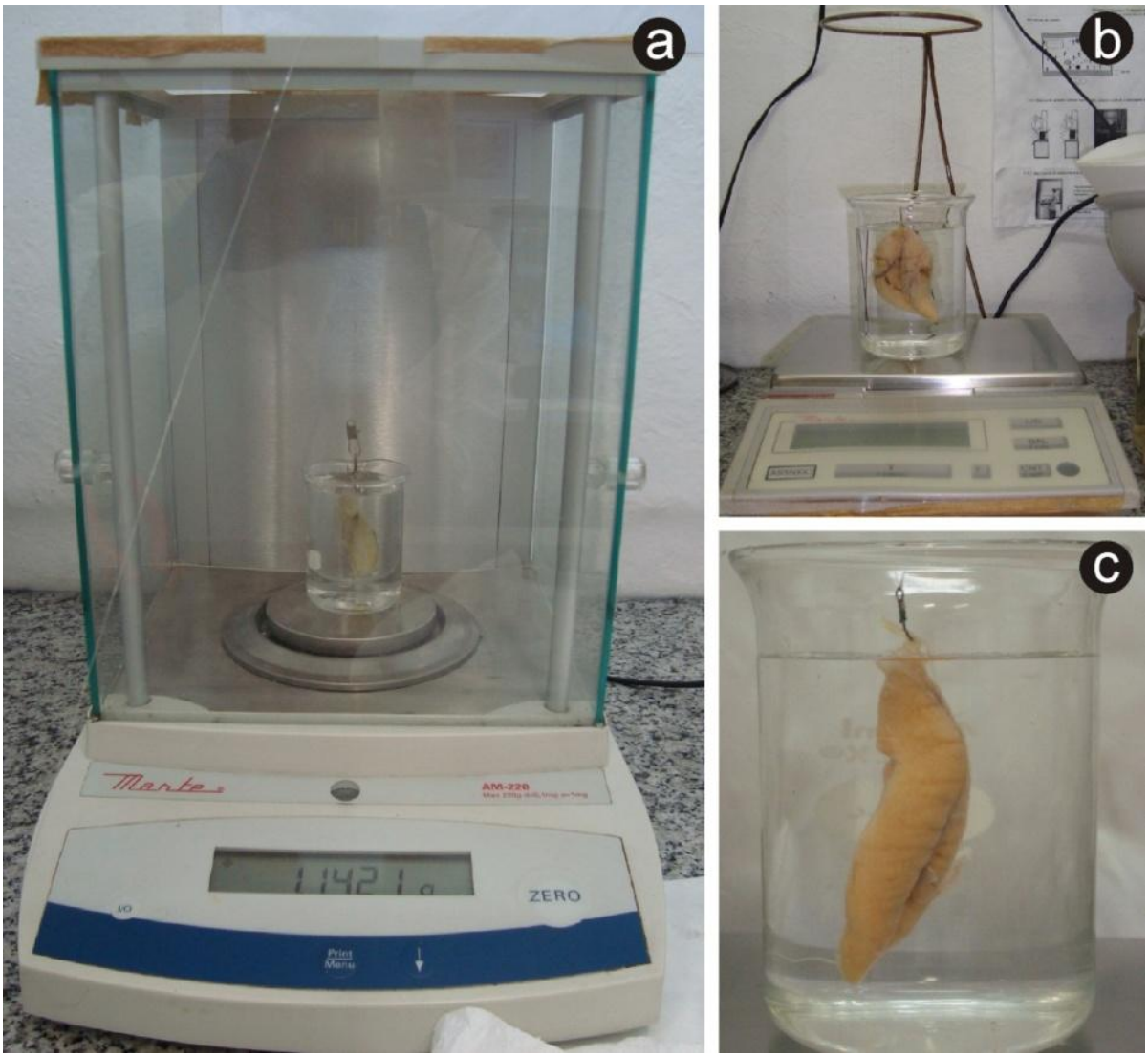

Figura 2. Estimating the volume of the fish ovaries. (a) The analytical balance showing the buoyancy value, (b) the semi-analytical balance used to estimate the volume of the heavier ovaries, (c) immersed ovary suspended by a tied hook to nylon.

11.3 Combined gravimetric method and image analysis for estimating batch fecundity

Three subsamples ranging from 10 to 525 mg were taken from the anterior, middle and posterior portion of the ovary. Each subsample was weighted in analytical balance to the nearest 0.0001 g. To separate the hydrated oocytes from the connective tissues and from those non-hydrated, each sub-sample was sieved (using a 500–300 µm mesh sieve) and sprayed with high-pressure water using a pipette filled with distilled water. Then, the oocytes were stained with Rose Bengal for at least 24 h, placed in a glass plate/ Petri dish and photographed with a digital camera attached to the stereomicroscope (Figure 3).

All hydrated oocytes present in each image were counted and measured in the free software ImageJ using an automated method proposed by Ganias et al. (2010)[6] modified. Thus, the software was first calibrated using an image of a stage micrometer of 50 mm = $5x10^4$ µm; the light background of each image was subtracted; then, the images were converted to 8-bit format, adjusted manually their brightness and contrast, selected the region of interest (oocytes) using the function "threshold", and separated the oocytes from each other using the function "Watershed". Finally, it was counted and measured the oocytes present in the image (Figure 4).

[6] Ganias, K. et al. Measuring batch fecundity using automated particle counting. *Fisheries Research*, v. 106, p. 570-574, 2010.

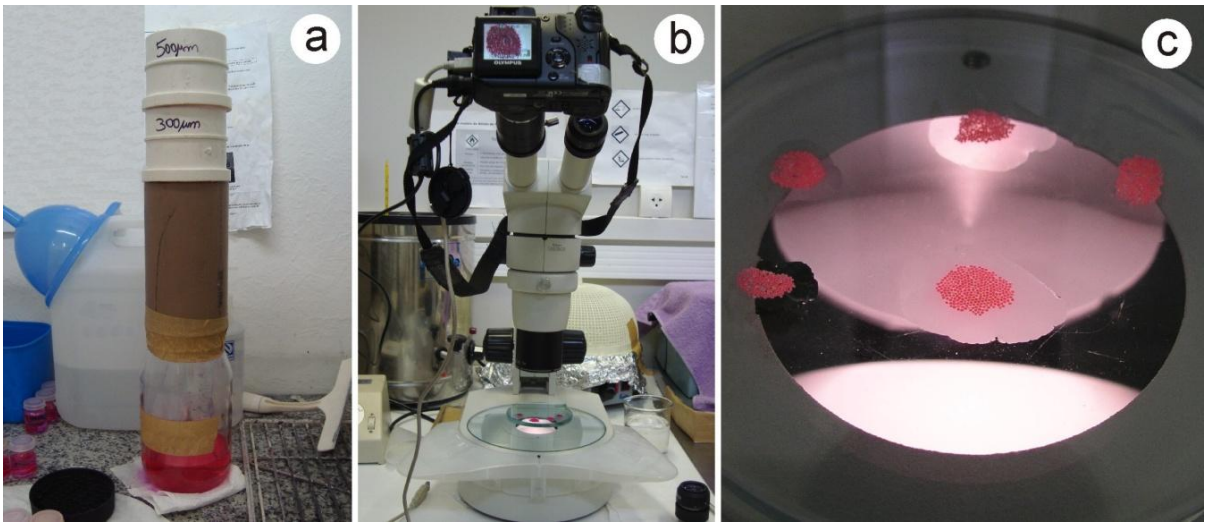

Figure 3. Procedure used to separate the hydrated oocytes for batch fecundity estimation. (a) Mesh sieves, (b) digital camera attached to the stereomicroscope, (c) separated oocytes stained with Rose Bengal.

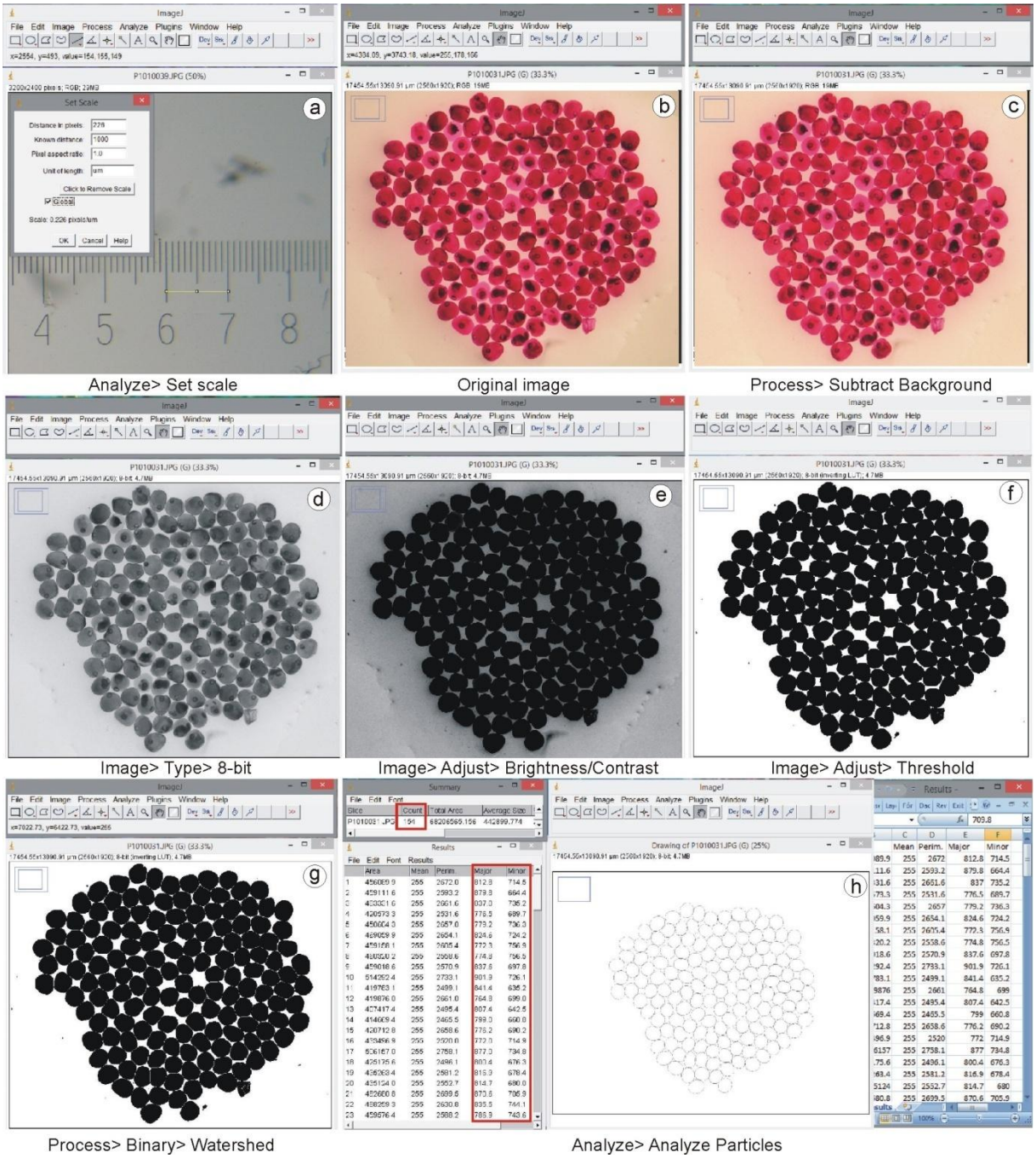

Figure 4. Processing steps for analysing the oocyte images using ImageJ software. (a) Setting scale, (b) original image, (c) subtracting the light background, (d) converting the image to 8 bit format, (e) adjusting the brightness and contrast, (f) indicating the region of interest, (g) separating the oocytes using the function "watershed", (h) ImageJ output showing the total number of oocytes counted and the longest (major) and shortest diameter (minor) of each oocyte measured, as well as the result saved in .xls format. The ImageJ commands are indicated below of each image.

12. APPENDIX B

12.1 Histological procedures and staining protocols

Fixation of the gonads: The tissues were fixed in 10% neutral buffered formalin. This solution had an approximate pH of 6.8 and was prepared as follows:

- Formaldehyde, 37%.....100 ml
- Distilled water.....900 ml
- Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$).....4 g
- Sodium phosphate dibasic (Na2H2PO4).....6.5g

Three to four subsamples from the anterior, middle and posterior portion of the fixed gonads were taken and placed in the tissue cassettes (Figure 5). Theses tissues were washed in running tap water for at least one hour to remove the fixative solution. Then, they were dehydrated through a series of graded ethanol baths, cleared in xylol and infiltrated with paraffin wax as follow**:**

Dehydration:

- 70% ethanol.....60 min
- 92.8% ethanol..... .....60 min
- 100% ethanol-I..... 60 min
- 100% ethanol-II.....60 min
- 100% ethanol-III.....60 min

Clearing:

- Ethanol+ xylol (50% ethanol/ 50% xylol).....45 min
- Xylol-I.....60 min
- Xylol-II.....60 min

Embedding:

- Paraffin wax-I (at 62 °C).....60 min
- Paraffin wax-II (at 62 °C).....60 min
- Paraffin wax-III (at 62 °C).....60 min

The blocks of paraffin containing the whole thickness of the ovarian wall were sectioned at 3-5 µm using a microtome. The tissues sectioned were placed onto the glass microscope slides with a thin layer of albumin. Then, the slides with paraffin sections were placed in an oven in a temperature of 62 °C for 30 min to melt the paraffin and to bond the tissue to the glass. Finally, the histological preparations were stained according to the staining protocols below.

12.1.1 Harris' Haematoxylin and Puttis' eosin (H&E)

Reagents and solutions

Harris' Haematoxylin

- Haematoxylin.....1 g
- 95% ethanol.....20 ml
- Potassium alum.....4 g
- Mercuric oxide…….0.6g
- Distilled water……..200 ml
- Glacial acetic acid…..…0.1 ml (0.1 ml of acid/ 100 ml of haematoxylin solution)

The haematoxylin was dissolved in the ethanol whilst the potassium alum in distilled water with heat. Then, the two solutions were mixed and heated to a boil. The final solution was removed from the heat and added the mercuric oxide. This solution was heated once again for 1 min, rapidly frozen and added the glacial acetic acid.

Puttis' eosin

- Eosin.....1 g
- 99.5% ethanol.....10 ml
- Potassium bichromate .....0.5 g
- Picric acid……1.3 g
- Distilled water……..180 ml

The eosin was dissolved in the ethanol, and the potassium bichromate in 80 ml of distilled water. The saturated solution of picric acid was made by dissolving the picric acid in 100 ml of distilled water. Then, the all solutions were mixed.

Procedure

Deparaffinisation and hydration

- Xylol-I.....13 min
- Xylol-II.....13 min
- Ethanol+ xylol (50% ethanol/ 50% xylol).....6 min
- 99.5% ethanol.....4 min
- 92.8% ethanol.....4 min
- 70% ethanol.....4 min
- Running tap water……4 min

H&E:

- Harris' haematoxylin…….....25 sec -1.30 min
- Running tap water….....4 min
- Acid ethanol solution.....rapidly rinsed
- Running tap water….....25 min
- Puttis' eosin……30 sec - 2 min

Dehydration

- Rinsed in tap water….....3x
- Rinsed in 92.8% ethanol.....2x
- Rinsed in 99.5%.....2x
- 99.5% Ethanol.....2 min
- Ethanol+ xylol (50% ethanol/ 50% xylol).....2 min
- Rinsed in xylol-I.....1x
- Rinsed in xylol-II.....1x

12.1.2 Periodic Acid-Schiff reaction (PAS) + Harris' Haematoxylin

Reagents and solutions

- Periodic acid…….....0.5 g
- Distilled water……..100 ml
- Schiff's reagent….....100 ml
- Harris' haematoxylin.....100 ml

The periodic acid solution was made by dissolving the periodic acid in distilled water.

Procedure

Deparaffinisation and hydration

- Xylol-I.....13 min
- Xylol-II.....13 min
- 99.5% ethanol.....4 min
- 92.8% ethanol.....4 min
- 70% ethanol.....4 min
- Running tap water……4 min

PAS reaction:

- Periodic acid solution (1%)…….....10 min
- Running tap water.....10 min
- Schiff's reagent.....60 min
- Running tap water….....10 min

Counterstain

- Harris' haematoxylin.....25 sec
- Rinsed in distilled water.....10 min

Dehydration

- Rinsed in 92.8% ethanol.....2x
- Rinsed in 99.5%.....2x
- 99.5% Ethanol.....2 min
- Ethanol+ xylol (50% ethanol/ 50% xylol).....2 min
- Rinsed in xylol-I.....1x
- Rinsed in xylol-II.....1x

12.1.3 PAS + Harris' Haematoxylin + Metanil yellow

Reagents and solutions

- Periodic acid solution (1%)…….....100 ml
- Distilled water……..230 ml
- Schiff's reagent….....100 ml
- Harris' haematoxylin.....100 ml
- Metanil yellow (MY).....0.1 g
- Glacial acetic acid……0.5 ml

The first solution was prepared by dissolving the metanil yellow in 100 ml of distilled water, and the second solution by mixing the glacial acetic acid with 100 ml of distilled water. Then, the MY solution was made by adding 20 ml of the first solution, 50 ml of the second solution to 30 ml of distilled water.

Procedure

Deparaffinisation and hydration

- Xylol-I.....13 min
- Xylol-II.....13 min
- 99.5% ethanol.....4 min
- 92.8% ethanol.....4 min
- 70% ethanol.....4 min
- Distilled water……4 min

PAS reaction:

- Periodic acid solution (1%)…….....10 min
- Running tap water.....10 min
- Schiff's reagent.....60 min
- Running tap water….....10 min

Counterstain

- Harris' haematoxylin.....25 sec
- Rinsed in distilled water.....10 min

MY

- Metanil yellow solution.....1.30-2 min

Dehydration

- Rinsed in 92.8% ethanol.....2x
- Rinsed in 99.5%.....2x
- 99.5% Ethanol.....2 min
- Ethanol+ xylol (50% ethanol/ 50% xylol).....2 min
- Rinsed in xylol-I.....1x
- Rinsed in xylol-II.....1x

12.2 Mounting the stained tissues

The permanent histological preparation was made by covering the stained tissues bonded in the glass slide with a glass coverslip. The Entellan mounting media was used to adhere the coverslip to the slide (Figure 5).

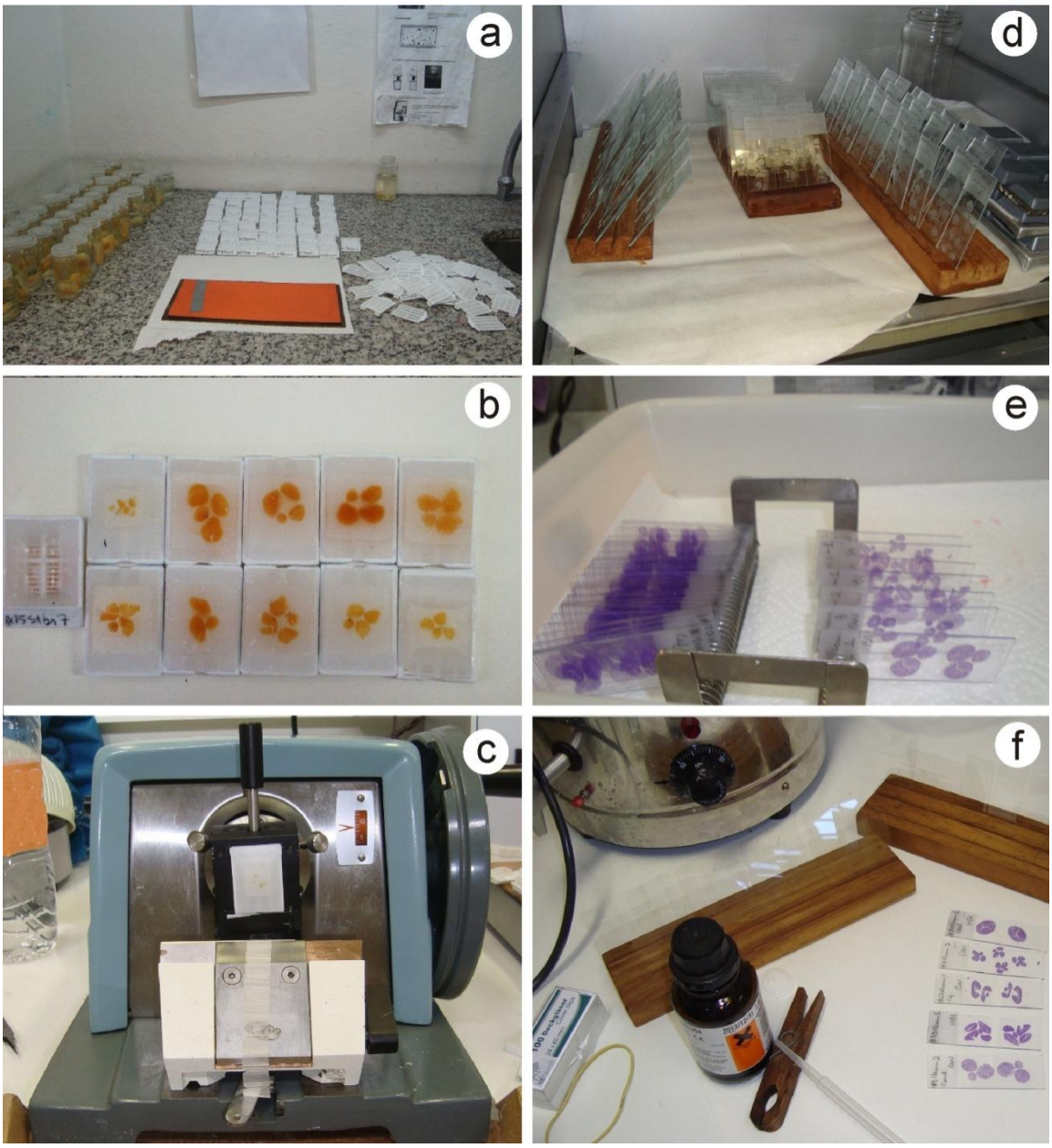

Figure 5. Histological procedures. (a) Taking the subsamples from the formalin-fixed gonads, (b) permanent blocks containing the ovary tissues embedded in paraffin, (c) sectioning the tissues, (d) the slides with paraffin sections in the oven to melt the paraffin and to bond the tissue to the glass, (e) stained tissues with Harris' Haematoxylin and Puttis' eosin, (f) mounting the histological preparations.

13. APPENDIX C

13.1. Table 1. Region, sampling date, time, season and environmental variables (temperature and salinity) measured* during the period of study in the inner shelf of Ubatuba and in the estuarine system of Cananéia, São Paulo State, Brazil.

Region	Sampling date (yyyy-mm-dd)	Sampling time (hh:mm)	Season	ST (°C)	BT (°C)	SS	BS	Sampling depth (m)
Ubatuba	2012-06-25	16:23	Autumn		22.6		35.0	20.60
Ubatuba	2012-06-25	09:00	Autumn		22.2		34.0	14.80
Ubatuba	2012-06-25	15:46	Autumn		22.3		36.0	13.10
Ubatuba	2012-06-27	09:30	Autumn		22.2		34.0	16.00
Ubatuba	2012-06-27	14:46	Autumn		22.0		35.0	15.50
Ubatuba	2012-06-28	08:43	Autumn	21.0	22.4		35.0	15.50
Ubatuba	2012-08-21	16:05	Winter	22.7	21.0	35.9	36.6	15.60
Ubatuba	2012-08-21	16:05	Winter	22.8	21.0			15.60
Ubatuba	2012-08-23	08:41	Winter	22.5	19.8	36.8	37.2	15.00
Ubatuba	2012-08-23	15:49	Winter	23.4	22.1	36.6	36.5	9.80
Ubatuba	2012-08-24	09:40	Winter	21.5	20.9	36.1	36.4	10.50
Ubatuba	2012-08-24	15:22	Winter	23.1	21.5	36.0	36.0	7.70
Ubatuba	2012-10-24	09:03	Spring	21.9	21.3	36.0	36.0	12.00
Ubatuba	2012-10-24	15:52	Spring	22.9	21.2	35.0	36.0	11.00
Ubatuba	2012-10-25	08:58	Spring	22.1	21.5	35.0	36.0	8.00
Ubatuba	2012-10-25	15:52	Spring	22.4	20.6	36.0	36.0	10.30
Ubatuba	2012-10-26	08:49	Spring	21.8	21.5	36.0	36.0	10.80
Ubatuba	2012-10-26	13:50	Spring	22.4	21.1	35.0	36.0	10.00
Ubatuba	2012-12-11	08:52	Spring	26.0	23.0	35.0	35.0	9.60
Ubatuba	2012-12-11	15:56	Spring	26.5	17.6	36.0	36.0	12.60
Ubatuba	2012-12-12	08:37	Spring	25.7	17.2	36.0	36.0	13.70
Ubatuba	2012-12-12	14:33	Spring	26.4	21.5	36.0	36.0	12.10
Ubatuba	2012-12-13	08:43	Spring	27.1	25.3	35.0	36.0	10.00
Ubatuba	2012-12-13	15:34	Spring	25.8	23.3	36.0	36.0	10.30
Ubatuba	2013-02-23	08:42	Summer	27.4	28.4	36.0	36.0	7.20
Ubatuba	2013-02-23	14:49	Summer	27.4	27.9	34.0	36.0	9.70
Ubatuba	2013-02-24	08:48	Summer	27.0	27.6	35.0	36.0	9.70
Ubatuba	2013-02-24	15:24	Summer	26.6	28.8	36.0	36.0	10.30
Ubatuba	2013-02-25	08:56	Summer	26.4	28.5	35.0	36.0	9.30
Ubatuba	2013-02-25	15:41	Summer	24.1	28.3	36.0	37.0	10.40
Ubatuba	2013-04-26	15:38	Autumn	24.1	24.0	36.0	36.0	9.90
Ubatuba	2013-04-29	09:08	Autumn	24.4	24.0	36.0	36.0	8.00
Ubatuba	2013-04-29	15:58	Autumn	24.6	24.3	36.0	36.0	8.40
Ubatuba	2013-04-30	08:30	Autumn	24.7	24.5	36.5	36.5	9.60
Ubatuba	2013-04-30	15:24	Autumn	25.2	24.5	36.5	35.8	8.60

continued

Region	Sampling date (yyyy-mm-dd)	Sampling time (hh:mm)	Season	ST (°C)	BT (°C)	SS	BS	Sampling depth (m)
Cananéia	2012-07-10	15:23	Winter	20.8	20.5	21.5	23.5	4.90
Cananéia	2012-07-11	08:27	Winter	20.5	20.5	25.1	27.2	3.70
Cananéia	2012-07-11	14:35	Winter	20.4	20.7	23.3	24.8	12.50
Cananéia	2012-07-12	08:31	Winter	20.1	20.2	19.4	26.7	5.20
Cananéia	2012-07-12	15:12	Winter	20.2	20.2	16.0	28.0	7.30
Cananéia	2012-07-13	08:27	Winter	18.9	19.9	18.0	23.0	5.50
Cananéia	2012-09-24	08:25	Winter	22.9	22.7	27.0	27.0	4.30
Cananéia	2012-09-24	15:40	Winter	23.4	22.7	26.0	29.0	4.30
Cananéia	2012-09-25	08:15	Winter	23.0	22.7	25.0	30.0	4.60
Cananéia	2012-09-25	15:50	Winter	22.5	22.5	31.0	30.0	3.70
Cananéia	2012-09-26	08:15	Winter	21.7	21.9	30.0	30.0	5.80
Cananéia	2012-09-26	15:40	Winter	21.5	21.0	34.0	34.0	6.40
Cananéia	2012-11-27	08:11	Spring	24.9	25.1	30.0	30.0	4.90
Cananéia	2012-11-27	15:33	Spring	24.8	24.6	33.0	33.0	5.50
Cananéia	2012-11-29	15:37	Spring	27.5	26.0	32.0	33.0	4.90
Cananéia	2012-11-30	08:08	Spring	25.5	25.5	33.0	33.0	3.70
Cananéia	2013-01-24	15:40	Summer	26.2	26.0	20.0	24.0	
Cananéia	2013-03-26	08:15	Summer	25.2	25.2	21.0	22.0	4.60
Cananéia	2013-03-26	15:34	Summer	27.8	25.7	32.0	34.0	6.10
Cananéia	2013-03-27	08:15	Summer	25.0	25.3	23.0	29.0	4.60
Cananéia	2013-03-27	14:51	Summer	25.5	23.4	33.0	34.0	6.10
Cananéia	2013-03-28	08:22	Summer	24.9	24.9	28.0	29.0	5.50
Cananéia	2013-03-28	14:08	Summer	25.4	25.4	31.0	32.0	4.90
Cananéia	2013-05-27	08:27	Autumn	23.5	22.4	29.0	29.7	4.30
Cananéia	2013-05-27	15:17	Autumn	25.7	22.5	30.0	31.8	4.60
Cananéia	2013-05-28	08:14	Autumn	25.5	24.9	28.5	29.8	4.00
Cananéia	2013-05-28	14:42	Autumn	27.9	27.7	27.0	29.0	3.70
Cananéia	2013-05-29	08:20	Autumn	22.0	22.2	30.8	32.8	5.20
Cananéia	2013-05-29	14:39	Autumn	22.8	22.3	26.0	29.0	1.90

* Surface temperature (SS) and bottom temperature (BT) were measured using a reversing thermometer attached to a bottle Nansen, and salinity (surface- SS and bottom- BS) with a help of a digital multiparameter meter and a refractometer. The depth was measured using an ecobathymeter coupled with a GPS.

13.2. Table 2. Spawning capable females in relation to region, species, season, sampling date, time, ovarian phase, total length (TL), total weight (TW), bottom temperature (BS) and bottom salinity (BS), sampled in the inner shelf of Ubatuba and in the estuarine system of Cananéia, São Paulo State, Brazil.

Region	Species*	Season	Sampling date (yyyy-mm-dd)	Sampling time (hh:mm)	Ovarian phase[#]	TL (mm)	TW (g)	BT (°C)	BS
Ubatuba	Anfi	Autumn	2012-06-27	09:30	GVM	105	8.3	20.2	34.0
Ubatuba	Anfi	Autumn	2012-06-28	08:43	GVM	105	8.6	22.4	35.0
Ubatuba	Anfi	Summer	2013-02-24	08:48	GVM	102	8.2	27.6	36.0
Ubatuba	Anfi	Autumn	2013-04-29	09:08	GVM	99	7.2	24.0	36.0
Ubatuba	Ceed	Summer	2013-02-24	08:48	GVM	172	51.3	27.6	36.0
Ubatuba	Ceed	Summer	2013-02-24	08:48	GVM	146	29.8	27.6	36.0
Cananéia	Cisp	Winter	2012-09-24	15:40	GVM	160	39.6	22.7	29.0
Cananéia	Cisp	Winter	2012-09-24	15:40	GVM	129	106.0	22.7	29.0
Cananéia	Cisp	Winter	2012-09-25	15:50	LGVM	161	40.7	22.7	29.0
Cananéia	Cisp	Winter	2012-09-25	15:50	LGVM	119	13.9	22.7	29.0
Ubatuba	Stbr	Spring	2012-10-25	08:58	Hyd	133	25.9	21.5	33.0
Ubatuba	Stbr	Spring	2012-10-25	08:58	Hyd	133	26.1	21.5	33.0
Ubatuba	Stbr	Spring	2012-10-25	08:58	Hyd	116	14.7	21.5	36.0
Ubatuba	Stbr	Spring	2012-10-26	13:50	Hyd	153	47.3	21.1	36.0
Ubatuba	Stbr	Spring	2012-10-26	13:50	Hyd	152	43.7	21.1	36.0
Ubatuba	Stbr	Spring	2012-12-13	08:43	Hyd	165	51.3	25.3	36.0
Ubatuba	Stbr	Spring	2012-12-13	08:43	Hyd	155	48.7	25.3	36.0
Ubatuba	Stbr	Spring	2012-12-13	08:43	Hyd	165	53.1	25.3	36.0
Ubatuba	Stbr	Spring	2012-12-13	08:43	Hyd	182	72.0	25.3	36.0
Ubatuba	Stbr	Spring	2012-12-13	08:43	Hyd	159	45.5	25.3	36.0
Ubatuba	Stbr	Spring	2012-12-13	08:43	Hyd	156	42.4	25.3	36.0
Ubatuba	Stbr	Summer	2013-02-24	15:24	LGVM	162	47.1	28.8	36.0
Ubatuba	Stbr	Summer	2013-02-24	15:24	Hyd	122	18.5	28.8	36.0
Ubatuba	Stbr	Summer	2013-02-24	15:24	GVM	169	51.8	28.8	36.0
Ubatuba	Stbr	Summer	2013-02-24	15:24	Hyd	146	34.3	28.8	36.0
Ubatuba	Stbr	Summer	2013-02-24	15:24	Hyd	150	37.7	28.8	36.0
Ubatuba	Stbr	Summer	2013-02-24	15:24	Hyd	115	15.6	28.8	36.0
Ubatuba	Stbr	Summer	2013-02-25	15:41	GVM	175	63.6	28.3	37.0
Ubatuba	Stbr	Summer	2013-02-25	15:41	GVM	148	34.9	28.3	37.0
Ubatuba	Stbr	Summer	2013-02-25	15:41	GVM	160	44.2	28.3	37.0
Ubatuba	Stbr	Summer	2013-02-25	15:41	GVM	164	55.9	28.3	37.0
Ubatuba	Stbr	Summer	2013-02-25	15:41	GVM	156	42.8	28.3	37.0
Ubatuba	Stbr	Autumn	2013-04-26	15:30	Hyd	157	42.1	24.0	36.0
Ubatuba	Stbr	Autumn	2013-04-30	15:24	LGVM	155	43.7	34.5	25.8
Cananéia	Stra	Summer	2013-03-26	08:15	GVM	145	42.0	25.2	22.0
Cananéia	Stra	Autumn	2013-05-28	14:42	Hyd	134	29.8	27.7	29.0
Cananéia	Stra	Autumn	2013-05-29	08:20	GVM	120	20.5	22.2	32.8
Cananéia	Meam	Winter	2012-09-24	15:40	Hyd	235	131.8	22.7	29.0
Cananéia	Meam	Summer	2013-03-26	08:15	GVM	275	233.7	25.2	22.0
Ubatuba	Meam	Spring	2012-12-13	08:43	GVM	225	121.2	25.3	36.0

** Anfi= Anchoa filifera, Ceed= Cetengraulis edentulus, Cisp= Citharichthys spilopterus, Stbr= Stellifer brasiliensis,Stra= S. rastrifer, Meam= Menticirrhus americanus.*

[#] GVM= germinal vesicle migration, LGVM= late germinal vesicle migration, Hyd= hydrated oocytes.

13.3. Table 3. List of some species captured by the trawlers during the period of study in the inner shelf of Ubatuba (Uba) and in the estuarine system of Cananéia (Can), São Paulo State, southeastern Brazil. n= total number of species captured.

Order	Family	Species	Classification	Region	Phases of maturation	n
Clupeiformes	Clupeidae	*Harengula clupeola* (Cuvier, 1822)	Pelagic	Uba	A,B	43
	Engraulidae	*Anchoa januaria* Hildebrand, 1943	Pelagic	Uba	A,B,C	14
		Anchoa spinifera (Valenciennes, 1848)	Pelagic	Uba/Can	B,C	4
		Anchoa filifera (Fowler, 1915)	Pelagic	Uba/Can	A,B,C,D,	99
		Anchoa marinii Hildebrand, 1943	Pelagic	Can	B,C	8
		Anchoa tricolor (Agassiz, 1829)	Pelagic	Uba/Can	B, C, D	18
		Anchoviella lepidentostole (Fowler, 1941)	Pelagic	Uba/Can	B,C	6
		Lycengraulis grossidens (Agassiz, 1829)	Pelagic	Uba/Can	A,B,C,D	136
		Cetengraulis edentulus	Pelagic	Uba/Can	B, C, D	84
Pleuronectiformes	Achiridae	*Achirus lineatus* (Linnaeus, 1758)	Demersal	Can	A,B,C,D	96
		Achirus declivis Chabanaud, 1940	Demersal	Uba/Can	A,B,C	46
	Cynoglossidae	*Symphurus tesselatus* (Quoy & Gaimard, 1824)	Demersal	Uba/Can	A,B,C	52
	Paralichthyiidae	*Syacium papillosum* (Linnaeus, 1758)	Demersal	Uba	A	2
		Etropus crossotus Jordan & Gilbert, 1882	Demersal	Uba/Can	A,B	82
	Paralichthyiidae	*Paralichthys brasiliensis* (Ranzani, 1842)	Demersal	Can	A	2
		Citharichthys spilopterus günther, 1862	Demersal	Uba/Can	A,B,C,D	294
Perciformes	Sciaenidae	*Ctenosciaena gracilicirrhus* (Metzelaar, 1919)	Demersal	Uba	A,B,C	18
		Larimus breviceps Cuvier, 1830	Demersal	Uba	B,C	54
		Menticirrhus americanus (Linnaeus, 1758)	Demersal	Uba/Can	A,B,C,D	240
		Menticirrhus litoralis (Holbrook, 1847)	Demersal	Can	A	2
		Nebris microps Cuvier, 1830	Demersal	Uba	A,B,C	20
		Ophioscion punctatissimus Meek & Hildebrand, 1925	Demersal	Uba	B,C	2
		Paralonchurus brasiliensis (Steindachner, 1875)	Demersal	Uba/Can	A,B,C,D	610
		Stellifer brasiliensis (Schultz, 1945)	Demersal	Uba	B,C,D	243
		Stellifer rastrifer (Jordan,1889)	Demersal	Uba/Can	A,B,C,D	509
		Stellifer stellifer (Bloch, 1790)	Demersal	Uba	A,B,C	18
	Serranidae	*Diplectrum radiale* (Quoy & Gaimard, 1824)	Demersal	Uba/Can	A,B,C	160
		Diplectrum formosum (Linnaeus, 1766)	Demersal	Uba	A	2
	Stromateidae	*Peprilus paru* Linnaeus, 1758	Pelagic	Uba	A	37
	Gerreidae	*Eucinostomus argenteus* Baird & Girard, 1855	Demersal	Uba/Can	A,B,C	125
		Eucinostomus gula (Quoy & Gaimard, 1824)	Demersal	Uba	A,B,C	59
		Diapterus rhombeus (Valenciennes, 1830)	Demersal	Uba/Can	A,B,C	60
	Haemulidae	*Haemulon steindachneri*	Demersal	Uba	A,B,C	44
		Orthopristis ruber (Cuvier, 1830)	Demersal	Uba	B	1
	Sphyraenidae	*Sphyraena guachancho* Cuvier, 1829	Pelagic	Can	A	1
Aulopiformes	Synodontidae	*Synodus intermedius* (Spix & Agassiz, 1829)	Demersal	Can	A	3

Macroscopical classification of the ovaries: A= immature, B= developing, C= spawning capable, D= regressing.

14. APPENDIX C

14.1. R Script for estimation of the size at which the oocytes are recruited to vitellogenesis (ORS).

```
#Libraries required for analysis

library(MASS)
library(psyphy)
library(boot)
library(RCurl)
library("ggplot2")
library(brglm)

#Set working directory and load data

setwd("D:/Doutorado/Dot_pojeto_B/Tese/R_cap_1/STBR")
dat_1<-read.csv("STBR_Dev_UBA.csv",dec=",",sep=";",header=T,as.is=T)
attach(dat_1)

#Figure resolution and plot configuration

tiff("STBR_UBA.tiff", width = 5, height = 4.5,units = 'in',res=300
plot(SpawCap_Diam,Mat_SpawCap,col="white",xaxt="n",xlab="",ylab="",yaxt="n",cex
.axis=1.5,font=1,las=2)
title(main="",font.main=3,xlab="",ylab="")
axis(2,las=2,tck=-.025,cex.axis=1.5,seq(from=0,to=1,by=.2))
axis(1,seq(from=0,to=800,by=40),las=2,cex.axis=1.5)

#Fitting bias-reduction generalised linear model (GLM) with a logit link function  for
#females in spawning capable phase (Dev). Binomial response= 0 for pre-vitellogenic
#and 1 for vitellogenic oocytes.

Dev_glm=brglm(Mat_Dev~Dev_Diam,family=binomial(logit),dat_1,method = "glm.fit")
curve(predict(Dev_glm,data.frame(Dev_Diam=x),type="resp"),add=TRUE,col="black"
,lty=1,lwd=1)

#Fitting bias-reduction generalised linear model (GLM) with a logit link function for
#females in spawning capable phase with post-ovulatory follicle (Spaw). Binomial
#response= 0 for pre-vitellogenic and 1 for vitellogenic oocytes.

dat_2<-read.csv("STBR_Spaw_UBA.csv",dec=",",sep=";",header=T,as.is=T)
attach(dat_2)
dat_2=as.data.frame(cbind(Spaw_Diam,Mat_Spaw))
Spaw_glm=brglm(Mat_Spaw~Spaw_Diam,family=binomial(logit),dat_2,method =
"glm.fit")
curve(predict(Spaw_glm,data.frame(Spaw_Diam=x),type="resp"),add=TRUE,col="bl
ue",lty=1,lwd=1)
```

```
#Fitting bias-reduction generalised linear model (GLM) with a logit link function  for #females in spawning capable phase with germinal vesicle migration oocytes #(SpawCap). Binomial response= 0 for pre-vitellogenic and 1 for vitellogenic oocytes.

dat_3<-ead.csv("STBR_SpawCap_UBA.csv",dec=",",sep=";",header=T,as.is=T)
attach(dat_3)
dat_3=as.data.frame(cbind(SpawCap_Diam,Mat_SpawCap))
SpawCap_glm=brglm(Mat_SpawCap~SpawCap_Diam,family=binomial(logit),dat_3, method = "glm.fit")
curve(predict(SpawCap_glm,data.frame(SpawCap_Diam=x),type="resp"),add=TRUE,col="red",lty=1,lwd=1)

#Fitting bias-reduction generalised linear model (GLM) with a logit link function for #females in spawning capable phase with germinal vesicle migration oocytes #(ActSpaw). Binomial response= 0 for pre-vitellogenic and 1 for vitellogenic oocytes.

dat_4<-read.csv("STBR_ActSpaw_UBA.csv",dec=",",sep=";",header=T,as.is=T)
attach(dat_4)
dat_4=as.data.frame(cbind(ActSpaw_Diam,Mat_ActSpaw))
ActSpaw_glm=brglm(Mat_ActSpaw~ActSpaw_Diam,family=binomial(logit),dat_4,method = "glm.fit")
curve(predict(ActSpaw_glm,data.frame(ActSpaw_Diam=x),type="resp"),add=TRUE,col="purple",lty=1,lwd=1)

# ORS function

L50_Dev<-dose.p(Dev_glm,p=c(0.5))
L50_SpawCap<- dose.p(SpawCap_glm,p=c(0.5))
L50_Spaw<-dose.p(Spaw_glm,p=c(0.5))
L50_ActSpaw<-dose.p(ActSpaw_glm,p=c(0.5))

#ORS results

L50_Dev
L50_Spaw
L50_SpawCap
L50_ActSpaw

#Adding ORS values to the plot

points(158.4227,.5,pch=19,col="Black",lwd=2,cex=0.7)#DEV
points(182.9187,.5,pch=19,col="blue",lwd=2,cex=0.7)#Spawned
points(147.74,.5,pch=19,col="red",lwd=2,cex=0.7)#SpawCap
points(171.12629024,.5,pch=19,col="purple",lwd=2,cex=0.7)#ActSpaw
abline(h=.5,col="black")
text(330,.70,"Vitellogenesis",font=1,cex=0.9)
text(330,.65,"and",font=1,cex=0.9)
text(330,.60,"Maturation",font=1,cex=0.9)
text(330,.43,"Pre-vitellogenesis",font=1,cex=0.9)
```

```
#Legends

legend('bottomright',inset=.03, c('SpCa','SpCa(GVM)','SpCa(Hyd)', 'SpCa(POF)'),
col=c("black","red",'purple',"blue"), pch=c(19,19,19,19), lty=c(1,1,1,1), lwd=c(1,1,1,1),
bty='L', cex=0.9)

##########################################################################
# Plotting confidence intervals using bootstrapping outputs.             #
# Modified from Harry et al. (2013)[1].                                  #
##########################################################################

#Functions for females in spawning capable phase (Dev)

boot.dev<-function(dat_1, i){
  dat_1 <- dat_1[i,]
  mod <- brglm(formula = Mat_Dev~Dev_Diam, family = binomial(link="logit"),data=
dat_1)
  L50<-dose.p(mod,p=c(0.5))
  L95<-dose.p(mod,p=c(0.95))
  Bootstats<-c(coefficients(mod),L50,L95)
}
R=1000
Maturity.Bootstraps<- boot(dat_1,statistic=boot.dev,R)
Confidence.Intervals<-envelope(Maturity.Bootstraps)
Confidence.Intervals$point
Confints<-function(dat_1,boot.out,mod,num.curves){
  Boot.results<-boot.out
  Length<-seq(min(Dev_Diam,na.rm=T),max(Dev_Diam,na.rm=T),1)
  Num.curves<- num.curves
  Model<-mod
  par1<-Boot.results$t[1:Num.curves,1]
  par2<-Boot.results$t[1:Num.curves,2]
  par1.vec = rep(par1,each=length(Length))
  par2.vec = rep(par2,each=length(Length))
  predicted.maturity<- 1/(1+exp(-(par1.vec+par2.vec*Length)))
  plotmatrix <- matrix(predicted.maturity,ncol=length(Length),byrow=T)
  fitted.par.1<-coefficients(Model)[[1]]
  fitted.par.2<-coefficients(Model)[[2]]
  fitted.maturity<- 1/(1+exp(-(fitted.par.1+fitted.par.2*Length)))
  bounds <- quantile(plotmatrix[,1],c(.025,.975))
  boundsmatrix <- matrix(bounds,nrow=2)
  for (i in 2:length(Length)){
    bounds <- quantile(plotmatrix[,i],c(.025,.975))
    boundsmatrix <- cbind(boundsmatrix,bounds)
  }
  return(list(Length=Length,boundsmatrix=boundsmatrix,
              fitted.maturity=fitted.maturity))
}
CI.Plotting<-Confints(dat_1,Maturity.Bootstraps,Dev_glm,R)
for(i in 1:2){lines(CI.Plotting$Length, CI.Plotting$boundsmatrix[i,],lty=2)}
```

```
#Functions for females in spawning capable phase with POF (Spaw)
boot.spaw<-function(dat_2, i){
  dat_2 <- dat_2[i,]
  mod <- brglm(formula = Mat_Spaw~Spaw_Diam, family =
binomial(link="logit"),data= dat_2)
  L50_Spaw<-dose.p(mod,p=c(0.5))
  L95_Spaw<-dose.p(mod,p=c(0.95))
  Bootstats<-c(coefficients(mod),L50_Spaw,L95_Spaw)
}
R=1000
Maturity.Bootstraps<- boot(dat_2,statistic=boot.spaw,R)
Confidence.Intervals<-envelope(Maturity.Bootstraps)
Confidence.Intervals$point
Confints<-function(dat_2,boot.out,mod,num.curves){
  Boot.results<-boot.out
  Length<-seq(min(Spaw_Diam,na.rm=T),max(Spaw_Diam,na.rm=T),1)
  Num.curves<- num.curves
  Model<-mod
  par1<-Boot.results$t[1:Num.curves,1]
  par2<-Boot.results$t[1:Num.curves,2]
  par1.vec = rep(par1,each=length(Length))
  par2.vec = rep(par2,each=length(Length))
  predicted.maturity<- 1/(1+exp(-(par1.vec+par2.vec*Length)))
  plotmatrix <- matrix(predicted.maturity,ncol=length(Length),byrow=T)
  fitted.par.1<-coefficients(Model)[[1]]
  fitted.par.2<-coefficients(Model)[[2]]
  fitted.maturity<- 1/(1+exp(-(fitted.par.1+fitted.par.2*Length)))
  bounds <- quantile(plotmatrix[,1],c(.025,.975))
  boundsmatrix <- matrix(bounds,nrow=2)
  for (i in 2:length(Length)){
    bounds <- quantile(plotmatrix[,i],c(.025,.975))
    boundsmatrix <- cbind(boundsmatrix,bounds)
  }
  return(list(Length=Length,boundsmatrix=boundsmatrix,
              fitted.maturity=fitted.maturity))
}
CI.Plotting<-Confints(dat_2,Maturity.Bootstraps,Spaw_glm,R)
for(i in 1:2){lines(CI.Plotting$Length, CI.Plotting$boundsmatrix[i,],lty=2,col="blue")}

#Functions for females in spawning capable phase with GVM (SpawCap)

boot.spawcap<-function(dat_3, i){
  dat_3 <- dat_3[i,]
  mod <- brglm(formula = Mat_SpawCap~SpawCap_Diam, family =
binomial(link="logit"),data= dat_3)
  L50_Spawcap<-dose.p(mod,p=c(0.5))
  L95_Spawcap<-dose.p(mod,p=c(0.95))
  Bootstats<-c(coefficients(mod),L50_Spawcap,L95_Spawcap)
}
R=1000
```

```
Maturity.Bootstraps<- boot(dat_3,statistic=boot.spawcap,R)
Confidence.Intervals<-envelope(Maturity.Bootstraps)
Confidence.Intervals$point
Confints<-function(dat_3,boot.out,mod,num.curves){
  Boot.results<-boot.out
  Length<-seq(min(SpawCap_Diam,na.rm=T),max(SpawCap_Diam,na.rm=T),1)
  Num.curves<- num.curves
  Model<-mod
  par1<-Boot.results$t[1:Num.curves,1]
  par2<-Boot.results$t[1:Num.curves,2]
  par1.vec = rep(par1,each=length(Length))
  par2.vec = rep(par2,each=length(Length))
  predicted.maturity<- 1/(1+exp(-(par1.vec+par2.vec*Length)))
  plotmatrix <- matrix(predicted.maturity,ncol=length(Length),byrow=T)
  fitted.par.1<-coefficients(Model)[[1]]
  fitted.par.2<-coefficients(Model)[[2]]
  fitted.maturity<- 1/(1+exp(-(fitted.par.1+fitted.par.2*Length)))
  bounds <- quantile(plotmatrix[,1],c(.025,.975))
  boundsmatrix <- matrix(bounds,nrow=2)
  for (i in 2:length(Length)){
    bounds <- quantile(plotmatrix[,i],c(.025,.975))
    boundsmatrix <- cbind(boundsmatrix,bounds)
  }
  return(list(Length=Length,boundsmatrix=boundsmatrix,
              fitted.maturity=fitted.maturity))
}
CI.Plotting<-Confints(dat_3,Maturity.Bootstraps,SpawCap_glm,R)
for(i in 1:2){lines(CI.Plotting$Length, CI.Plotting$boundsmatrix[i,],lty=2,col="red")}

#Functions for females in spawning capable phase with hydrated oocytes
#(ActSpaw)

 (dat_4, i){
  dat_4 <- dat_4[i,]
  mod <- brglm(formula = Mat_ActSpaw~ActSpaw_Diam, family =
binomial(link="logit"),data= dat_4)
  L50_ActSpaw<-dose.p(mod,p=c(0.5))
  L95_ActSpaw<-dose.p(mod,p=c(0.95))
  Bootstats<-c(coefficients(mod),L50_ActSpaw,L95_ActSpaw)
}
R=1000
Maturity.Bootstraps<- boot(dat_4,statistic=boot.actspaw,R)
Confidence.Intervals<-envelope(Maturity.Bootstraps)
Confidence.Intervals$point
Confints<-function(dat_4,boot.out,mod,num.curves){
  Boot.results<-boot.out
  Length<-seq(min(ActSpaw_Diam,na.rm=T),max(ActSpaw_Diam,na.rm=T),1)
  Num.curves<- num.curves
  Model<-mod
  par1<-Boot.results$t[1:Num.curves,1]
```

```
  par2<-Boot.results$t[1:Num.curves,2]
  par1.vec = rep(par1,each=length(Length))
  par2.vec = rep(par2,each=length(Length))
  predicted.maturity<- 1/(1+exp(-(par1.vec+par2.vec*Length)))
  plotmatrix <- matrix(predicted.maturity,ncol=length(Length),byrow=T)
  fitted.par.1<-coefficients(Model)[[1]]
  fitted.par.2<-coefficients(Model)[[2]]
  fitted.maturity<- 1/(1+exp(-(fitted.par.1+fitted.par.2*Length)))
  bounds <- quantile(plotmatrix[,1],c(.025,.975))
  boundsmatrix <- matrix(bounds,nrow=2)
  for (i in 2:length(Length)){
    bounds <- quantile(plotmatrix[,i],c(.025,.975))
    boundsmatrix <- cbind(boundsmatrix,bounds)
  }
  return(list(Length=Length,boundsmatrix=boundsmatrix,
              fitted.maturity=fitted.maturity))
}
CI.Plotting<-Confints(dat_4,Maturity.Bootstraps,ActSpaw_glm,R)
for(i in 1:2){lines(CI.Plotting$Length, CI.Plotting$boundsmatrix[i,],lty=2,col="purple")}

dev.off()
```

[1]Harry et al (2013) Age, growth, and reproductive biology of the spot-tail shark, *Carcharhinus sorrah*, and the Australian blacktip shark, *C. tilstoni*,from the Great Barrier Reef World Heritage Area, north-eastern Australia. *Marine and Freshwater Research*, v. 64, n.4, p. 277-293. Doi: http://dx.doi.org/10.1071/MF12142

ANNEX

15. ANNEX A

15.1. Ethical statement.

CEEA-IOUSP
Comissão de Ética em Experimentação Animal
Instituto Oceanográfico - USP

Name: Eudriano Costa
Supervisor: Profa. Dra. June Ferraz Dias

Doctoral project: Reproductive strategies of marine fishes from the Southwest Atlantic Ocean: an application of histological and image processing techniques

Species	Number of specimens sampled
Anchoa filifera (Fowler, 1915)	104
Cetengraulis edentulus (Cuvier, 1829)	86
Citharichthys spilopterus Günther, 1862	427
Stellifer brasiliensis (Schultz, 1945)	281
Stellifer rastrifer (Jordan, 1889)	742
Menticirrhus americanus (Linnaeus, 1758)	410
Paralonchurus brasiliensis (Steindachner, 1875)	827
Diplectrum radiale (Quoy and Gaimard, 1824)	287
Total	3.164

The fishes were sampled for histological studies of the gonads. They were captured bimonthly, from June (2012) to May (2013), during research cruises using an otter trawl in the inner shelf of Ubatuba and estuarine system of Cananéia, São Paulo state, Brazil. Therefore, no laboratory experiments were performed. The captures were authorized by ICMBIO-MMA (Chico Mendes Institute for Biodiversity Conservation of the Brazilian Ministry of Environment)/ SISBIO (Authorization system and biodiversity information). The authorization number is 18493-1.
The most specimens captured were already dead after the trawling, but those alive were rapidly euthanised by direct destruction of the brain tissue (brainstem) causing the cessation of the brain and respiratory activity. All study and animal care were following the euthanasia practice guidelines of the National Council for the Control of Animal Experimentation, Brazil.
After analysis, the fishes were incinerated and the ovaries of females were preserved in 10% buffered formalin for future studies.
The formalin solution, alcohol, xylol and paraffin used during the histological processing were discarded adequately by the Institute of Oceanography of the University of São Paulo (IOUSP), Brazil.

The Ethics Committee on Animal Experiments of IOUSP approved this research protocol, once it is compatible with the ethical principles of animal experimentation prepared by the National Council of Ethics in Research (CONEP) and the Brazilian College of Animal Experimentation (COBEA).

São Paulo, 23/02/2015

Profa. Dra. Rosalinda Carmela Montone
Comissão de Ética em Experimentação Animal do IOUSP
presidente