LIFE10 NAT/IT/000239 “RARITY”

Eradicate invasive Louisiana red swamp
and preserve native white clawed crayfish
in Friuli Venezia Giulia

Az. A2

Monitoraggio della riproduzione di *P. clarkii* nel canale Brancoletto

Authors: Luca Peruzza[1] email: peruzzaluca@gmail.com,
Federica Piazza[1] email: federicapiazza1983@gmail.com,
Chiara Manfrin[1] email: chiaramanfrin@gmail.com,
Lucrezia Celeste Bonzi[1] email: lucrezia.bonzi@gmail.com,
Silvia Battistella[1] email: battiste@units.it,
Piero Giulio Giulianini[1]* email: giuliani@units.it.

[1]Department of Life Sciences, University of Trieste, via L. Giorgieri, 5, 34127, Trieste, Italy.

Abstract

In this study *Procambarus clarkii*'s (Girard, 1852) reproductive biology from a population living in a peculiar habitat with cold spring waters is investigated through a yearly sampling activity by monitoring Gonado-Somatic and Hepato-Somatic Indexes (GSI and HSI) and by performing cytology on ovaries. Despite its known preference for habitats with water temperature from 21 to 30 °C, our results clearly confirm the adaptation of this population at the peculiar characteristics of this habitat characterised by an annual mean water temperature value of 13.32 ±0.08 °C. The maximum gonadal development is reached in August (with maximum GSI median values of 0.64 instead of reported values even 10 times higher) and ovigerous females are found in autumn with a mean realized fecundity of 35 ±7, rather than values ranging from 285 to 995 reported for other habitats. In addition, histological analysis was concordant with other results and allowed us to follow ovarian development at cytological level. The importance of all these results is not to be underestimated: to our knowledge these findings are the first report of the coolest habitat successfully colonized by this species at the present time and so they have to be taken as a warning about the possible range expansion of *P. clarkii* also to northern and colder habitats that have few things in common with the native habitat of the species and, up to now, were considered “safe” from the invasion of the Louisiana crayfish.

Keywords: *Procambarus clarkii*, biological plasticity, GSI HSI, reproductive cycle, ovarian development

Introduction

The red swamp crayfish, *Procambarus clarkii* (Girard, 1852), is a Decapod crustacean coming from the South-eastern United States, proved to be an invasive species because “it is an agent of change and threatens native biological diversity (IUCN, 2014)”.
It was brought in Europe in the early 70s for aquaculture purposes and nowadays it is the most cosmopolitan crayfish, being found in all continents except Australia and Antarctica (Scalici and Gherardi 2007). *P. clarkii* was introduced in North Italy in 1989 and since 2006 its presence is attested also in Friuli Venezia Giulia with natural populations in almost all water courses.
P. clarkii is an r-selected species showing a wide plasticity of its life cycle (Gherardi et al. 2000a; Scalici and Gherardi 2007; Chucholl 2011a) which allows these animals to survive in almost all freshwater habitats, under the most diversified physical and chemical conditions (i.e. in polluted habitats, in brackish water or in dens partially outside the water).
The ecological success of this species is also granted by its reproductive biology: in its natural range (Louisiana, USA) *P. clarkii* breeds year round, with different recruitment peaks in summer, early winter and spring (Chucholl 2011a). Several studies led in Europe, have investigated the reproductive biology of such species, but when compared among them, they show different results. In Southern Spain *P. clarkii* populations under favourable conditions has up to three-four recruitment periods per year, as in Louisiana and California, while in Southern Germany, under unfavourable conditions, *P. clarkii* populations exhibit only one recruitment period from autumn to winter (Chucholl 2011a). In Italy, the majority of the studies have focussed in Central Italy (Barbaresi and Gherardi 2000; Dörr et al. 2006; Gherardi 2006; Scalici and Gherardi 2007; Scalici et al. 2010) and the Italian populations showed a reproductive pattern intermediate between the Spanish and German populations cited above (Chucholl 2011a).
In North Italy, up to date, no study has been conducted on the reproductive biology of this species despite its presence in many watercourses. Thanks to a large scale analysis of freshwater crustacean's populations living in Friuli Venezia Giulia (FVG, North-East Italy) promoted by the Life RARITY Project (LIFE 10 NAT / IT / 000239) some reports attested the strong presence of *P.*

clarkii living in a channel with peculiar water temperature values, near the Brancolo's river (Go, Italy) and inside the Brancolo's reclamation area. In the light of this fact, within the reclamation area a sampling site in one river has been chosen to investigate the population and to determine the reproductive biology of this invasive species on yearly scale. The findings obtained may provide information for successfully controlling and managing *P. clarkii* populations, lastly they can be compared to the other reproductive patterns studied in Europe, so far. The Gonado-Somatic Index (GSI) and the Hepato-Somatic Index (HSI) were monitored during a year and light microscopical and ultra-structural cytology were performed on the ovaries of female crayfish following the different stages of the ovarian development (Kulkarni et al. 1991; Ando and Makioka 1998).

Materials and methods

Sampling Area

The selected population inhabited an artificial canal inside the "Bonifica del Brancolo" ("Brancolo's reclamation area", 45°46' N, 13°30' E, GO, Italy). The area is bounded on the north by the Brancolo's canal, which flows parallel to the shoreline, on the south and east by the Adriatic Sea and is located one meter below the sea level. Various resurgences can be found on the river bed of all water channels inside the reclamation area which is also subjected to tides. For these reasons the area comprises artificial canals linked to a series of de-watering pumps which remove excess waters. Usually de-watering pumps work for 20h/day during the summer, removing water at 800 l/sec, but can reach 5400 l/sec during heavy rainfall.

Sampling activity

The sampling activity lasted for a year, from March 2013 with a total of 24 sampling sessions (except for the first two months, one sampling was performed every 15 days). Sampling was always conducted in the same place, in a bank of the river 120m long, by intensively capturing crayfish using 20 lobster pots with canned food left in the river bed for 48 h.
During every sampling date, water temperature (°C ±0.01) was determined with an outdoor thermometer data-logger (Tinytag Aquatic 2 TG-4100, Gemini Data Loggers, Chichester UK) at least 24 hours before the specimens harvesting. The instrument recorded the temperature in the river bed once every 10 minutes. Data collected were used to control trend in the water temperature on a monthly scale.
During several samplings, salinity measurements (μS/cm ±1) were taken using a conductivity probe Hanna HI 8633 (Hanna instruments, Woonsocket, RI). It was made sure to measure salinity on the bottom of the channel binding the instrument probe with sinkers. For every spot, measures were done three times. Measures during different sampling dates were conducted to compare salinity values with the tidal cycle.
Captured crayfish were counted, sexed and their carapax total length (CTL) was determined to a precision of ±0.1 mm.
From ovigerous females, pleopodal eggs or offspring were counted in order to determine the realized fecundity of the studied population (FR) (Ma et al., 1998).
The reproductive stage of captured males has been determined according to Taketomi (Taketomi et al. 1996). Sexually mature males have been staged E, on the base of the presence of reversed spines on the third and fourth walking legs, while sexually immature males have been collectively staged D (comprehending Taketomi's A – D stages). The classification used by Taketomi et al. is only based on secondary sexual characteristics on the copulatory appendages and their relation with development of testis and androgenic gland; although no actual correlation between Taketomi's

classification and commonly used Form I and Form II classification is present, actually only Stage E males have the anatomical characteristics needed to successfully complete copula.
For each sampling date 14 females with CTL between 44 and 70 mm were dissected after anaesthetization, their HSI (HSI= wet weight hepatopancreas / wet weight female *100) and GSI (GSI= wet weight ovary / wet weight female *100) were determined and their ovaries were fixed for 24 h at room temperature in a modified SPAFG fixation solution (Ermak and Eakin 1976) (2.5% glutaraldeide, 0.8% paraformaldehyde and 7.5% saturated aqueous solution of picric acid in 0.1 M phosphate buffered saline, pH 7.4, with 1.5% sucrose). Samples for optical microscopy were embedded in LR-White resin (London Resin Company, UK) after dehydration in ethanol (50%,70%,95% and absolute). Semithin sections (1-2μm) cut on a Pabisch TOP Ultra 150 (*Pabish*, Germany) were stained with toluidin blue. Samples used for TEM were post-fixed in 1% osmium tetroxide, dehydrated in ethanol and embedded via propylene oxide in Epon 812-Araldite mixture (Electron Microscopy Sciences, USA). Sections on 200-300 mesh nickel grids were stained with uranyl acetate and lead citrate and viewed under a Philips EM 208 TEM (Philips) at 100 kV.
All data are expressed as mean ± SEM. Statistical analysis were performed using R software version 3.0.1 RC 2014, while histological measures on samples were performed using ImageJ software (ver. 1.48b) (Schneider et al. 2012).

Ethical Note

The experiments comply with the current laws of Italy, the country in which they were done. No specific permits were required for the studies that did not involve endangered or protected species. Individuals were maintained in appropriate laboratory conditions to guarantee their welfare and responsiveness. After the experiments were completed, crayfish were sacrificed by hypothermia.

Results

Monitoring activity

As cited by De Luise, the first evidences of *P. clarkii* in the reclamation area “Bonifica del Brancolo” went back to 2007 and its introduction probably was due to its use as live bait by local fishermen (De Luise 2009). The sampling site is located on a reclamation area where de-watering pumps remove excess waters coming from several resurgences (present on the river bed) and from the sea. The sampling site is bounded on the north by the Brancolo river (which has salty and colder waters and where *P. clarkii* is not present), on the south and east by the Adriatic sea and by an extensive network of drainage canals (with same water characteristics as in our site) on the west that isolate the studied population inside the reclamation area.
From March 2013 a total of 24 sampling campaigns were done. A total of 3090 animals were captured, with an overall sex ratio of 1:1.15 (F:M). Relative abundance of males and females crayfishes for every sampling date is showed in Figure 1. From June to December captured males are always more abundant than females, ultimately reaching a sex ratio of 2.85:1 on 5 September. From January the situation is inverted and females reach a maximum sex ratio value of 1.55:1 on 7 March 2014.
CTL measurements of the total number of animals captured show that, the smallest animal is 19.5 mm long, the longest is 77.1 mm long, mean CTL of males and females respectively are 48.94 ±0.23 mm and 49.89 ±0.23 mm.
The reproductive stage of captured males is shown in Figure 2. Males with fully developed copulatory organ (with CTL ranging from 26.60 mm to 71.90 mm, mean 50.31 ±0.40 mm) are present with maximum abundances during the summer period and then their abundance decrease until the next Spring.

Trend in water temperature over the year on the sampling site is showed in Figure 3. Because of the cold spring waters, temperature recorded on the river bed are always low and a relatively small thermal excursion is found across different seasons. Mean values over the 24 h range from 8.65 ±0.08 °C to 16.19 ±0.12 °C, around an annual mean value of 13.32 ±0.08 °C. The minimum water temperature value of 7.16 °C has been recorded on December 2013. Also during the summer, while mean air temperature can reach 27.9°C (Aeronautica Militare, climatic tables http://clima.meteoam.it/AtlanteClimatico/pdf/%28110%29Trieste.pdf), mean water temperature stands under 19 °C (with maximum mean water temperature value over 24 h of 16.17 ±0.08 °C – November 2013 and maximum water temperature measured value of 19.13 °C – June 2013).
Conductivity measurements report oscillations in values between a minimum of 732 µS/cm and a maximum of 1770 µS/cm, with a mean value of 1128 ±48 µS/cm. Tidal cycle, de-watering pumps activity, spring waters and precipitations are the main factors that account for the difference between the conductivity values recorded.
GSI and HSI values obtained from females during the monitoring activity are shown in Figure 4.
The mean GSI value across the year is 0.38 ±0.02 (bold line, Figure 4A) with a minimum value of 0.04, recorded on 19th November 2013, and a maximum value of 4.24, recorded on 9th April 2014. During the summer period it is reached the maximum median GSI value of 0.64 on 7th August. Only 4 medians out of 24 (from July to September) are found above the mean GSI value. In the same period of time it is found a high inter-individual variability, visualised by the wideness of the boxplots which, during the rest of the sampling activity, are much more compressed indicating homogeneity in GSI values. Pairwise comparisons of GSI between dates using Wilcoxon rank sum test with Bonferroni's correction (data not presented) showed that the sampling occurred on 7th August is statistically different (p-value <0.05) from 10 (out 23) other sampling dates, followed by 22nd August and 4th December that are statistically different from 4 other dates each.
The mean HSI value across the year is 7.46 ±0.07 (bold line, Figure 4B) with a minimum value of 3.56, recorded on 9th April 2014, and a maximum value of 10.70, recorded on 4th December 2013. A high inter-individual variability is found across all sampling dates. In general, median HSI values tend to rise up until the beginning of June, after that, during the summer period, they decrease, ultimately reaching the minimum median HSI value of 5.67 on October, and then they gradually start rising again. Pairwise comparisons of HSI's among dates using Wilcoxon rank sum test with Bonferroni's correction showed that only the sampling occurred on 10th October, where the lowest HSI values have been collected, shows a statistical difference (p-value <0.05) with 8 other sampling dates.
From the end of September to the beginning of February a total of 30 ovigerous females were captured, 13 of them with eggs in different developmental stages and 17 of them with offspring still attached to the pleopods. The mean realized fecundity was 35 ±7 eggs/female. The smallest ovigerous females captured was 39.50 mm (CTL), while the biggest was 63.30 mm (CTL); the mean CTL value for ovigerous females was 53.29 ±0.99 mm.

Histological analysis

Histological analysis have been performed to describe the reproductive biology of the species in this peculiar habitat. Histological specimens have been classified according to the classification given by Kulkarni (Kulkarni et al. 1991). The more immature ovary observed is between Stage 3 – Avitellogenic and Stage 4 – Early Vitellogenic (Figure 5A). The mean oocyte diameter is 196.3 µm ±78.2 (n=9) with a mean nuclear diameter of 47.5 µm ±19.3 (n=7). The nucleus is centrally located, it has a circular shape and usually 4 nucleoli of 1.9 µm2 ±0.5 (n=30) are present just beneath the nuclear membrane. The ooplasm is homogeneous, without yolk granules. A 4.7µm ±1.1 thick layer of flat follicle cells is present around oocyte membrane, creating the oogenetic pouch.

Oocytes in Early Vitellogenic (Kulkarni et al. 1991) phase exhibit a mean diameter of 232.1 μm ± 77.3 (n=18) with a nuclear diameter of 57.2 μm ±17.8 (n=8). Up to five nucleoli are present immediately under the nuclear membrane. During this phase, in the ooplasm, some small yolk granules (maximum diameter < 4 μm) start storing in the perinuclear area (Figure 5B).
Early during the secondary vitellogenesis, mean oocytes diameter is 361.4 μm ±79.6 (n=12), nuclear diameter is 60.2 μm ±17.1 (n=4) and about 6 nucleoli are present next to the nuclear membrane. Immediately under the cell membrane small yolk granules of 5.9 μm ±1.3 (n=55) are homogeneously stored in the ooplasm (Figure 5C).
During their development, oocytes gradually increase their size , reaching a mean diameter of 420.6 μm ±116.8 (n=9), the nuclear mean diameter is 71.0 μm ±18.7 (n=4) and usually 4 nucleoli are present. Oocytes tend to lose their round shape in cross sections and appear squarish. In the ooplasm yolk granules have increased their number (but not their size, 5.3 μm ±1.1, n=55) and are interposed between lipid droplets which appear as white vesicles (Figure 5D).
When oogenesis is almost complete, oocytes reach a mean diameter of 852.3 μm ±179.4 (n=5); yolk granules and lipid droplets occupy the majority of the cell, with ooplasm reduced to a narrow band surrounding the nucleus. In this stage of the oogenesis, yolk granules have a diameter ranging from 17 to 83 μm, due to merging events where small numerous granules become a single bigger one (Figure 5E).
In post-ovulatory ovaries, some oocytes at various developmental stage are still present in the ovary even if empty spaces (left by laid oocytes) appear and large zones made only by ovarian epithelium, follicle cells and hemocytes are visible. Follicle cells not any more associated to an oocyte appear small, circular and are patchy disposed in the ovarian lumen. Atretic oocytes, surrounded by follicular cells can be found; these auxiliary cells appear thicker (up to 20 μm) because of the uptake of yolk granules and lipid droplets from apoptotic oocyte's ooplasm (Figure 5F).
Ultra-structural analysis on the germinative portion of the ovary is shown in Figure 6. This region of the ovary is essentially composed by oogonia, follicular cells and some previtellogenic oocytes. Although still immature, oogonia are always surrounded by one or more follicular cells which will later expand and create a monolayer of epithelial cells, the so-called oogenetic pouch. In cross sections oogonia exhibit different forms, ranging from polygonal to elliptical; during their development they increase in dimensions (oogonia shown have diameters from 14 to 28 μm); they have a large and circular euchromatic nucleus who can account for as much as 66% of the area of the cell. The ooplasm appears not homogeneous and numerous small mitochondria (with mean diameter of 474.62 nm ±43.65, n=17) are present; in addition, some mitochondria with shelflike and tubular cristae can be seen. Follicular cells have an irregular shape, that in the majority of the cases is elongated, embracing the oocyte. They have a minimum thickness of 0.88 μm and a maximum of 4.36 μm in the region of the nucleus. The nucleus is heterochromatic and its shape resembles that of the cell.

Discussion

Monitoring activity

While the overall sex ratio of the population is not different from 1:1, is interesting that from June to November males are always more abundant than females, while from December to May the situation is inverted, splitting the year in two periods where one of the two sexes is much more active than the other. In general, males are active during the reproductive season (pushed by the search for a sexually mature female) which lies in the summer, while females are more active during winter-spring, as they have to feed and accumulate energy in the hepatopancreas. It is noteworthy that no problem in specimen capture was ever encountered also during the winter; this is in contrast with observations made on other populations. In fact in the North-eastern Italy

(Vicenza) and as reported in one case in Central Italy (Dörr et al. 2006; Dörr & Scalici 2013), in rivers or lakes that have a high difference in water temperature between winter and summer, the wintry sampling activity results with no captures because crayfishes are inhibited by low temperatures. The same inhibitory pattern has been found in Southern Germany in lentic habitats with high thermal excursion where traps were exposed in the water up to two weeks during winter samplings (Chucholl 2011a; Chucholl 2011b).
High variability in males reproductive size is confirmed by our data; in fact, CTL values from stage E males cover an interval which is almost equal to the whole male sampled population (ranging from 19.50 to 73.70 mm) in our site. Data from the reproductive stage show that mature males are always present in the habitat, although with different percentages across the seasons. The constant 100% percentage of stage E males during the summer indicates that reproduction occurs during this season and could be possibly extended up to the end of October (where more than 60% males are still in stage E); these results are similar to those reported in Southern Germany (Chucholl 2011a), but differ from data reported in Central Italy (Gherardi et al. 2000b; Scalici and Gherardi 2007) and in Spain (Anastácio and Marques 1995): in fact, during the Spring both in Central Italy and in Spain waters are warmer than those in Germany and in our study area, allowing *P. clarkii*'s populations to begin their reproductive cycle in April, from 2 to 3 months earlier than Northern populations. Moreover, the higher abundance of males captured during the summer than females is concordant with the beginning of the reproductive period: in fact, sexually mature males, are more active during the reproductive period because they are pushed by the research of a mating companion and they can cover long distances (Gherardi and Barbaresi 2000). On the other side, the presence of stage E males also during the winter, could be justified by the peculiar water characteristics, with particular reference to the temperature that is almost constant during the year. In fact, the annual mean water temperature is 13.32 ±0.08 °C with a few thermal excursion during the year: even when median values are relatively distant from the annual mean value, one of the two whiskers of the boxplot is always found near or at least 3 °C far from that mean value. In the light of this, the presence of *P. clarkii* in rivers with this water temperatures is quite uncommon; indeed many researches have underlined that this species has a strong preference for temperatures ranging from 21 °C to 30 °C, with an optimum at 23.5°C (Espina et al. 1993; Bückle Ramírez et al. 1994; Huner and Holdich 2002; Lagerspetz and Vainio 2006) while Brancolo's population never experiences such water temperatures, even during the summer period because of the continuous input of cold waters in the channel; even the populations studied in Germany are found on habitats with lentic waters that during the summer can reach 20 °C (Chucholl 2011b; Chucholl 2011a). Moreover when in winter mean temperature were respectively 8.65 °C, 10.81 °C, 8.92 °C and 11.80 °C no inhibition in crayfish activity (intended as decrease in number of specimens captured with the same catch per unit effort, Figure 1) has been recorded.
In our sampling area mean salinity was 1g/L and doesn't seem a limiting factor for these animals (both in terms of growth and reproduction); in fact, our data are concordant with what was previously stated by Huner and Meineri, who reported that reproduction is limited to water less than 5g/L salinity (Huner & Barr 1991; Meineri et al. 2014).
The studied population presents low GSI values across all the year, with a mean value of 0.38 ±0.02 (bold line, Figure 4A) while in other habitats *P. clarkii* can reach GSI values of 8 (Alcorlo, Geiger, & Otero, 2008) with annual mean values near 1 (Chucholl 2011a). In our study area, the highest GSI median values have been recorded between July and September and reached a maximum median value of 0.64 (7 August); moreover, GSI samplings in August compared with 14 (out 23) other sampling dates show statistically significant differences (p-value < 0.05). All these results bring to the conclusion that, in our population, the maximum gonadal development is reached in August, although GSI values for mature females are far below from the ones reported in literature. Furthermore, in contrast from what reported in literature (Niquette and d' Abramo 1991; Daniels et al. 1994) in our study area the temperature doesn't seem to be the overriding factor that regulates

whether or not ovarian development proceeds or, in other words, in this population, the threshold temperature is lower than for other populations reported; in fact, also with mean water temperatures between 14 and 17 °C, ovarian development is active and ovigerous females appear later in September.
CTL values from ovigerous females ranged between 39.50 and 63.30 mm, an interval which comprises almost half CTL's values of the female population (from 21.00 to 77.10 mm); in comparison, data reported by Chucholl (CTL's ovigerous females from 44.9 to 56.1 mm) show that ovigerous females are found in a narrower CTL range in comparison with our population where females up to 10 mm smaller can be reproductive. The realized fecundity of 35 ±7 eggs per female is extremely low in comparison with the reported values of 285 in Germany (Chucholl 2011a), 313 in Louisiana (Penn Jr 1943), 433 in Kenya (Oluoch 1990) and 995 in Spain (Alcorlo et al. 2008). It is likely that the peculiar low temperatures of this area, far below from the optimum of this species, stress the animals that can devote a relatively low amount of energy to reproduction, resulting in really low GSI and fecundity values.

Histological analysis

Histological analysis have been performed to describe the reproductive biology of the species in this peculiar habitat by following ovarian development through different stages, across a whole year. The classifications for ovarian development of *P. clarkii* are based on ovarian macroscopic characteristics (Romaire and Lutz 1989), or on histological ones (Kulkarni et al. 1991).
It is noteworthy that also fully developed oocytes (as the one presented in Figure 5E, dissected in August) have been always found in ovaries with a low GSI (Figure 4A), with only 1.86% of GSI values above the value of 1.75 and none above 4.2. Both, observations made on macroscopic and on histological characteristics confirm the reproductive activity of *P. clarkii* in Brancolo's channel: all ovarian stages from Romaire and Lutz system have been found basing on ovary's colour and aspect, but not on GSI; indeed black ovaries (fully developed) from our specimens could have a GSI value ranging from 0.16 - 4.24, instead of the reported range of 2.3 - 8. Taken togheter, all these observations, support the statement that the population living in the Brancolo's channel is able to complete its reproductive cycle, but females are not able to carry on the development of a great amount of oocytes, accordingly to the low GSI and fecundity values recorded.

In conclusion, from the monitoring activity it has been shown that the population living in the Brancolo's channel has unique biological adaptations/characteristics and it is able to complete its life cycle at temperatures that, according to other studies, should put a stop to growth (Chucholl 2011a) and inhibit the reproduction (Niquette and d' Abramo 1991). In fact, abundance data, male reproductive stage and histological analysis data, confirm these conclusions that could be seen collectively as the greatest example of biological plasticity of this species.
The importance of all these results is not to be underestimated: in fact they have to be taken as a warning about the possible range expansion of *P. clarkii* also to northern and colder habitats that have few things in common with the native habitat of the species and, up to now, were considered "safe" from the invasion of the Louisiana crayfish.

References

Alcorlo P, Geiger W, Otero M (2008) Reproductive biology and life cycle of the invasive crayfish *Procambarus clarkii* (Crustacea: Decapoda) in diverse aquatic habitats of South-Western Spain: Implications for population control. Fundamental and Applied Limnology / Archiv für Hydrobiologie 173:197–212. doi: 10.1127/1863-9135/2008/0173-0197

Anastácio P, Marques J (1995) Population biology and production of the red swamp crayfish *Procambarus clarkii* (Girard) in the lower Mondego river valley, Portugal. Journal of Crustacean Biology 156–168.

Ando H, Makioka T (1998) Structure of the Ovary and Mode of Oogenesis in a Freshwater Crayfish, *Procambarus clarkii* (Girard). Zoological Science 15:893–901. doi: 10.2108/zsj.15.893

Barbaresi S, Gherardi F (2000) The Invasion of the Alien Crayfish *Procambarus Clarkii* in Europe, with Particular Reference to Italy. Biological Invasions 2:259–264. doi: 10.1023/A:1010009701606

Bückle Ramírez LF, Diaz Herrera F, Correa Sandoval F, et al. (1994) Diel thermoregulation of the crawfish Procambarus Clarkii (crustacea, cambaridae). Journal of Thermal Biology 19:419–422. doi: 10.1016/0306-4565(94)90041-8

Chucholl C (2011a) Population ecology of an alien “warm water” crayfish (*Procambarus clarkii*) in a new cold habitat. Knowledge and Management of Aquatic Ecosystems 29. doi: 10.1051/kmae/2011053

Chucholl C (2011b) Disjunct distribution pattern of *Procambarus clarkii* (Crustacea, Decapoda, Astacida, Cambaridae) in an artificial lake system in Southwestern Germany. Aquatic Invasions 6:109–113. doi: 10.3391/ai.2011.6.1.14

Daniels WH, D’Abramo LR, Graves KF (1994) Ovarian Development of Female Red Swamp Crayfish (Procambarus clarkii) as Influenced by Temperature and Photoperiod. Journal of Crustacean Biology 14:530. doi: 10.2307/1548999

Dörr AJM, La Porta G, Pedicillo G, Lorenzoni M (2006) Biology of *Procambarus clarkii* (GIRARD, 1852) in lake Trasimeno. Bulletin Français de la Pêche et de la Pisciculture 1155–1168. doi: 10.1051/kmae:2006018

Dörr AJM, Scalici M (2013) Revisiting reproduction and population structure and dynamics of *Procambarus clarkii* eight years after its introduction into Lake Trasimeno (Central Italy). Knowledge and Management of Aquatic Ecosystems:10.1051/kmae/2013045

Ermak TH, Eakin RM (1976) Fine structure of the cerebral and pygidial ocelli in Chone ecaudata (polychaeta: sabellidae). Journal of Ultrastructure Research 54:243–260. doi: 10.1016/S0022-5320(76)80154-2

Espina S, Diaz Herrera F, Bückle R. LF (1993) Preferred and avoided temperatures in the crawfish *Procambarus clarkii* (Decapoda, Cambaridae). Journal of Thermal Biology 18:35–39. doi: 10.1016/0306-4565(93)90039-V

Gherardi F (2006) Crayfish invading Europe: the case study of *Procambarus clarkii*. Marine and Freshwater Behaviour and Physiology 39:175–191. doi: 10.1080/10236240600869702

Gherardi F, Barbaresi S (2000) Invasive crayfish: activity patterns of *Procambarus clarkii* in the rice fields of the Lower Guadalquivir (Spain). Archiv für Hydrobiologie 150:153–168.

Gherardi F, Barbaresi S, Salvi G (2000a) Spatial and temporal patterns in the movement of *Procambarus clarkii*, an invasive crayfish. Aquatic Sciences 62:179–193.

Gherardi F, Raddi A, Barbaresi S, Salvi G (2000b) Life history patterns of the red swamp crayfish, *Procambarus clarkii*, in an irrigation ditch in Tuscany, Italy. Crustacean Issues 12:99–108.

Huner JV, Barr JE (1991) Red swamp crawfish: biology and exploitation: Louisiana State University. Center for Wetland Resources, Louisiana Sea Grant College Program, Baton Rouge, LA

Huner JV, Holdich DM (2002) *Procambarus*. Biology of freshwater crayfish 541–584.

IUCN 2014. The IUCN Red List of Threatened Species. Version 2014.2.

Kulkarni GK, Glade L, Fingerman M (1991) Oogenesis and effects of neuroendocrine tissues on in vitro synthesis of protein by the ovary of the red swamp crayfish *Procambarus clarkii* (Girard). Journal of Crustacean Biology 513–522.

Lagerspetz KYH, Vainio LA (2006) Thermal behaviour of crustaceans. Biological Reviews 81:237–258. doi: 10.1017/S1464793105006998

Luise G De (2009) Studio preliminare sulla presenza del gambero rosso della Louisiana in Friuli Venezia Giulia. Ente Tutela Pesca - Friuli Venezia Giulia , http://www.entetutelapesca.it/images/pdf/studi_ricerche/gambero_rosso_2009.pdf

Ma Y, Kjesbu OS, Jłrgensen T (1998) Effects of ration on the maturation and fecundity in captive Atlantic herring (*Clupea harengus*). Canadian Journal of Fisheries and Aquatic Sciences 55:900–908.

Meineri E, Rodriguez-Perez H, Hilaire S, Mesleard F (2014) Distribution and reproduction of *Procambarus clarkii* in relation to water management, salinity and habitat type in the Camargue. Aquatic Conserv: Mar Freshw Ecosyst 24:312–323

Niquette DJ, d' Abramo LR (1991) Population dinamics of red swamp crawfish, *Procambarus clarkii* (Girard, 1852) and white river crawfish, *P. acutus acutus* (Girard, 1852), cultured in earthen ponds. Journal of Shellfish Research, Maine 10:179–186.

Oluoch AO (1990) Breeding biology of the Louisiana red swamp crayfish *Procambarus clarkii* Girard in Lake Naivasha, Kenya. Hydrobiologia 208:85–92. doi: 10.1007/BF00008447

Penn Jr GH (1943) A study of the life history of the Louisiana red-crawfish, *Cambarus clarkii* Girard. Ecology 24:1–18.

Romaire RP, Lutz CG (1989) Population dynamics of *Procambarus clarkii* (Girard) and *Procambarus acutus acutus* (Girard) (Decapoda: Cambaridae) in commercial ponds. Aquaculture 81:253–274. doi: 10.1016/0044-8486(89)90151-8

Scalici M, Chiesa S, Scuderi S, et al. (2010) Population structure and dynamics of *Procambarus clarkii* (Girard, 1852) in a Mediterranean brackish wetland (Central Italy). Biological invasions 12:1415–1425.

Scalici M, Gherardi F (2007) Structure and dynamics of an invasive population of the red swamp crayfish (*Procambarus clarkii*) in a Mediterranean wetland. Hydrobiologia 583:309–319.

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Meth 9:671–675. doi: 10.1038/nmeth.2089

Taketomi Y, Nishikawa S, Koga S (1996) Testis and Androgenic Gland during Development of External Sexual Characteristics of the Crayfish *Procambarus clarkii*. Journal of Crustacean Biology 16:24. doi: 10.2307/1548927

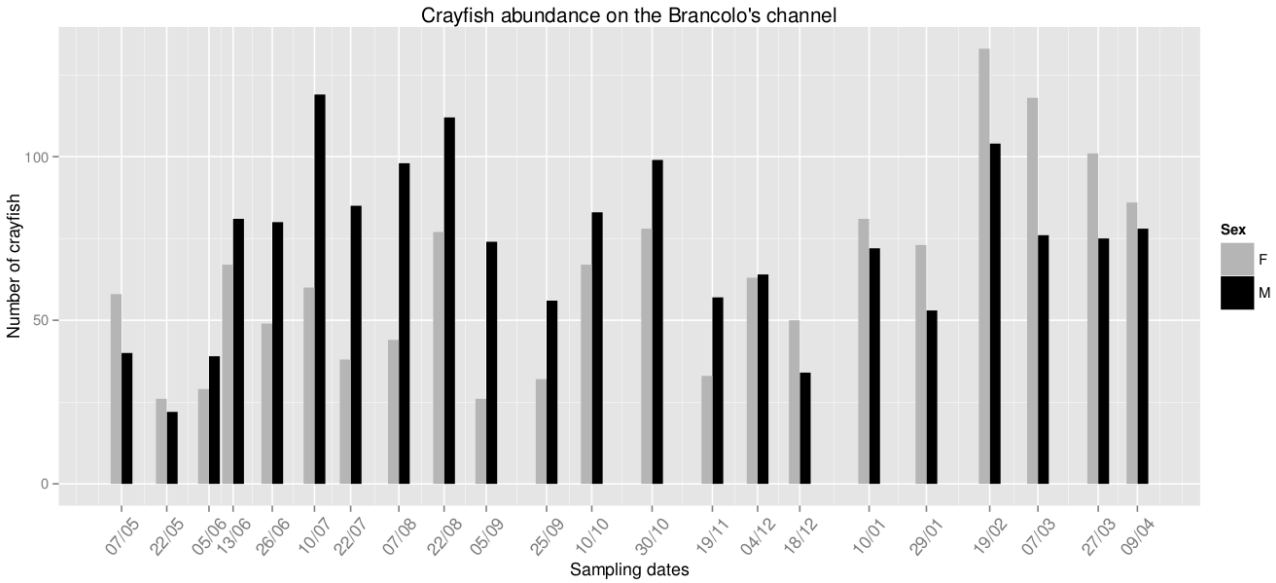

Figure 1 *P. clarkii*'s abundance of males and females crayfish captured during the monitoring activity of the population living in the Brancolo's channel. Distance between two adjacent dates on the X-axis is proportional to the number of days between them.

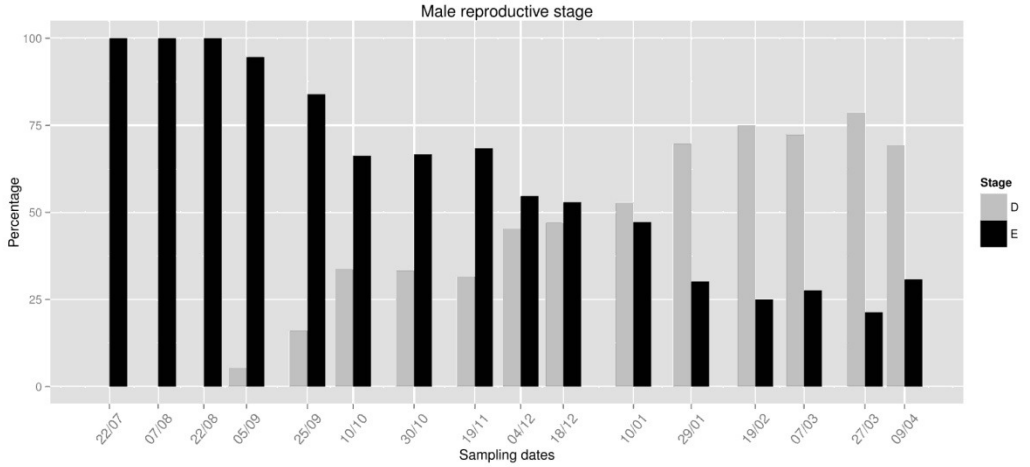

Figure 2 Reproductive stage (according to Taketomi et al., 1996) of captured males. See text for morphological distinction between Stage D and Stage E. Distance between two adjacent dates on the X-axis is proportional to the number of days between them.

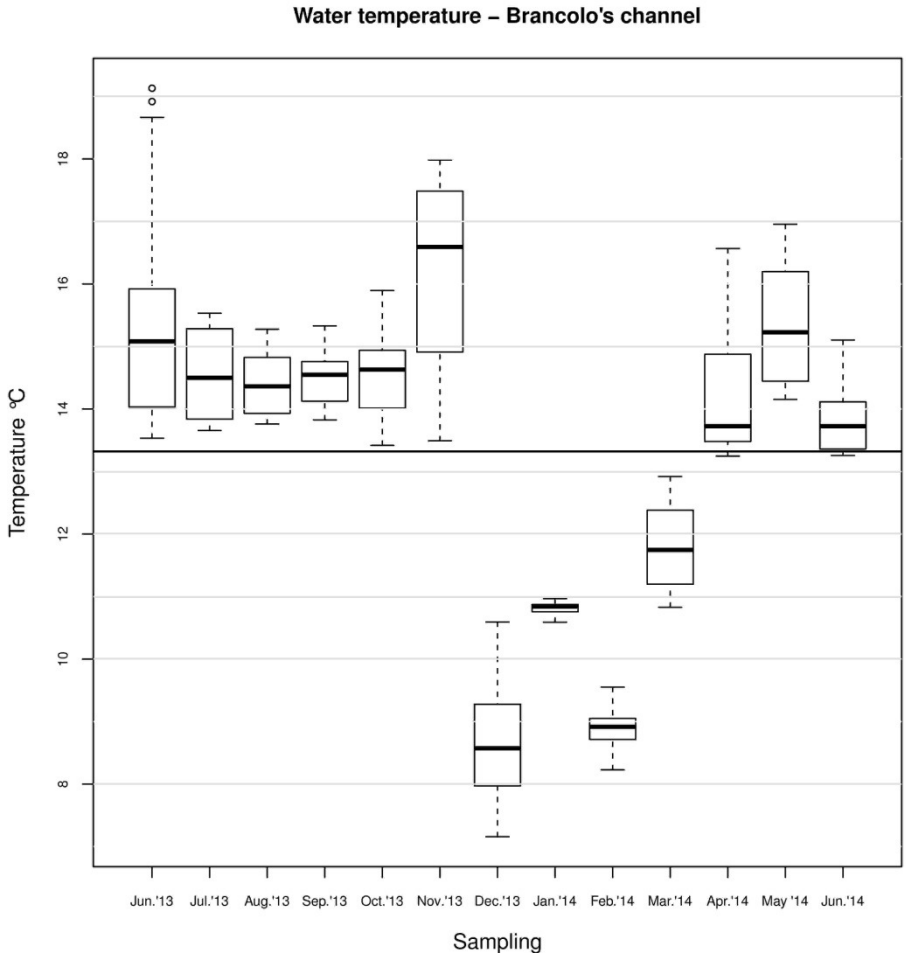

Figure 3 Trend in water temperature on the river bed of the sampling area. Bold line represents the mean annual water temperature value (13.32 ±0.08 °C). Circles beyond whisker are out-layer values.

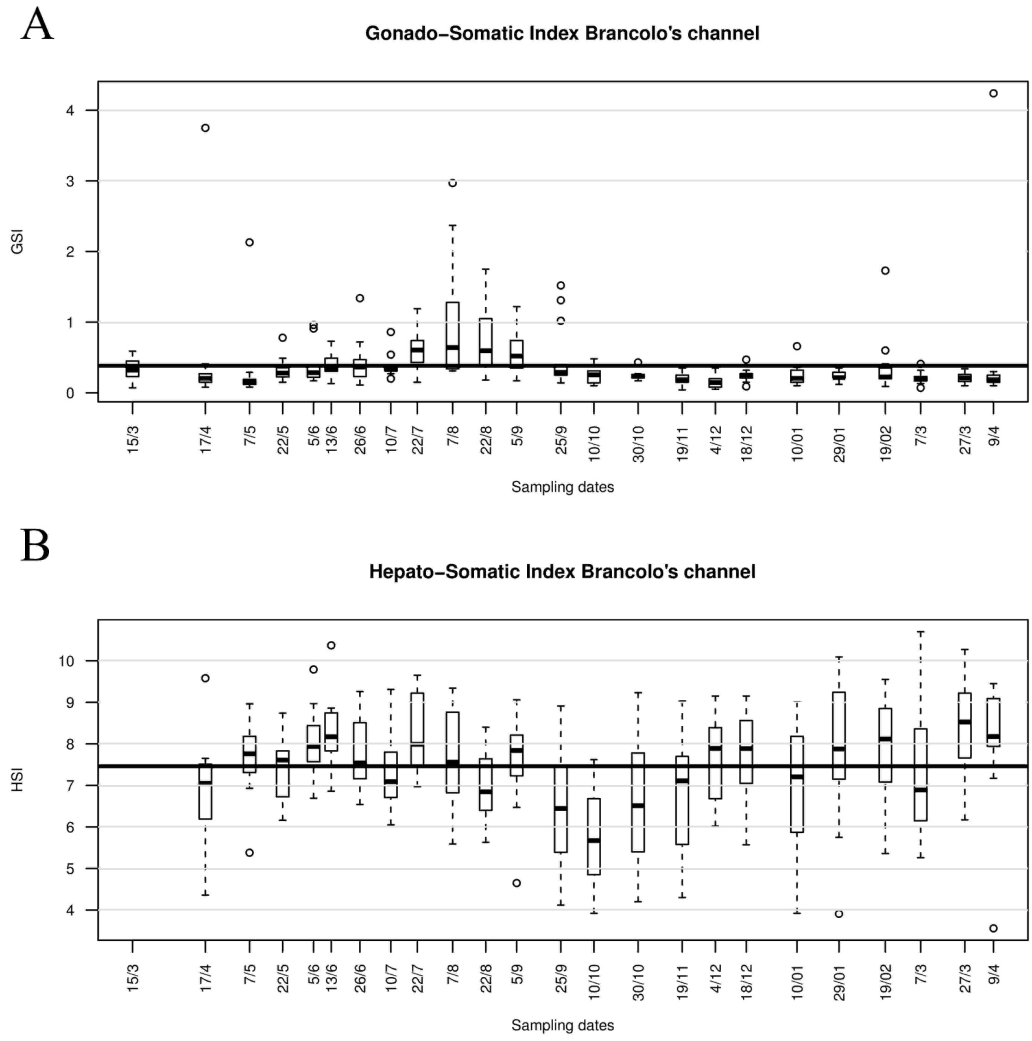

Figure 4 Gonado-Somatic (A) and Hepato-Somatic (B) Indexes from captured female crayfishes sampled in Brancolo's channel. Bold line represents the mean GSI and HSI annual value (respectively 0.38 ±0.02 and 7.46 ±0.07). Circles beyond whisker are out-layer values. Distance between two adjacent dates on the X-axis is proportional to the number of days between them.

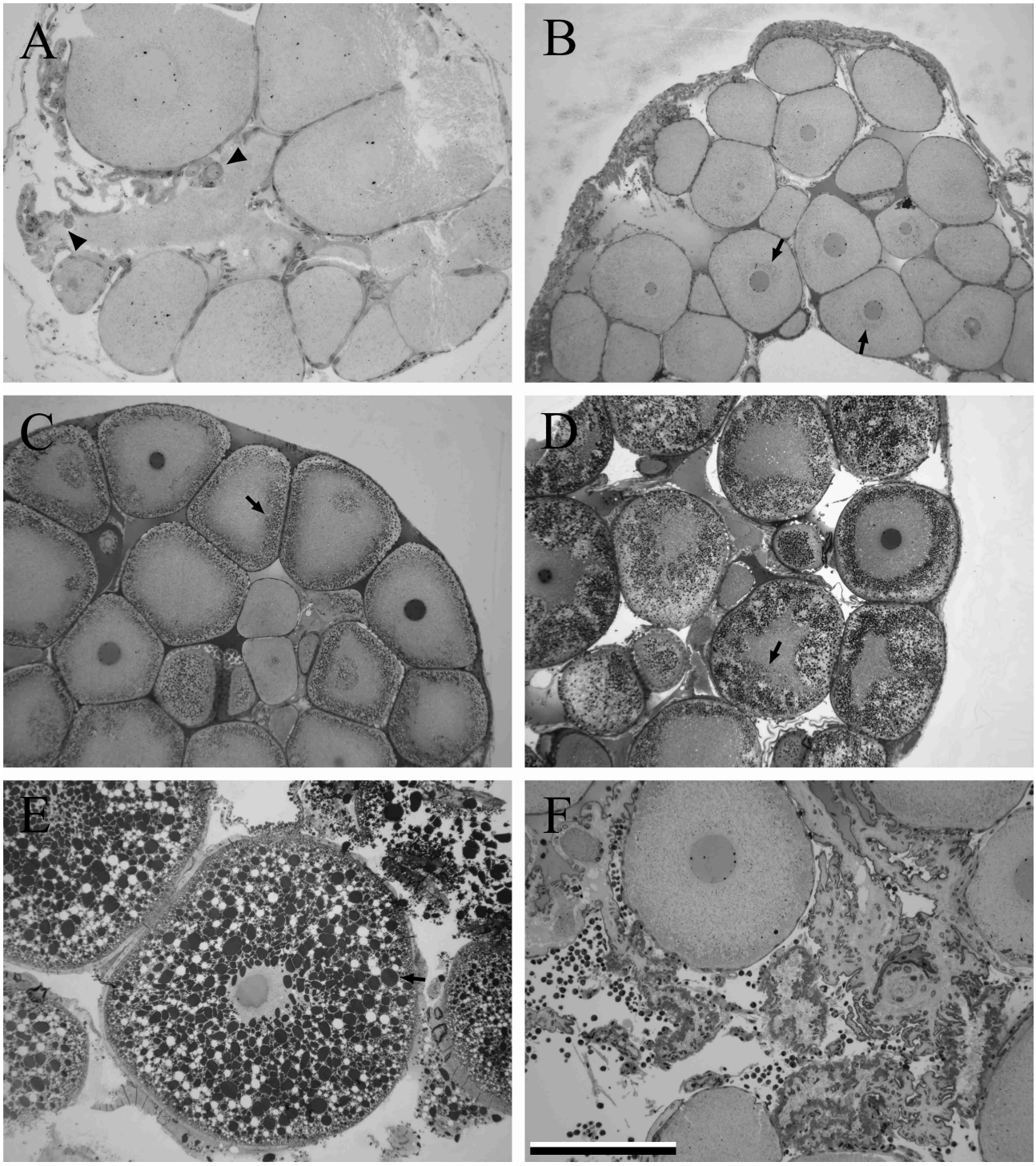

Figure 5 Optical microscopy images of ovaries dissected from females sampled on the Brancolo's channel. **a**: 20x section of immature ovary showing some Germinaria (arrowhead). **b**, **c**, **d**, **e**: 10x section of different ovaries at increasing ovarian development, showing the gradual storage of yolk granules (arrow) in the oocyte's ooplasm. It is noteworthy the increase in dimensions of these granules as result of merging events. **f**: 20x section of post-ovulatory ovary showing a large central portion without oocytes as a result of spawning, with follicle cells patchy disposed in the ovarian lumen. Calibration bar: 500 μm for micrographs b, c, d and e. 250 μm for micrographs a and f.

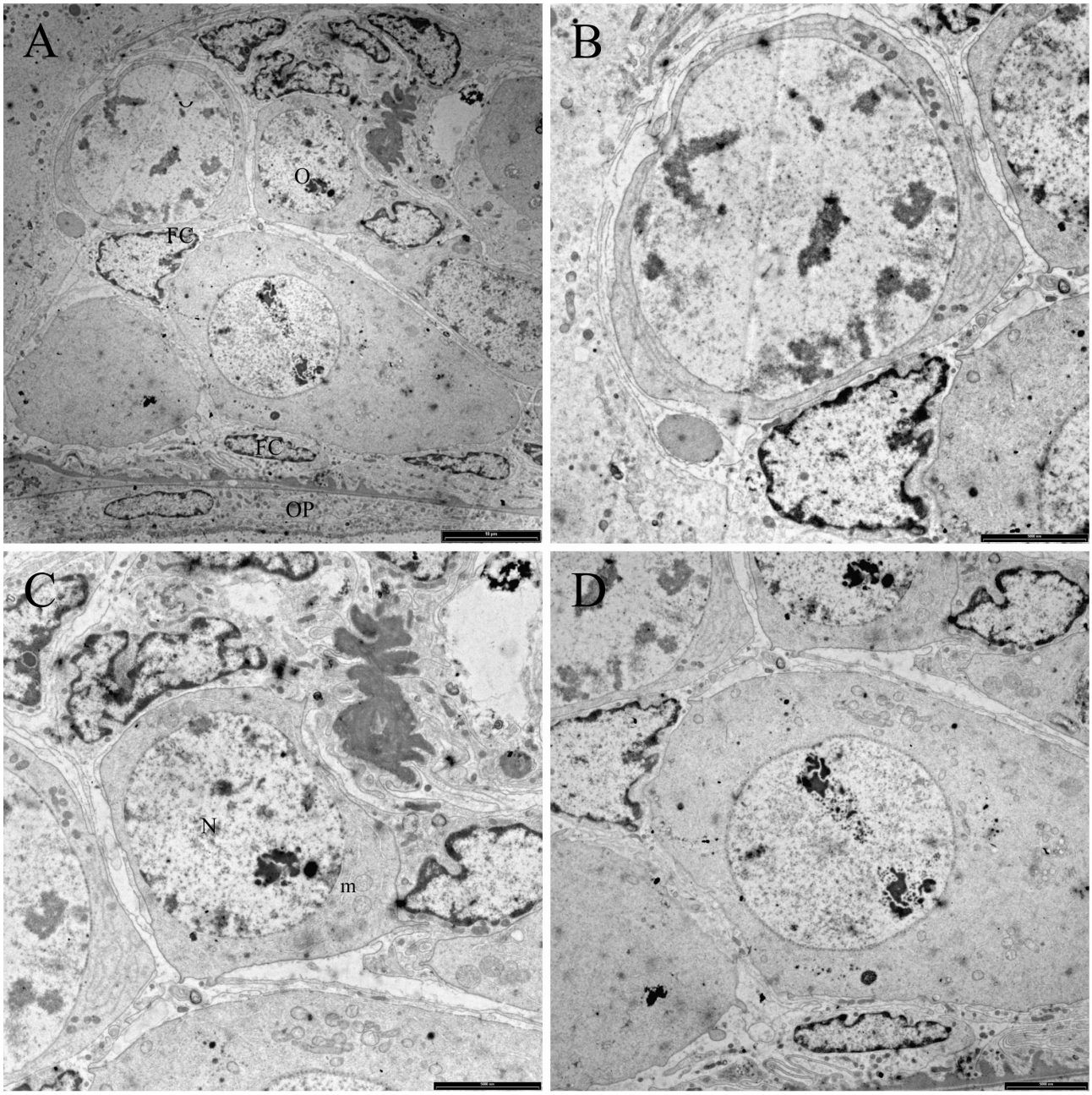

Figure 6 Electron microscopical images of the germinative portion in the ovary, known as Germinarium. **a**: 1100x micrograph showing 3 oogonia (O) with a large, euchromatic nucleus and several follicular cells (FC) next to them. Follicular cells arranged to form an oogenetic pouch (OP) with an oocyte are found next to the Germinarium. **b**, **c**: 2800x micrograph showing 2 oogonia at early stage of their development with a nucleus (N) who can account for as much as 66% of the area of the cell. In the ooplasm some small mitochondria with shelflike and tubular cristae (m) can be seen. **d**: 2200x micrograph of an older oogonium with a bigger ooplasm and surrounded by some follicular cells.