MACROSCOPIC AND MICROSCOPIC MATURITY STAGES. LIVING WORKING DOCUMENT FOR SMALL TUNA SPECIES

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SUMMARY

Maturity ogives are usually estimated using different methods, including macroscopical and microscopical maturity data. Differences in maturity ogives estimations are found for species and by area/stock. So those differences may be a consequence of the use of different methodological techniques (criteria) or due to different spawning tactics. Taking this into account is essential to guarantee that the maturity criteria for each species are consistent across the laboratories and countries involved in stock assessment. The objective of this document is to show a large amount of detailed photos (macro and microphotographs) of the different gonad stages of Auxis rochei, Sarda sarda *and* Euthynnus alletteratus, *for females and males, which will be an enhancement to the descriptions of maturity stages given in the maturity tables.*

RÉSUMÉ

*Les ogives de maturité sont généralement estimées à l'aide de différentes méthodes, notamment des données de maturité macroscopiques et microscopiques. Des différences dans les estimations des ogives de maturité ont été observées entre les espèces et les zones / stocks. Ces différences peuvent donc être une conséquence de l'utilisation de techniques méthodologiques différentes (critères) ou dues à différentes stratégies de reproduction. Il est essentiel de prendre cela en compte pour garantir la cohérence des critères de maturité pour chaque espèce dans les laboratoires et les pays participant à l'évaluation des stocks. L'objectif du présent document consiste à montrer une grande quantité de photos détaillées (macro et microphotographies) des différents stades gonadiques d'*Auxis rochei, Sarda sarda *et* Euthynnus alletteratus, *pour les femelles et les mâles, ce qui permettra d'améliorer la description des stades de maturité inclus dans les tableaux de maturité.*

RESUMEN

Las ojivas de madurez se suelen estimar utilizando diferentes métodos, incluyendo datos de madurez macroscópica y microscópica. Se han detectado diferencias en las estimaciones de ojivas de madurez para las especies y por área/stock. Por lo tanto, esas diferencias pueden ser consecuencia del uso de diferentes técnicas metodológicas (criterios) o de diferentes estrategias de desove. Tener esto en cuenta es esencial para garantizar que los criterios de madurez para cada especie sean coherentes en todos los laboratorios y países implicados en la evaluación de stocks. El objetivo de este documento es mostrar una gran cantidad de fotos detalladas (macro y microfotografías) de los diferentes estadios de gónadas de Auxis rochei, Sarda sarda y Euthynnus alletteratus, *para hembras y machos, lo que supondrá una mejora en la descripción de las fases de madurez que aparecen en las tablas de madurez.*

KEYWORDS

Auxis rochei, Sarda sarda, Euthynnus alletteratus, *macroscopic maturity, microscopic maturity, photographs*

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1. Introduction

Fish maturity ogives are usually estimated through macroscopical maturity data, i.e. the maturity stage of fish is based on macroscopic observation (visual stating method) of the gonads. This is a relatively inexpensive and quick method; however, it should be noted that the naked human eye has shortcomings particularly with colour interpretation and in noticing the presence of small particles such as cells (Kjesbu, 2016). An accurate evaluation of the reproductive gonad stages needs to be based on histological examinations (Hunter and Macewicz, 1985). The most important issue is to differentiate between immature and mature stages, which may be a difficult task when studying species with an indeterminate reproductive strategy, such as tuna, particularly when they are sampled before or after the spawning season and only using macroscopic criteria.

The objective of this document is to show macroscopical and histological images in order to facilitate the interpretation of the different gonad stages of *Auxis rochei* (bullet tuna), *Sarda sarda* (Atlantic bonito) and *Euthynnus alletteratus* (little tunny), for females and males, which will be an enhancement to the descriptions of maturity stages given in the maturity tables.

2. Material and methods

2.1. Sampling collection

Individuals of *A. rochei*, *S. sarda* and *E. alletteratus* were collected throughout the Mediterranean Sea (Spanish coast) and Atlantic Ocean (south of the Iberian Peninsula, both Portuguese and Spanish waters). The individuals of the three species were caught by both commercial and recreational fishermen. For each fish, straight fork length (SFL) was measured either to the nearest 0.1 cm (when fish was measured in the lab or at landings) or to the nearest 0.5 cm (fish measured on board). The gonads were removed and gonad weights (GW) were recorded to the nearest 0.01 g. The sex was classified as male, female and undetermined (i.e., sex is not distinguished by naked eye, because the gonads are very small, ribbon-like). Macroscopic maturity stages of ovaries and testes (visual stating method) were assigned using similar criteria to those developed for *S. sarda*, *E. alletteratus* and *Scomberomorus tritor* by Diouf (1981). The five macroscopic stages are: (1) immature (virgin); (2) developing and regenerating; (3) spawning capable; (4) spawning; (5) regressing (see description of stages in **Table 1**).

Immediately after taking a photograph of the gonads, a 2-3 cm cross- section of the central part of the right or left lobe was cut and fixed in Bouin's fluid for 4 hours, and then preserved in 70% ethanol. For small gonads, the whole gonads were preserved.

2.2. Histological processing and microscopic maturity staging of gonads

A representative portion (from the *tunica albuginea* to the ovarian lumen) of the preserved gonad tissue was dehydrated in ascending concentrations of ethanol, cleared with n-butanol, and embedded in paraffin. Sections were cut at 10 μm and stained with Mallory's trichrome stain. Classification of gonadal development was based on a modification of the microscopic criteria of Schaefer (1998) and Farley *et al*. (2013).

The most advanced group of oocytes (MAGO) was recorded for each ovary: primary growth (PG), lipid-stage (LP), early or primary vitellogenic (VT1), mid or secondary vitellogenic (VT2), advanced or tertiary vitellogenic (VT3), migratory nucleus (MG) and hydrated (HY) oocyte stages (see description of oocyte stages in **Table 2**). Ovarian stages were assigned based on the MAGO, the presence/absence of postovulatory follicles (POFs), the level of alpha and beta atresia of vitellogenic oocytes, the presence/absence of late stages of atresia (gamma/delta) and, the observation of the thickness of the *tunica albuginea* (gonad wall). Ovaries were classified into six stages: (1) immature (virgin); (2) developing; (3) spawning capable; (4) spawning; (5) regressing and regenerating (postspawning), and finally, the stage (6) abnormal (**Table 3**). In the present study, the developing stage was split into two sub-stages depending on the MAGO present within the ovary, (2a) LP- developing, including the lipid-stage oocytes and (2b) developing, including the early vitellogenic oocytes. The objective of splitting the developing stage into two was to estimate the length at first maturity (L_{50}) taking into account different criteria in future studies. Furthermore after the spawning season the gonad is first regressing (5a) and thereafter regenerating (5b) (see details in **Table 3**).

Four cellular stages (spermatogonia, spermatocytes, spermatids, and spermatozoa) were differentiated and recorded in testes. Testes were group into six stages (**Table 4**) based on histological examination of: the relative abundance of cysts containing the several cellular stages, the presence or absence of spermatozoa within

seminiferous tubules, and the amount of sperm (when present) within the central longitudinal sperm duct (vas deferens).

3. Results and discussion

The results of the present work are the macro and microphotographs for *A. Rochei* (**Figures 1-12**), *S. sarda* (**Figures 13-24**) and *E. alletteratus* (**Figures 25-32**).

The authors observed that when staging the maturity of gonads using visual method a wide range of colors where found for each stage. It is recommendable to cut the gonad to observe the size of oocytes and the amount of sperm in the spermatic duct (if it is present). It was also evident that there are problems with separating immature fish from early developing fish, and separating immature fish from mature inactive fish (regenerating stage). Therefore, although histology is expensive, the examination of histological sections allows to accurately distinguishing the different oocyte developmental stages, as well as the presence of other structures and maturity markers and thereby, allows a characterization of the reproductive stage.

Finally, it should be noted that macroscopic maturity staging criterion may vary depending on the reader and so, it is important that the maturity scale or criteria used for each species is consistent not only within readers of the same laboratory but also across the laboratories and countries involved in stock assessment.

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References

Diouf T., 1981. Premieres donnees relatives a l'exploitation et a la biologie de quelques "petits thonides et species voisines": Euthynnus, Sarda, Scomberomous au Senegal. Collective Volume of Scientific Papers ICCAT 15(2), 327–336.

Farley J.H., Williams A.J., Hoyle S.D., Davies C.R., Nicol S.J., 2013. Reproductive dynamics and potential annual fecundity of South Pacific albacore tuna (*Thunnus alalunga*). PloS ONE 8 (4), e60577. doi: 10.1371/journal.pone.0060577

Hunter, J.R., Macewicz, B.J., 1985. Measurement of spawning frequency in multiple spawning fishes. In: Lasker, R. (Ed.,) An Egg Production Method for Estimating Spawning Biomass of Pelagic Fish: Application to the Northern Anchovy, *Engraulis mordax*: NOAA Technical Report NMFS, 36, pp. 79–94.

Kjesbu, O.S., 2016. Applied fisheries reproductive biology: contribution of individual reproductive potential to recruitment and fisheries management, in: Jakobsen, T., Fogarty, M.J., Megrey, B.A., Moksness, E. (Eds.), Fish Reproductive Biology: Implications for Assessment and Management (second edition). John Wiley and Sons Ltd, Chichester, United Kingdom, pp. 321–366.

ICES, 2014. Report of the Workshop for maturity staging chairs (WKMATCH), 11-15 June 2012, Split, Croatia. ICES CM 2012/ACOM:58, 57pp.

Saber S., Macías D., Ortiz de Urbina J., Kjesbu, O.S., 2015. Stereological comparison of oocyte recruitment and batch fecundity estimates from paraffin and resin sections using spawning albacore (*Thunnus alalunga*) ovaries as a case study. Journal of Sea Research 95, 226–238.

Schaefer K.M., 1998. Reproductive biology of yellowfin tuna (*Thunnus albacares*) in the eastern Pacific Ocean. Inter-American Tropical Tuna Commission Bulletin 21, 205–272.

Table 1. Maturity stages of ovaries (visual stating method). Based on the criteria of Diouf (1981).

Reproductive stage	Females	Males
1. Immature (virgin)	Ovaries are small; more or less translucent-pinkish.	Testes are small, thin flattened and ribbon-like; more or less translucent - lightly pink.
2. Developing (early developing) and regenerating (recovering)	Ovaries are increasing in size; pink-orange colour. External blood vessels start to develop around the gonads (vascularisation).	Testes are increasing in size and triangular in cross section; whitish - pinkish colour. Sperm does not flow with pressure.
3. Spawning capable (late developing)	Ovaries are well developed and firm; yellow - orange colour. Opaque oocytes are visible.	Testes are well developed; whitish - pinkish colour. Accumulation of sperm in the spermatic ducts, sperm flows with low pressure
4. Spawning	Ovaries are greatly enlarged; orange - reddish colour, with conspicuous superficial blood vessels. Opaque oocytes are visible and large translucent hydrated oocytes may be visible.	Testes are greatly enlarged with conspicuous superficial blood vessels; pinkish colour. Large amount of sperm flows freely under very lightly pressure.
5. Regressing	Ovaries are bloody and flaccid, show a wrinkled wall; reddish colour.	Testes are flaccid and bloodshot; pinkish colour. Sperm may still flow (only small quantity).

Table 2. Description of the oocyte stages in *Auxis rochei*, *Sarda sarda* and *Euthynnus alletteratus* ovaries according to their histological characteristics (Saber *et al*., 2015).

Stage oocytes	Histological characteristics
Primary growth oocytes (PG)	Homogeneous basophilic cytoplasm with no cytoplasmic inclusions.
Lipid-stage (LP) or cortical alveoli (CA) oocyte	Small lipid droplets in the cytoplasm but still no yolk granules.
Early or primary vitellogenic oocytes (VT1)	Yolk granules in the periphery of the cytoplasm and lipid droplets occupy more of the cytoplasmic area than the yolk granules;
Mid or secondary vitellogenic (VT2)	Yolk granules and lipid droplets are spread throughout the cytoplasm.
Advanced or tertiary orvitellogenic (VT3)	Larger yolk granules than that of VT2 stage. Lipid droplets fuse and are distributed around the nucleus.
Migratory nucleus (MG)	Lipid droplets fuse into 1−3 large droplets. Migration of the nucleus toward the animal pole. In a later phase, yolk granules fuse progressively.
Hydrated (HY)	The nucleus has disintegrated. All yolk granules fuse into a homogeneous yolk mass and the oocyte increases in size due to hydration. The oocyte is still surrounded by the follicle layer, i.e. ovulation has yet not taken place.

Table 3. Microscopic classification criteria for *Auxis rochei*, *Sarda sarda* and *Euthynnus alletteratus* females based on a modification of the criteria of Schaefer (1998) and Farley *et al*. (2013). POFs, postovulatory follicles; MAGO, the most advanced group of oocytes.

Maturity stage. Females	Microscopic characteristics
1. Immature (virgin, immature)	Only oogonia and primary growth oocytes present. No atresia. Absence of POFs. Thin ovarian wall and little space between oocytes.
2. Developing	
2a. LP-Developing (immature or mature)*	Lipid-stage oocytes present as MAGO. Some atresia may be present. Absence of POFs.
2b. Developing (immature or mature)*	Early vitellogenic oocytes present as MAGO. Some atresia may be present. Absence of POFs.
3. Spawning capable (mature)	Mid or advanced vitellogenic oocytes present as the MAGO. Atresia (<50%) can be present. Absence of POFs.
4. Spawning (mature)	POFs present and /or migratory nucleus or hydrated oocytes present as MAGO. Atresia, when present at all, only in limited amounts.
5. Regressing and regenerating (postspawning)	
5a. Regressing (mature)	Lipid-stage or early vitellogenic oocytes as MAGO. Abundant alpha and/or beta atresia. Absence of POFs. Disorganization of ovary structures, with some spaces. Thick and/or wrinkled gonad wall is observed (in some ovaries).
5b. Regenerating (mature)	Only primary growth oocytes as MAGO present, with some spaces. Absence of POFs. Late stages of atresia. Thick and/or wrinkled gonad wall is observed (in some ovaries).
6. Abnormal**	Intersex, both oocytes and spermatogonia are present at the same time; sclerosis, the ovary is dominated by atretic oocytes and large amounts of connective tissue; infections; necrosis (atrophy).

* The maturity status is immature or mature depending on the staging criteria.
**Abnormal stage was defined according to the International Council for the Exploration of the Sea (ICES) (ICES, 2014).

Table 4. Microscopic classification criteria for *Auxis rochei*, *Sarda sarda* and *Euthynnus alletteratus* males based on a modification of the criteria of Schaefer (1998).

Maturity stage. Males	Microscopic characteristics
1. Immature (virgin)	Only spermatogonia present. No sperm in the sperm duct.
2. Developing	Spermatocytes, spermatids, and spermatozoa.
3. Spawning capable	Abundant spermatids and some spermatozoa within seminiferous tubules. Sperm duct relatively full of sperm.
4. Spawning	Some spermatids and abundant spermatozoa. Greatly enlarged tubules, sperm duct full of sperm.
5. Regressing and regenerating (postspawning)	In regressing, residual spermatozoa. In regenerating stage only spermatogonia present.
6. Abnormal*	Intersex, both oocytes and spermatogonia are present at the same time; infections; necrosis (atrophy).

*Abnormal stage was defined according to the International Council for the Exploration of the Sea (ICES) (ICES, 2014).

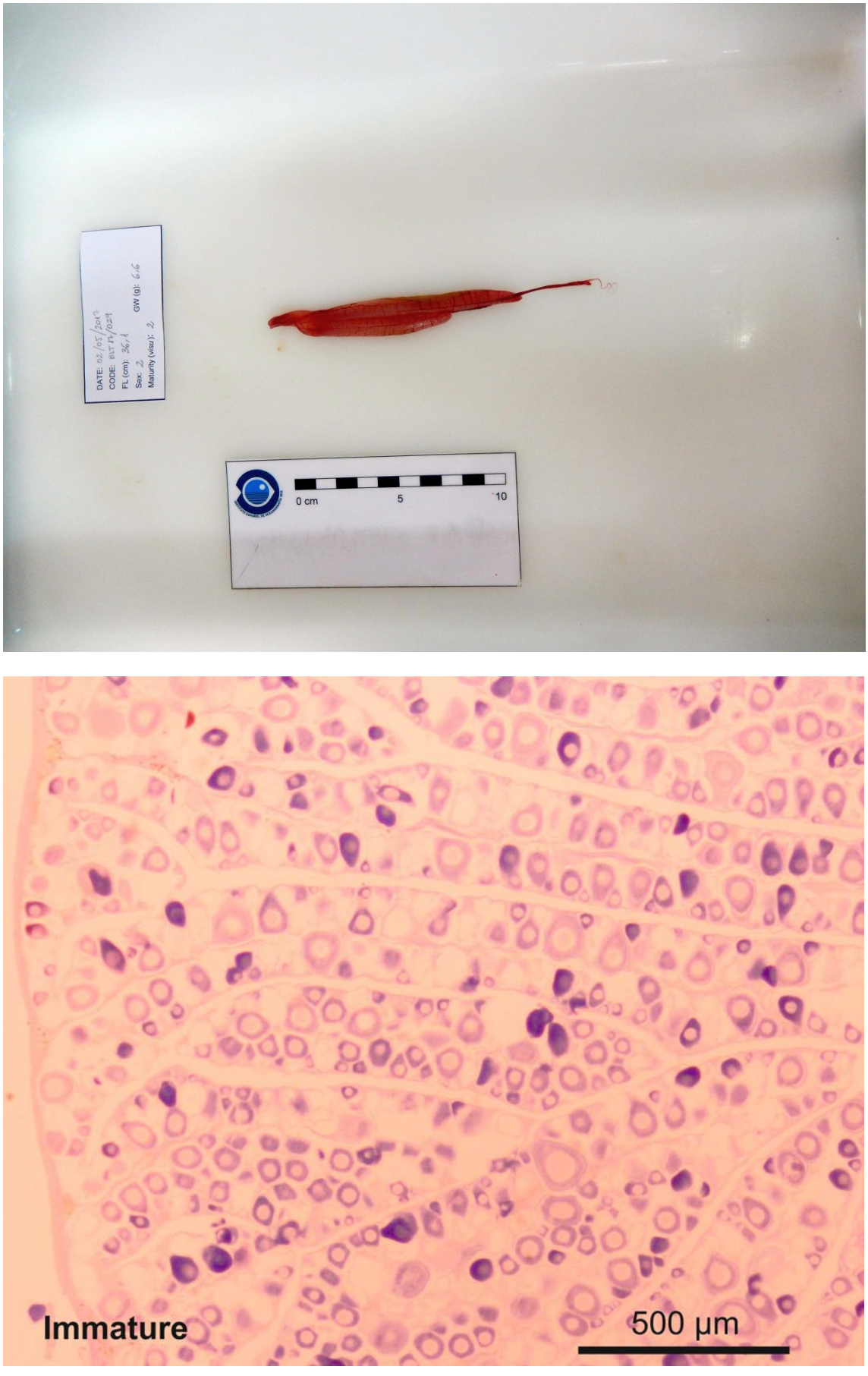


Figure 1. Macroscopic and microscopic photographs for immature stage of female *A. rochei*.

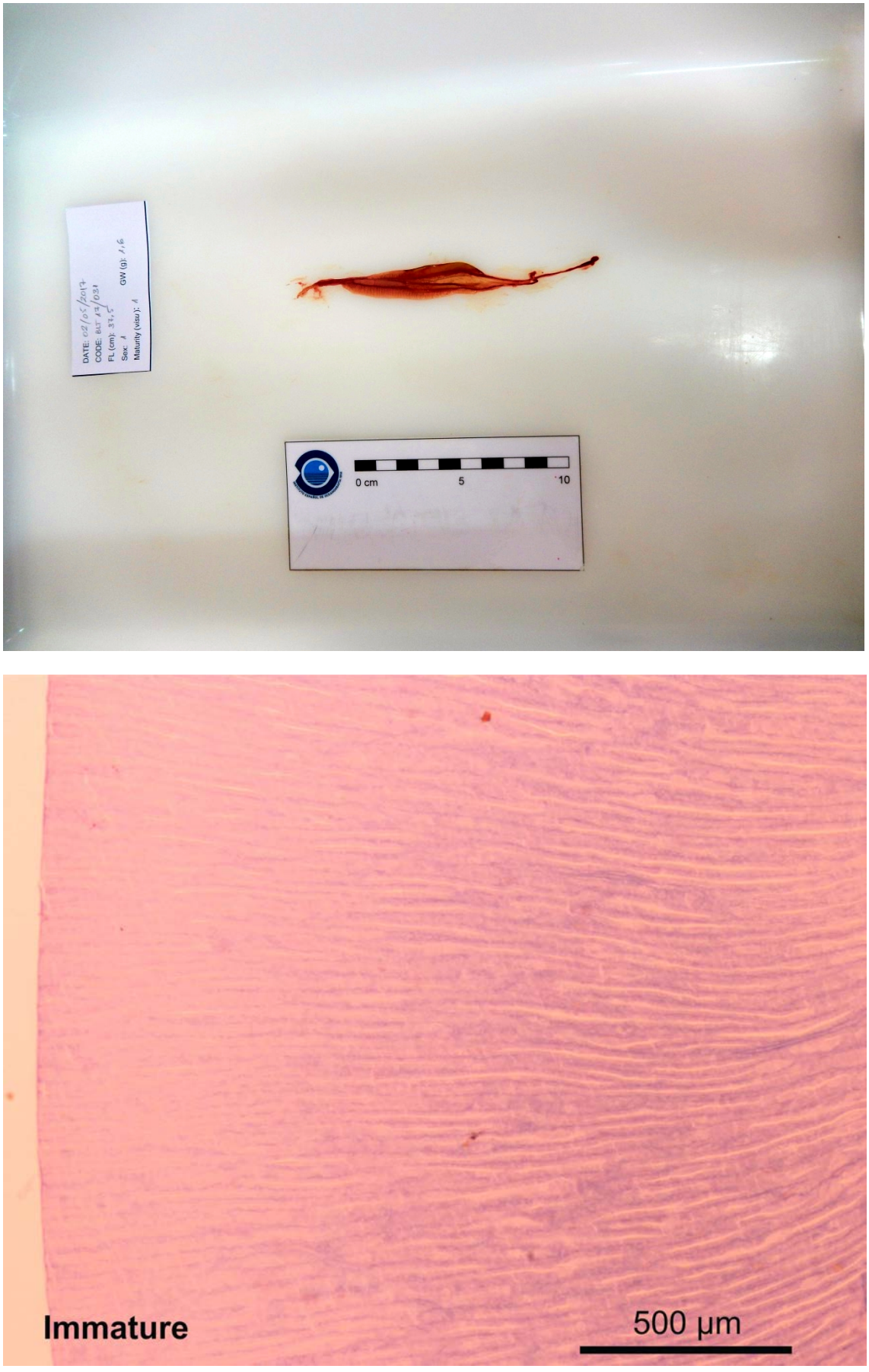


Figure 2. Macroscopic and microscopic photographs for immature stage of male *A. rochei.*

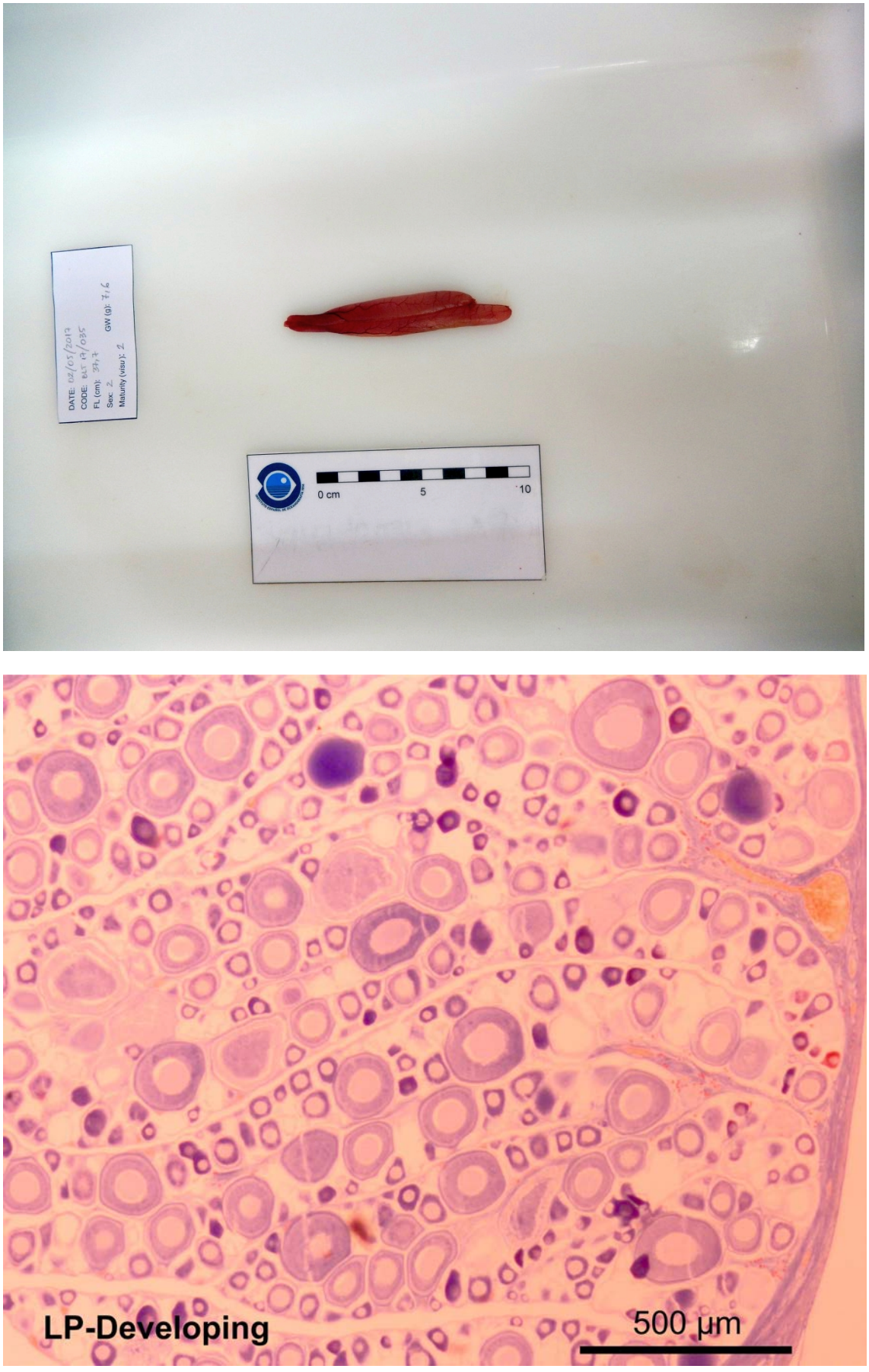


Figure 3. Macroscopic and microscopic photographs for developing stage (2a) of female *A. rochei*.

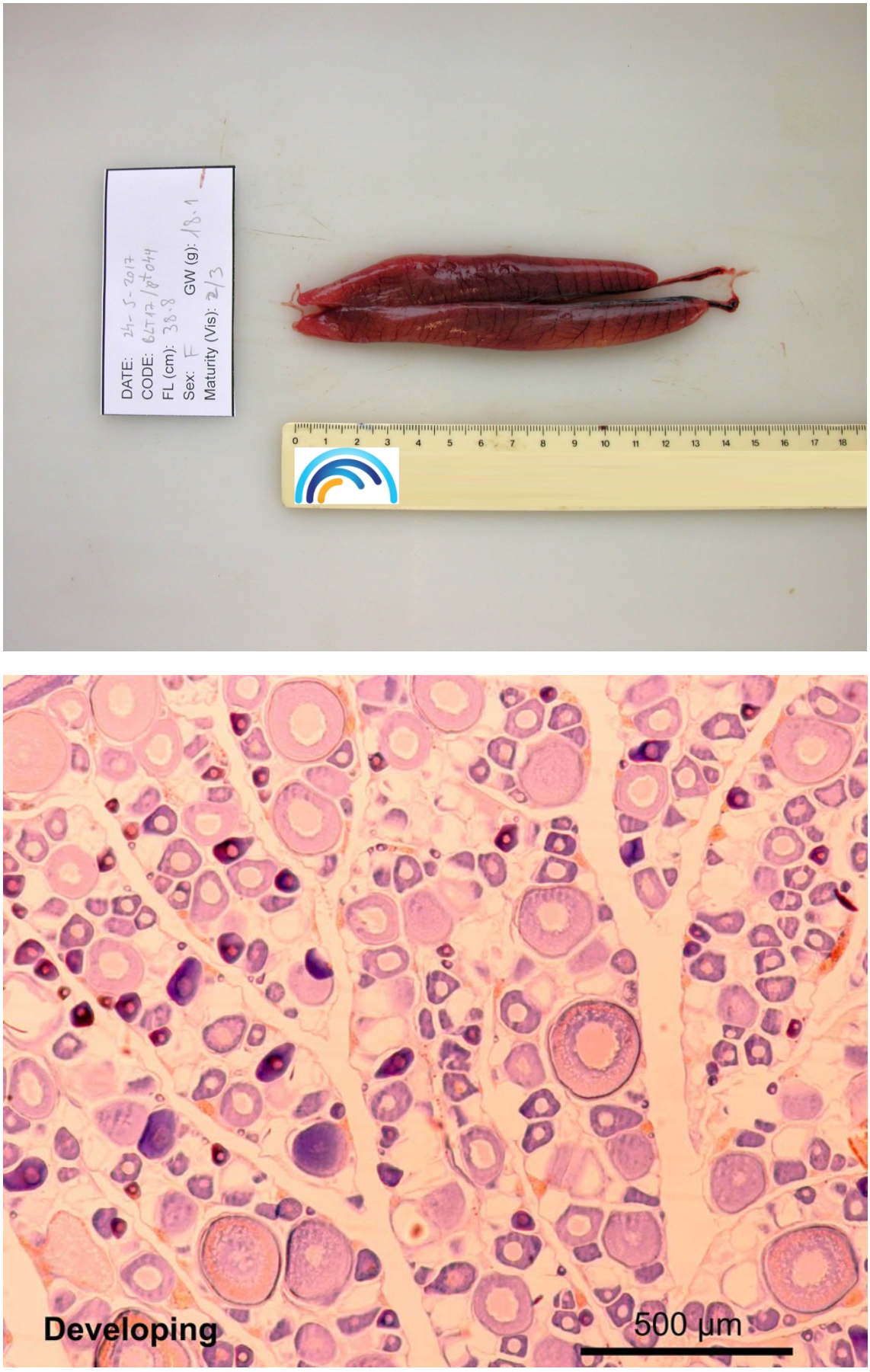


Figure 4. Macroscopic and microscopic photographs for developing stage (2b) of female *A. rochei*.

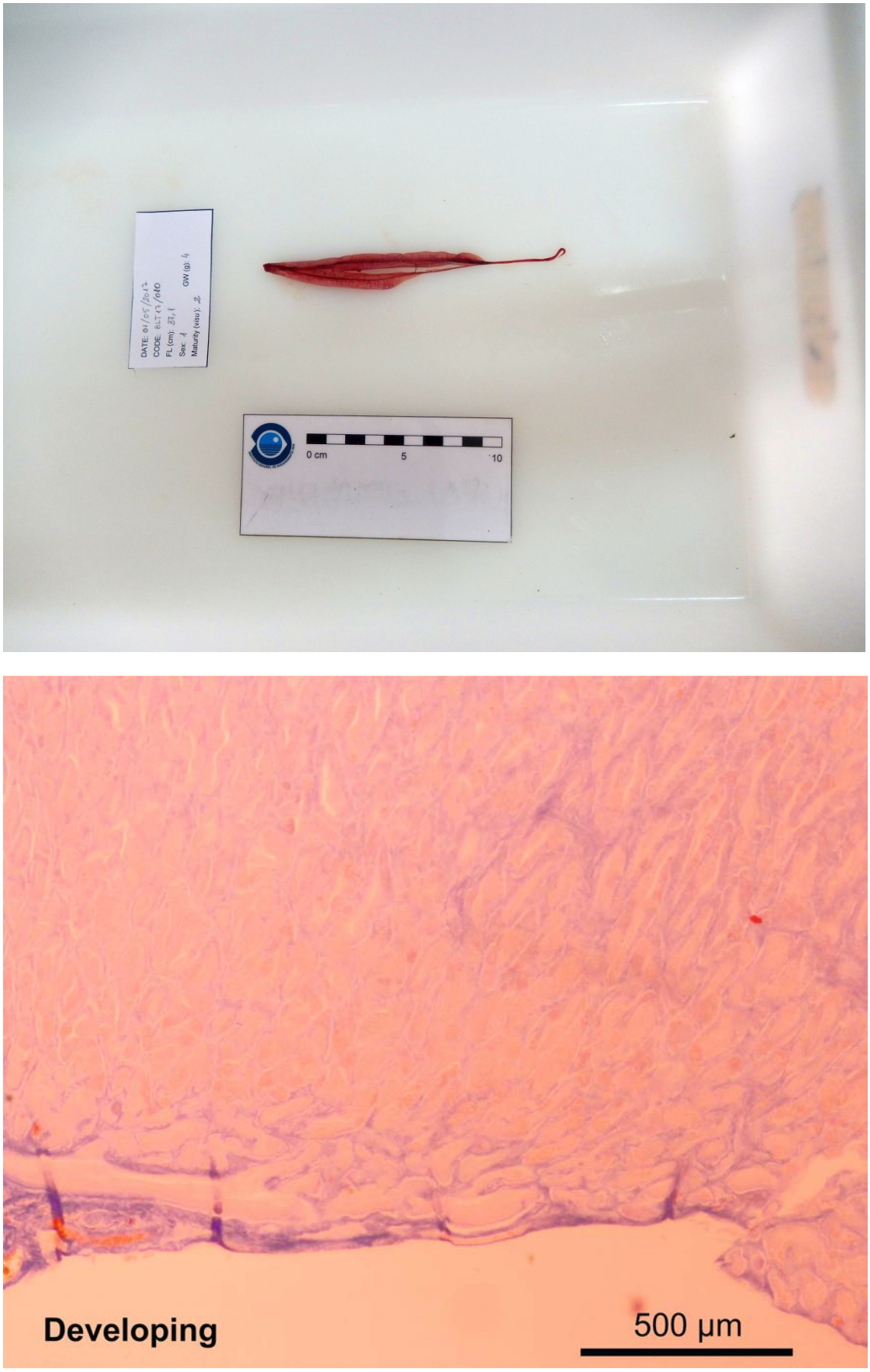


Figure 5. Macroscopic and microscopic photographs for developing stage of male *A. rochei*.

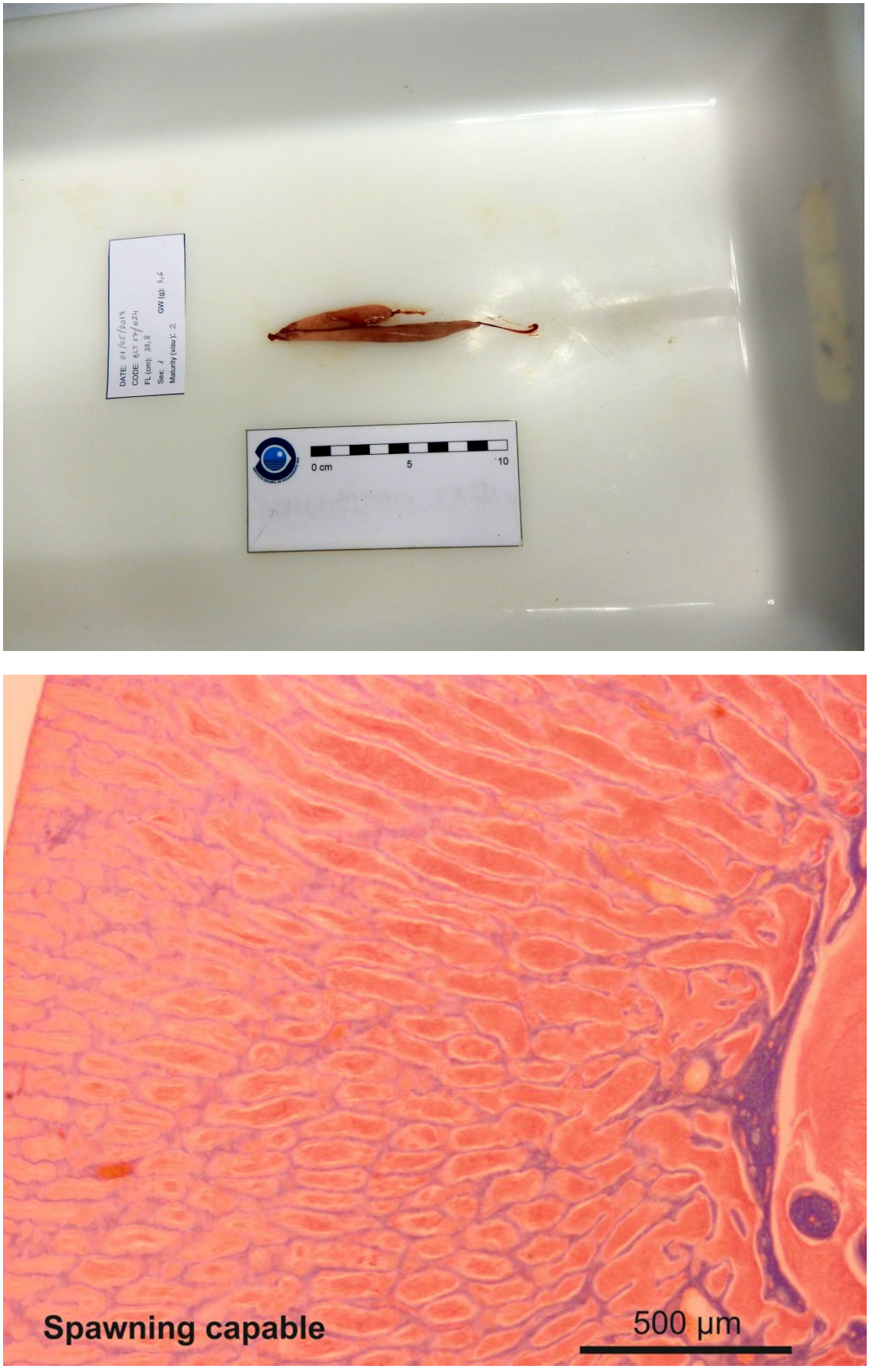
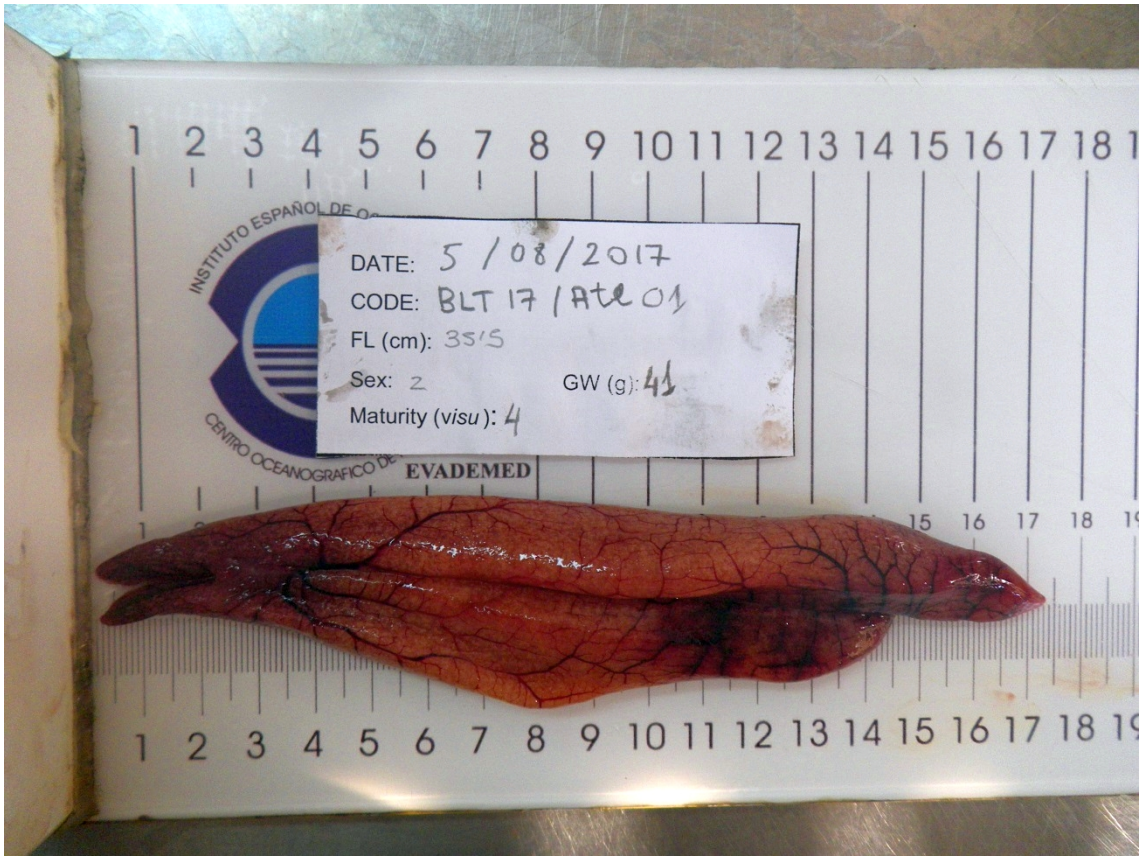


Figure 6. Macroscopic and microscopic photographs for spawning capable stage of male *A. rochei*.



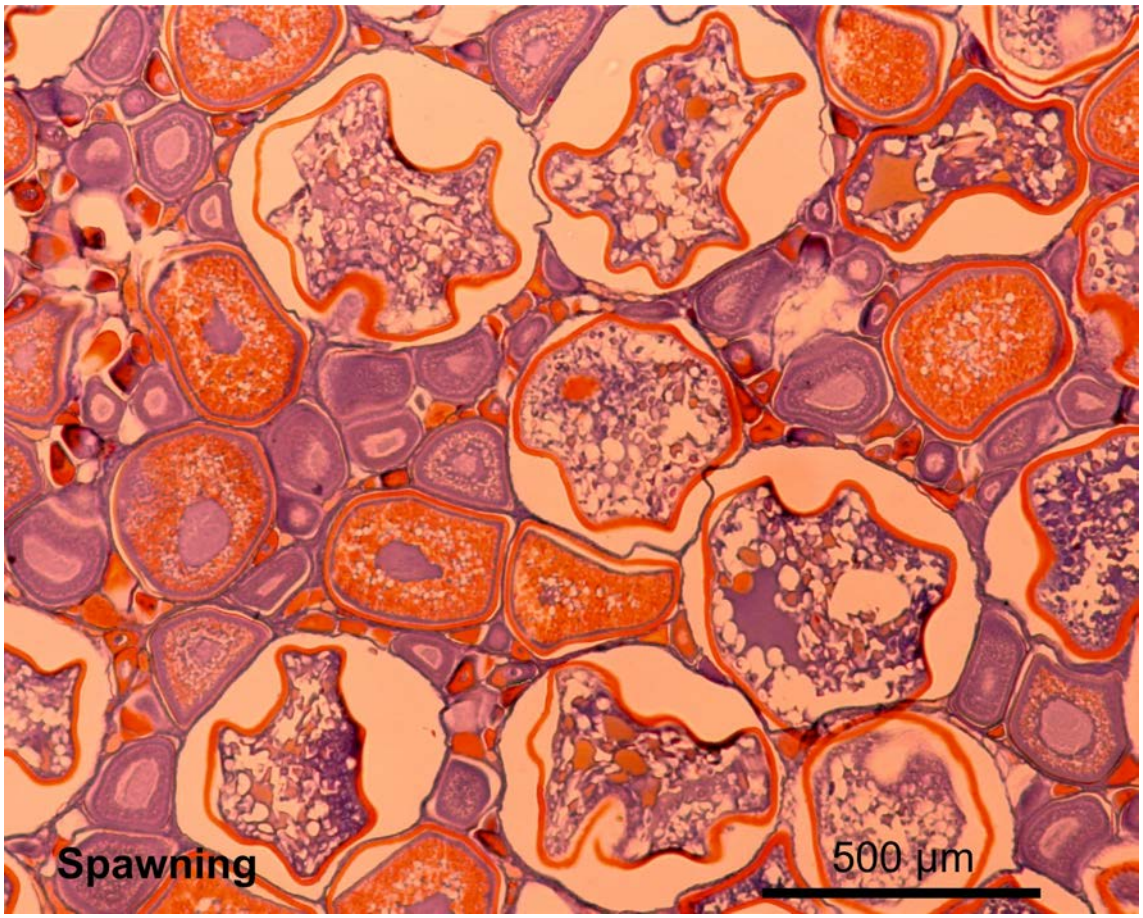


Figure 7. Macroscopic and microscopic photographs for spawning stage of female *A. rochei*.

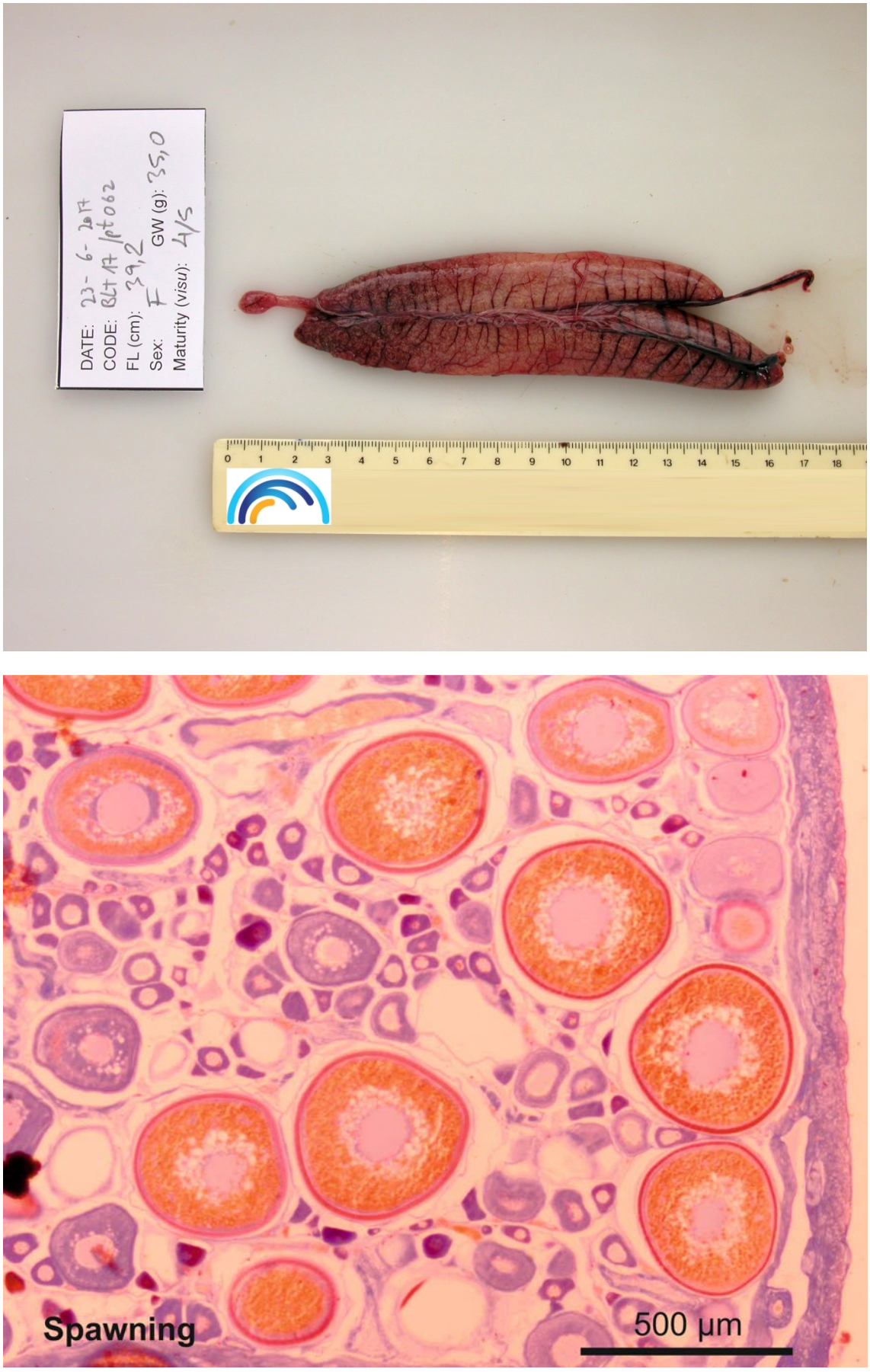


Figure 8. Macroscopic and microscopic photographs for spawning stage of female *A. rochei*.

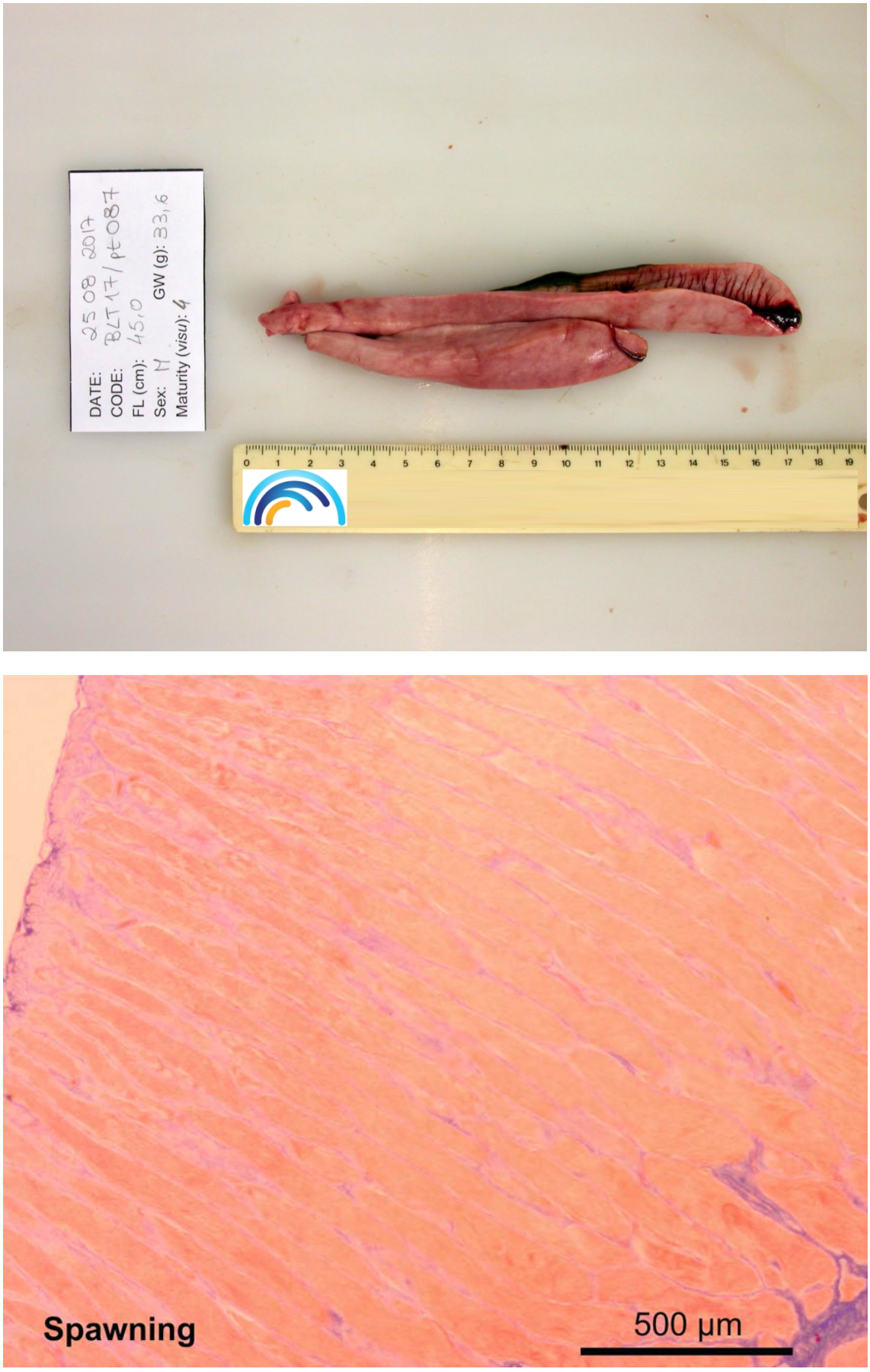


Figure 9. Macroscopic and microscopic photographs for spawning stage of male *A. rochei*.

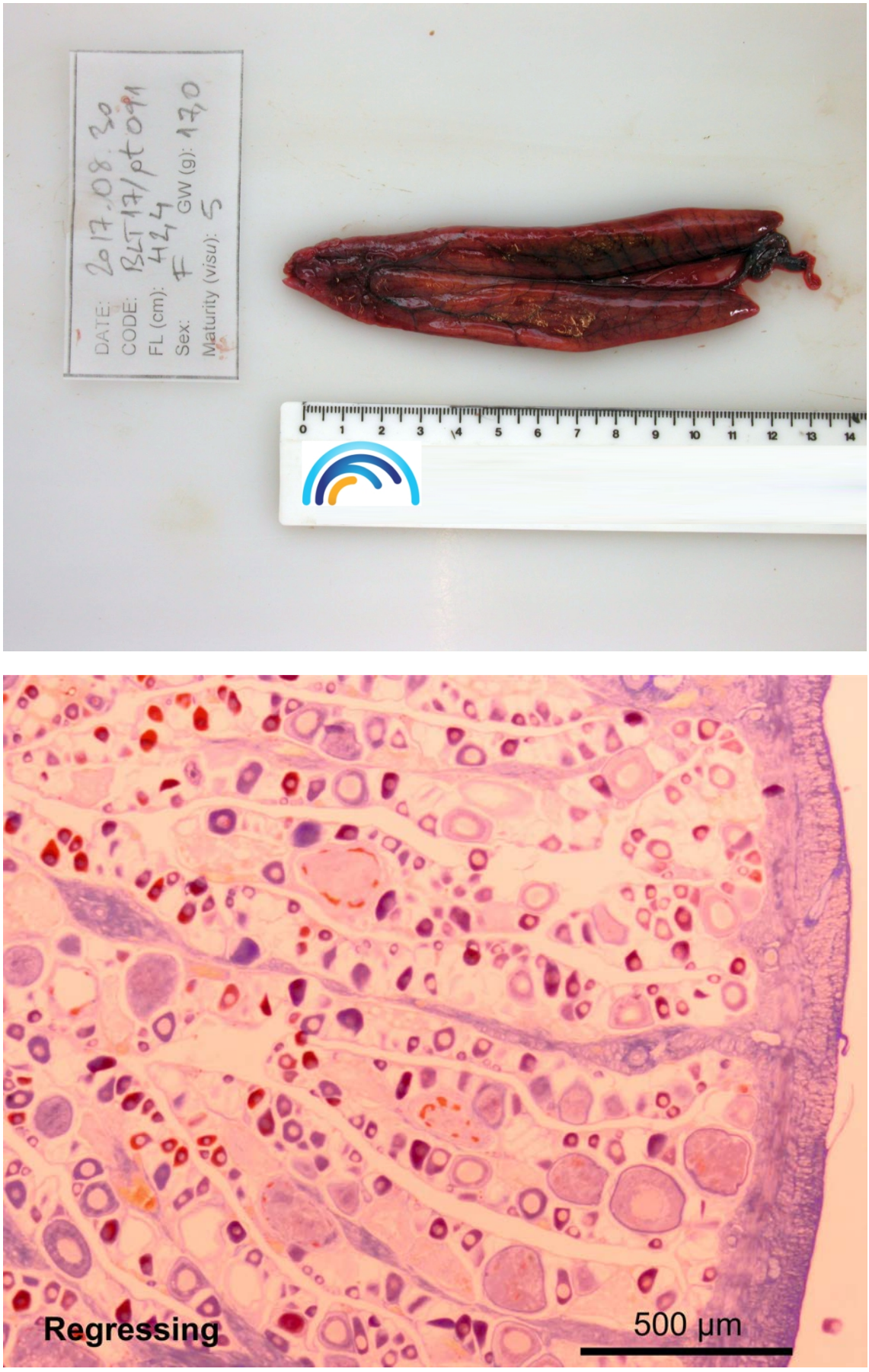


Figure 10. Macroscopic and microscopic photographs for regressing stage of female *A. rochei*.

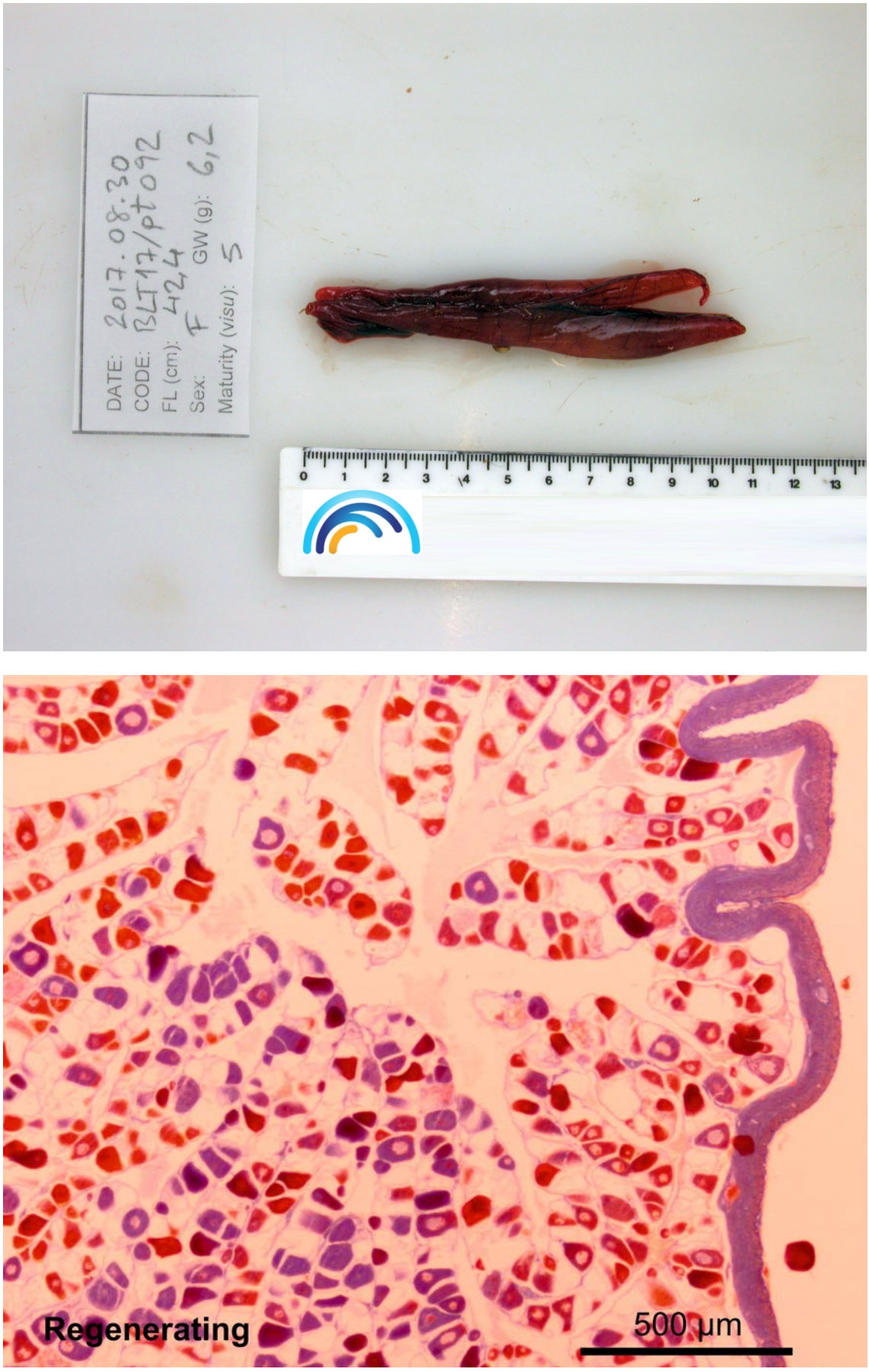


Figure 11. Macroscopic and microscopic photographs for regenerating stage of female *A. rochei*.

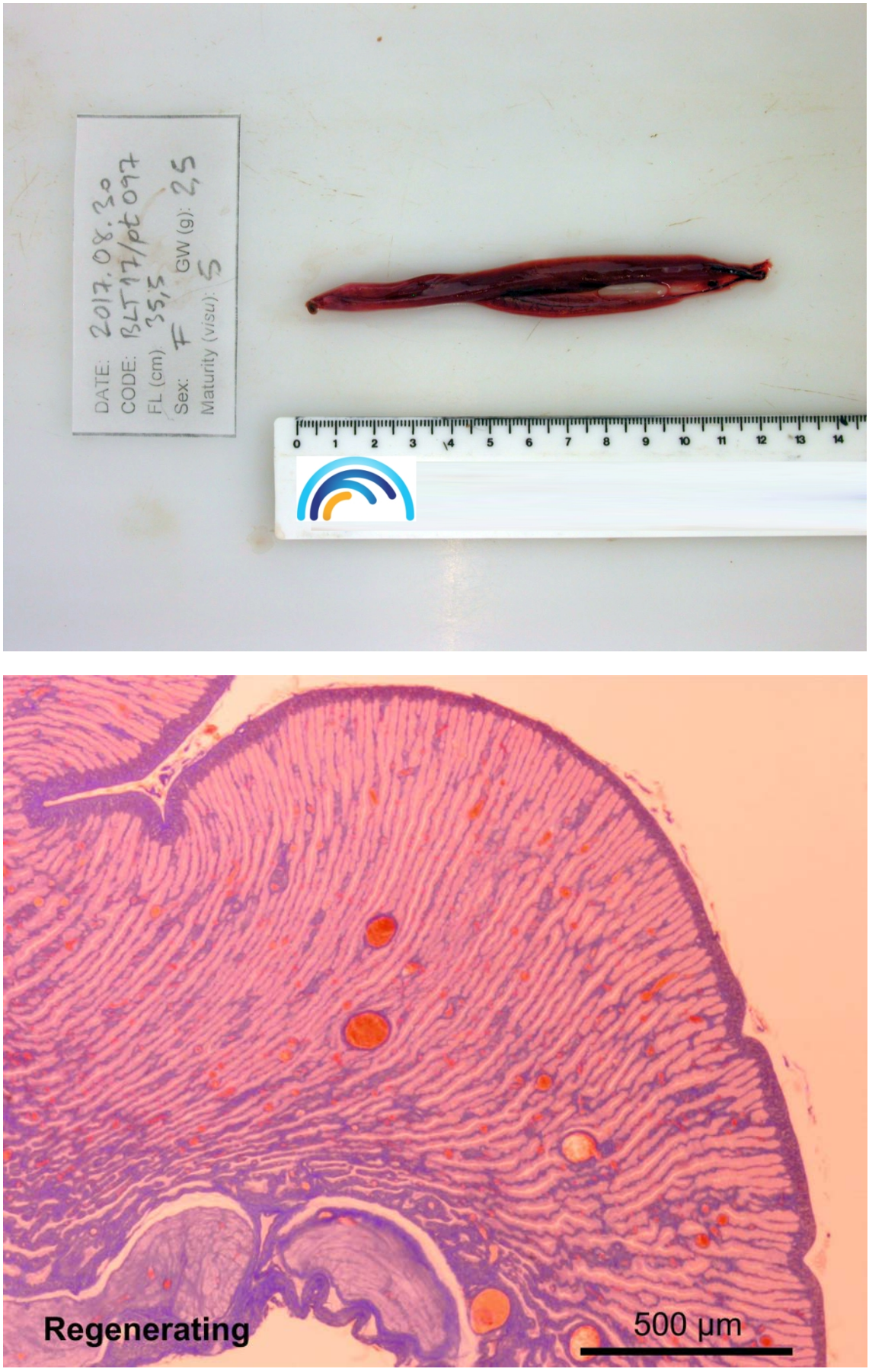


Figure 12. Macroscopic and microscopic photographs for regenerating stage of male *A. rochei*.

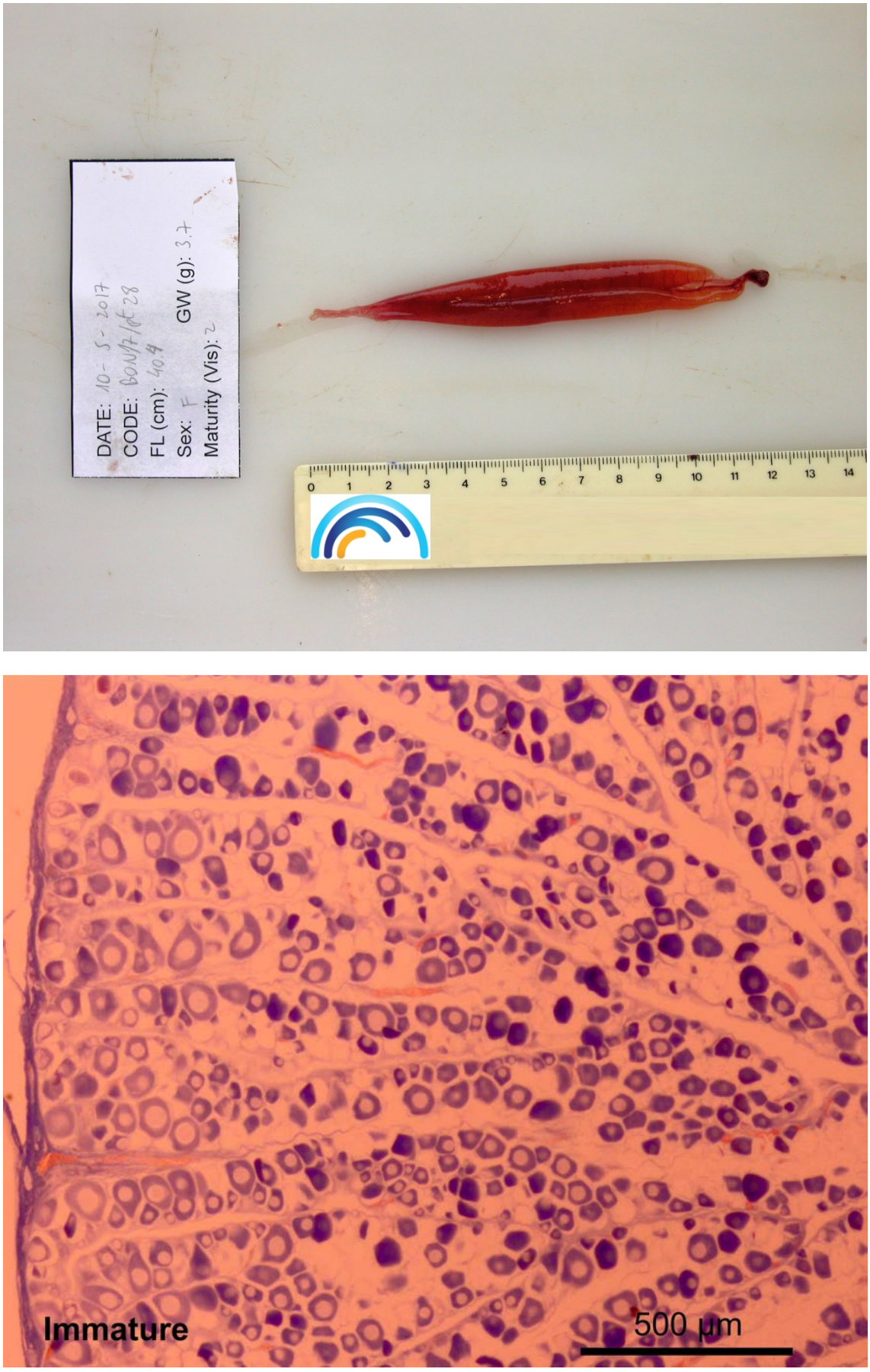


Figure 13. Macroscopic and microscopic photographs for immature stage of female *S. sarda*.

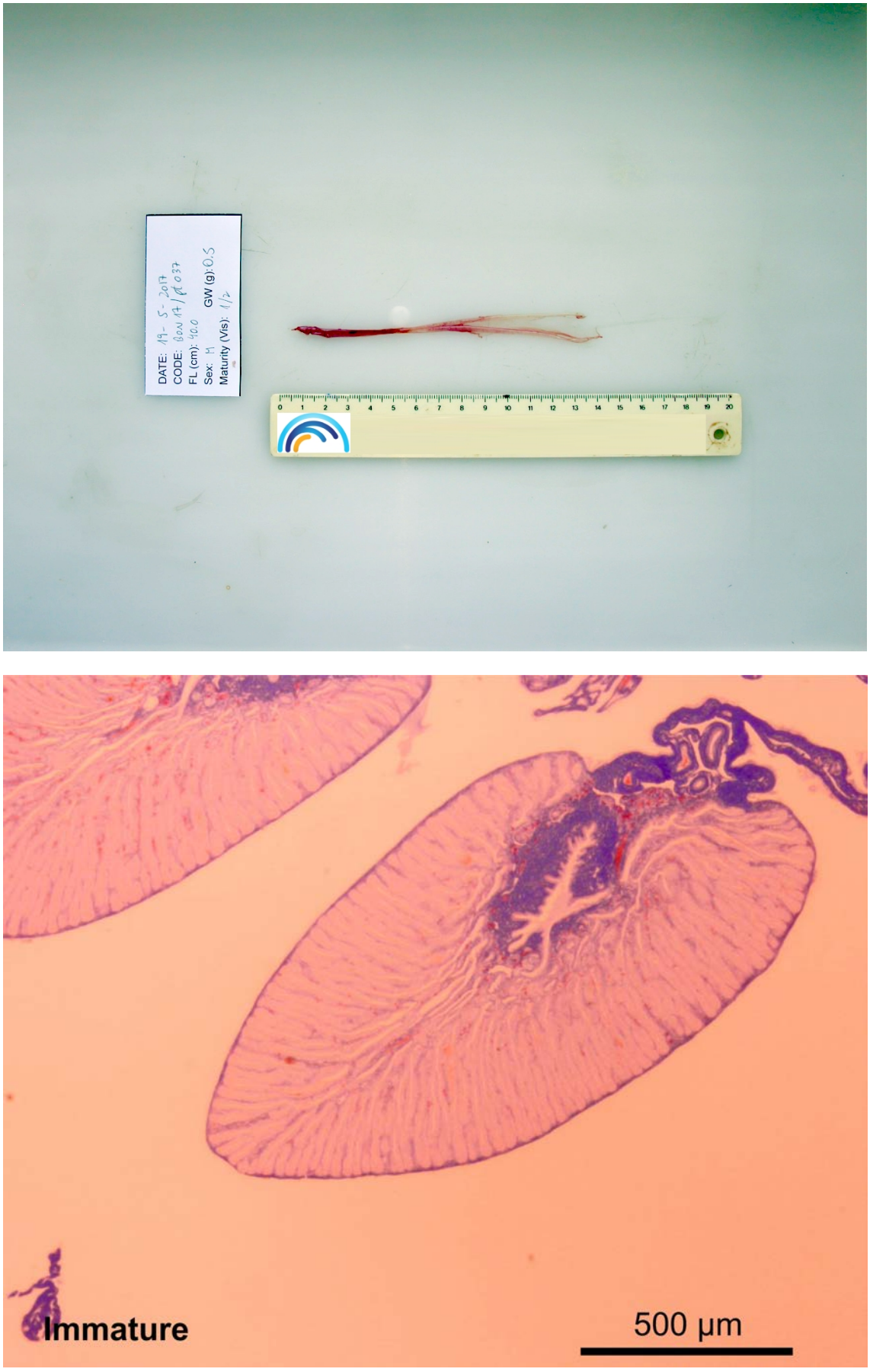


Figure 14. Macroscopic and microscopic photographs for immature stage of male *S. sarda*.

Figure 15. Macroscopic and microscopic photographs for developing stage (2a) of female *S. sarda*.

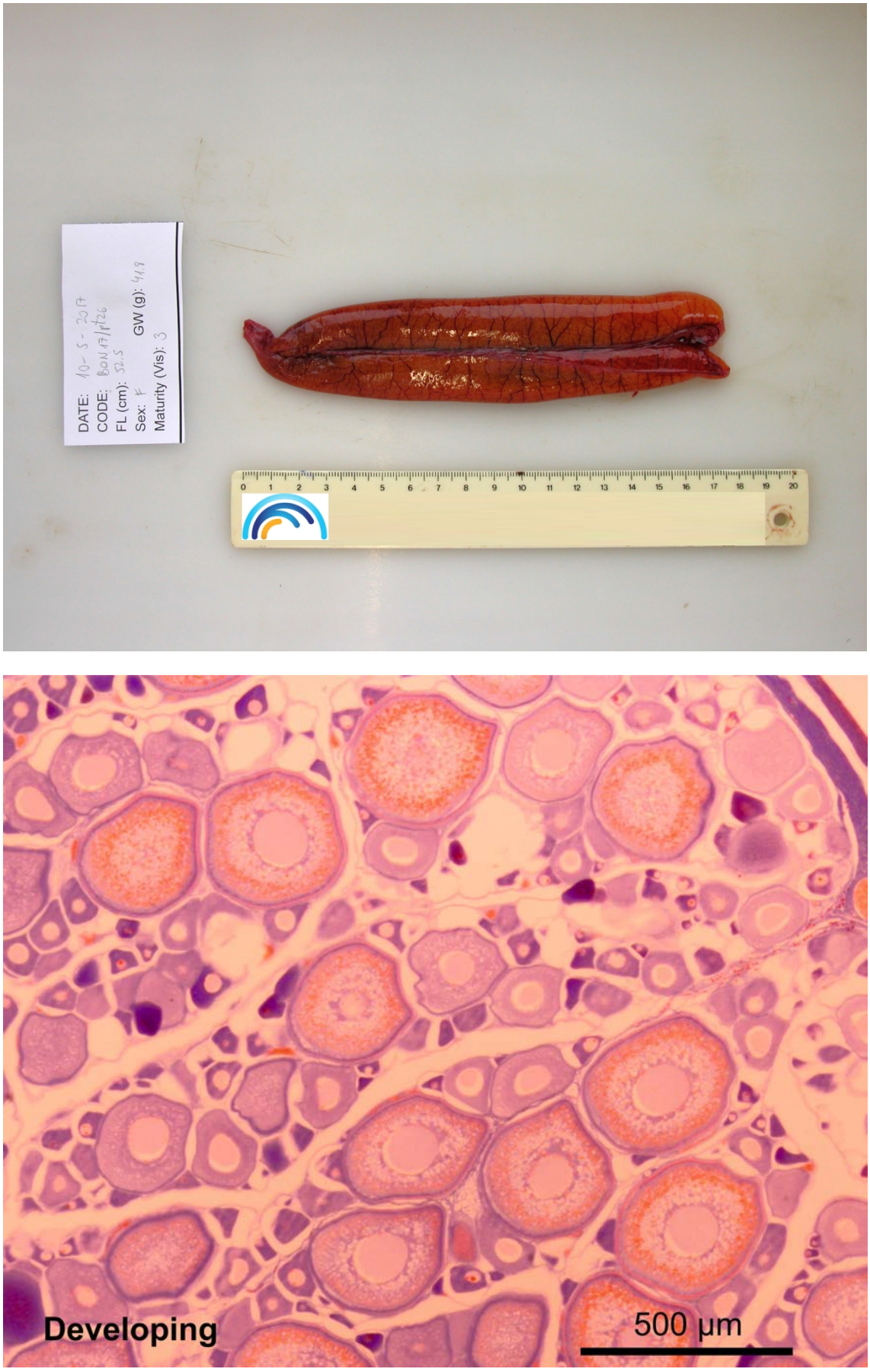


Figure 16. Macroscopic and microscopic photographs for developing stage (2b) of female *S. sarda*.

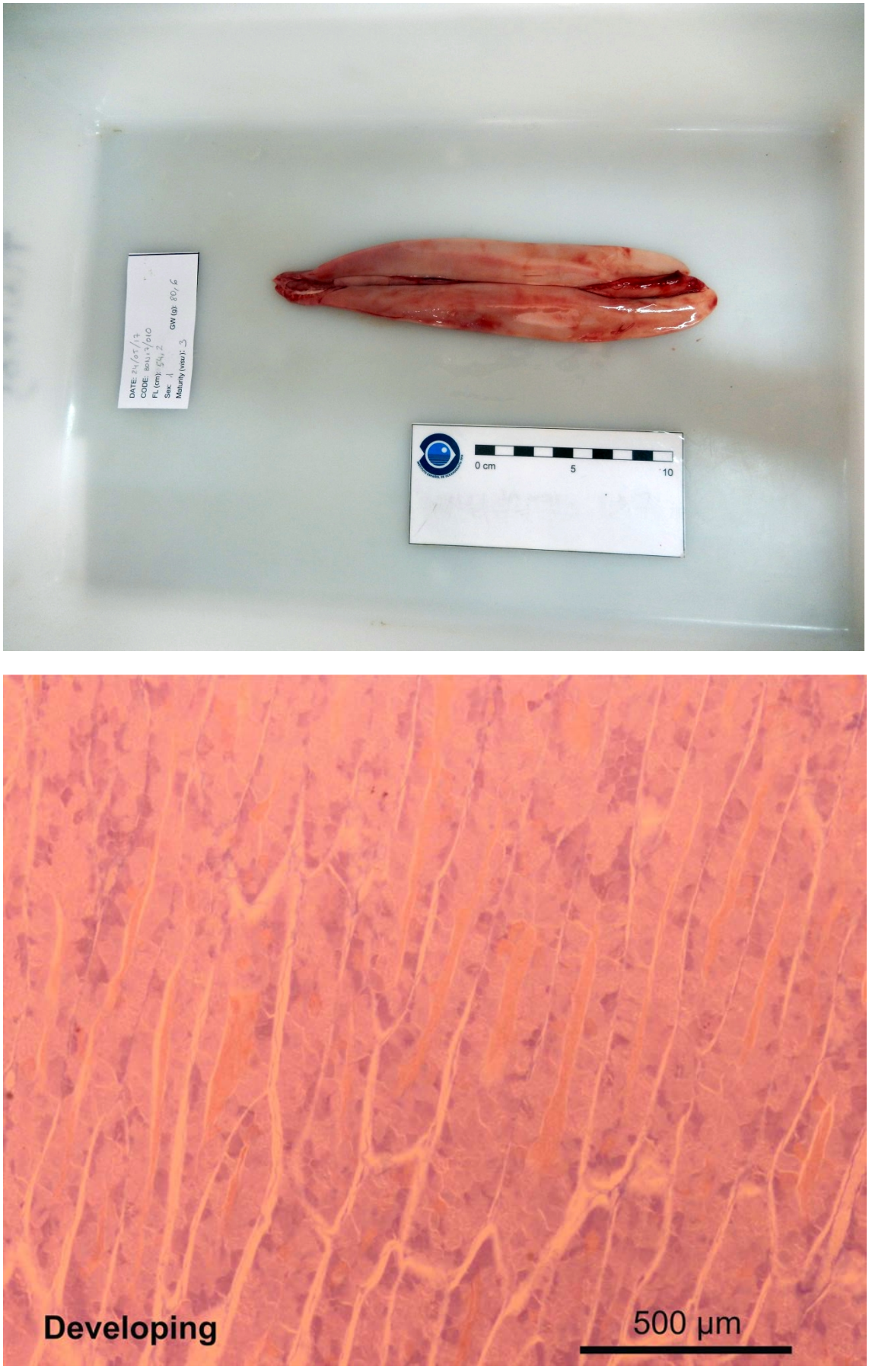


Figure 17. Macroscopic and microscopic photographs for developing stage of male *S. sarda*.

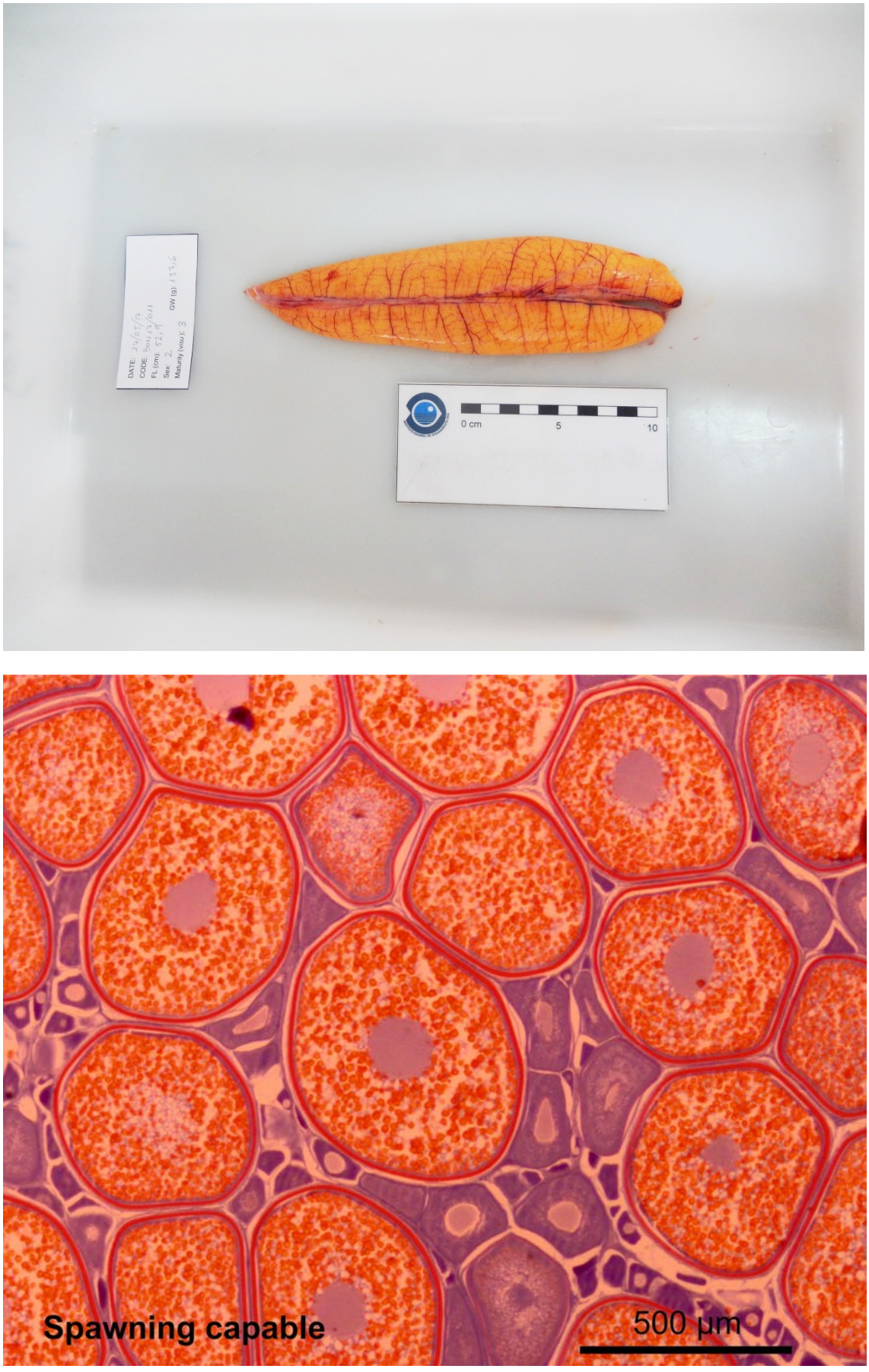


Figure 18. Macroscopic and microscopic photographs for spawning capable stage of female *S. sarda*.

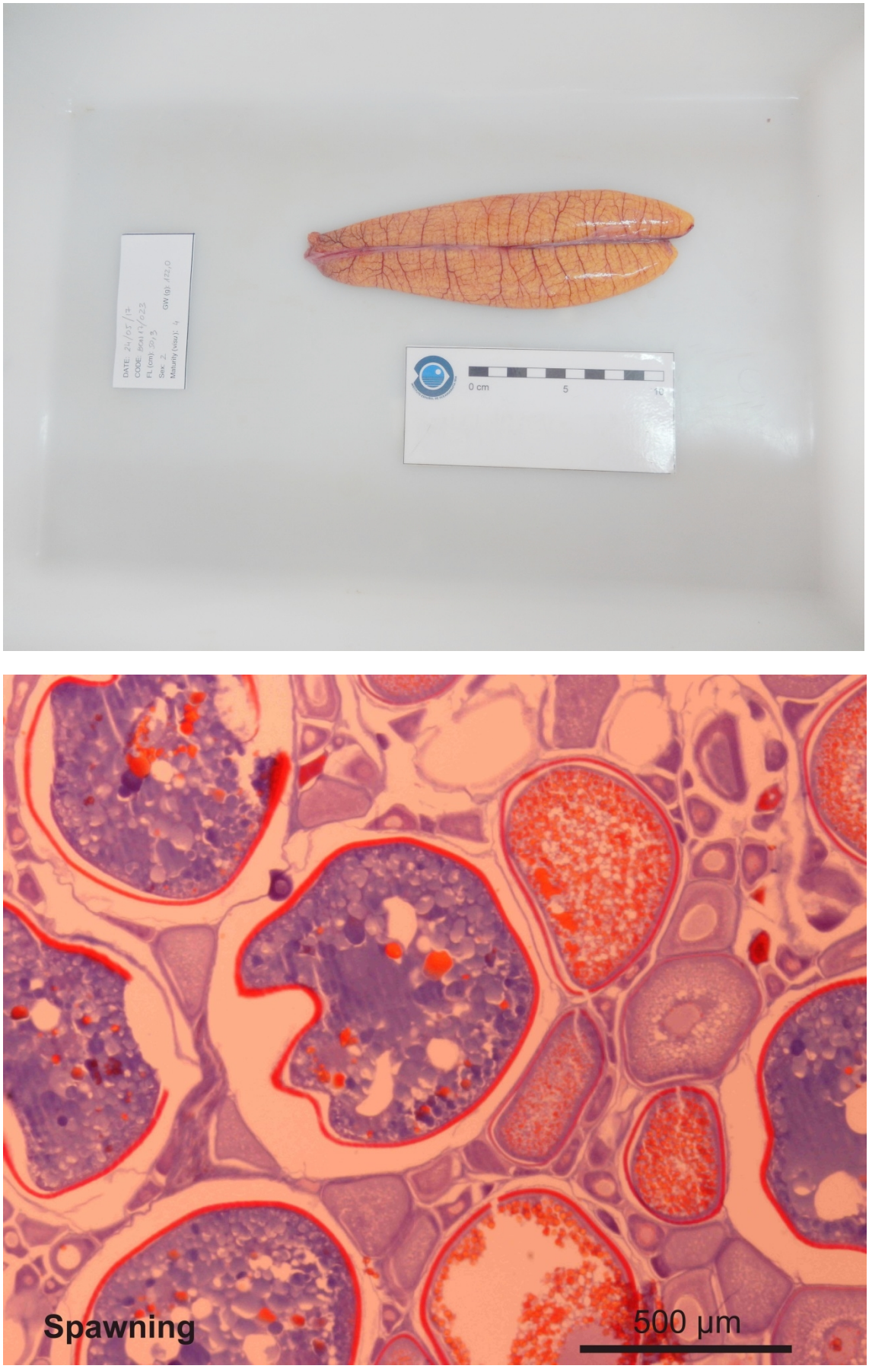


Figure 19. Macroscopic and microscopic photographs for spawning stage of female *S. sarda*.

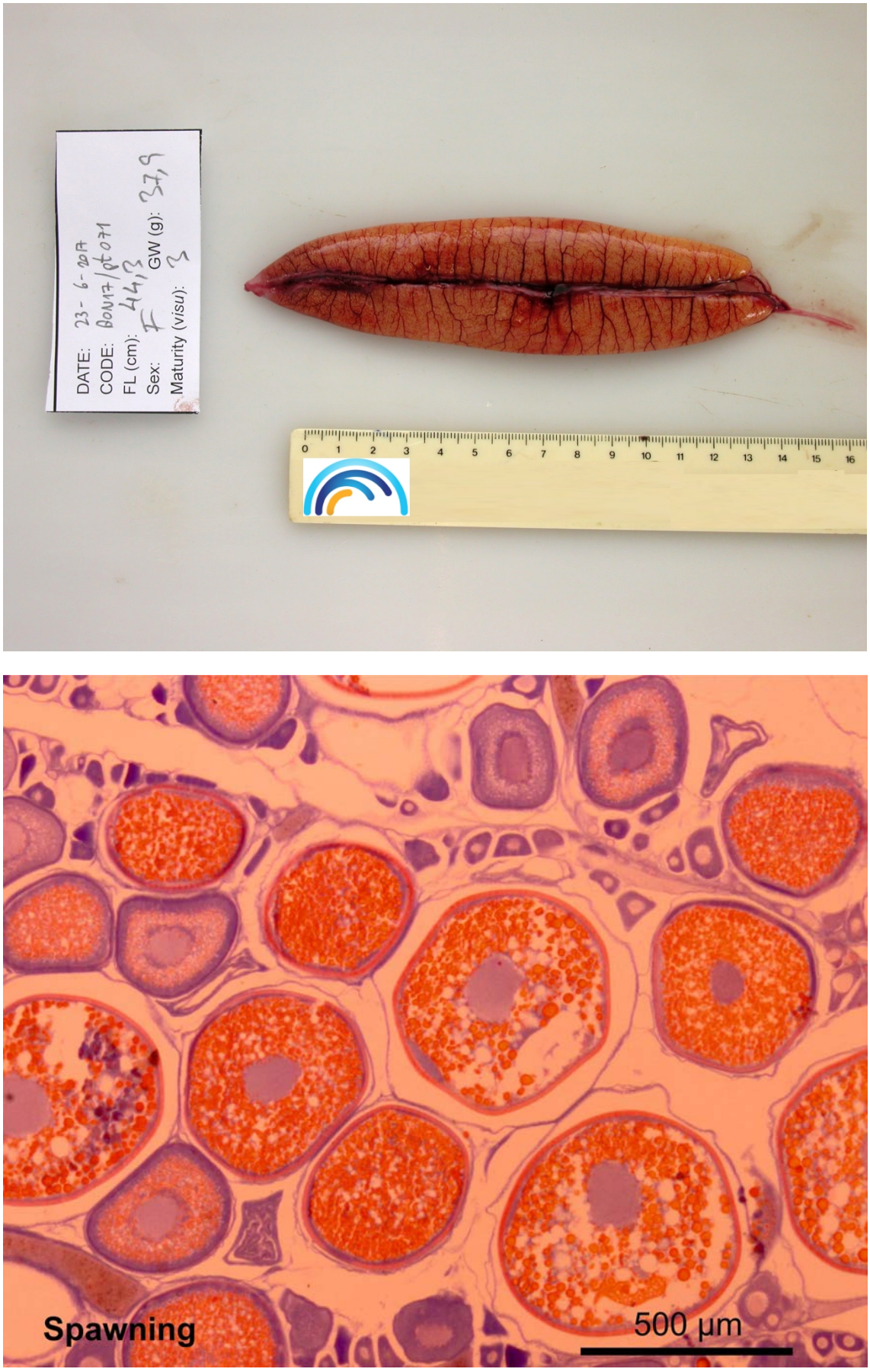


Figure 20. Macroscopic and microscopic photographs for spawning stage of female *S. sarda*.

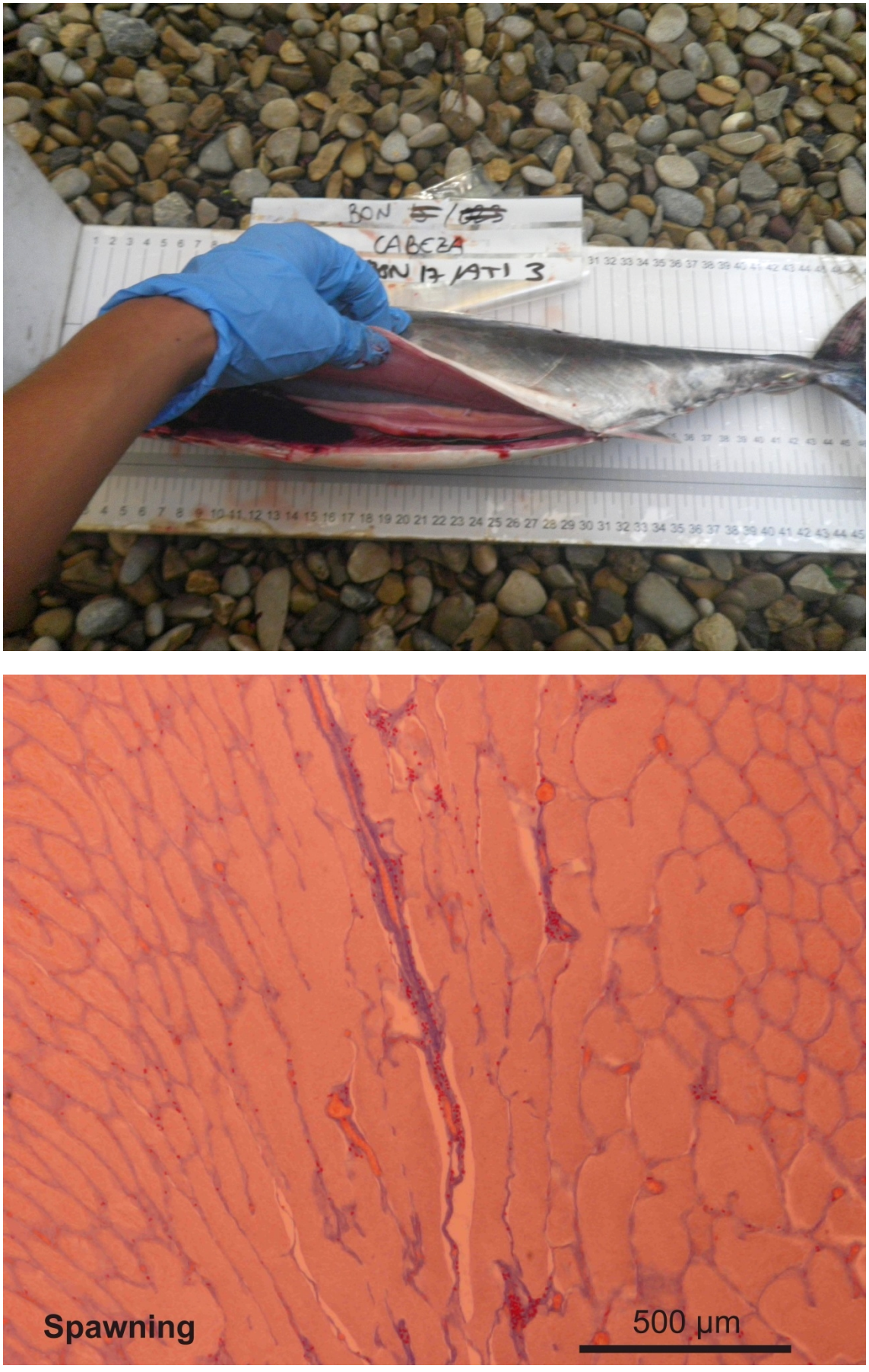


Figure 21: Macroscopic and microscopic photographs for spawning stage of male *S. sarda*.

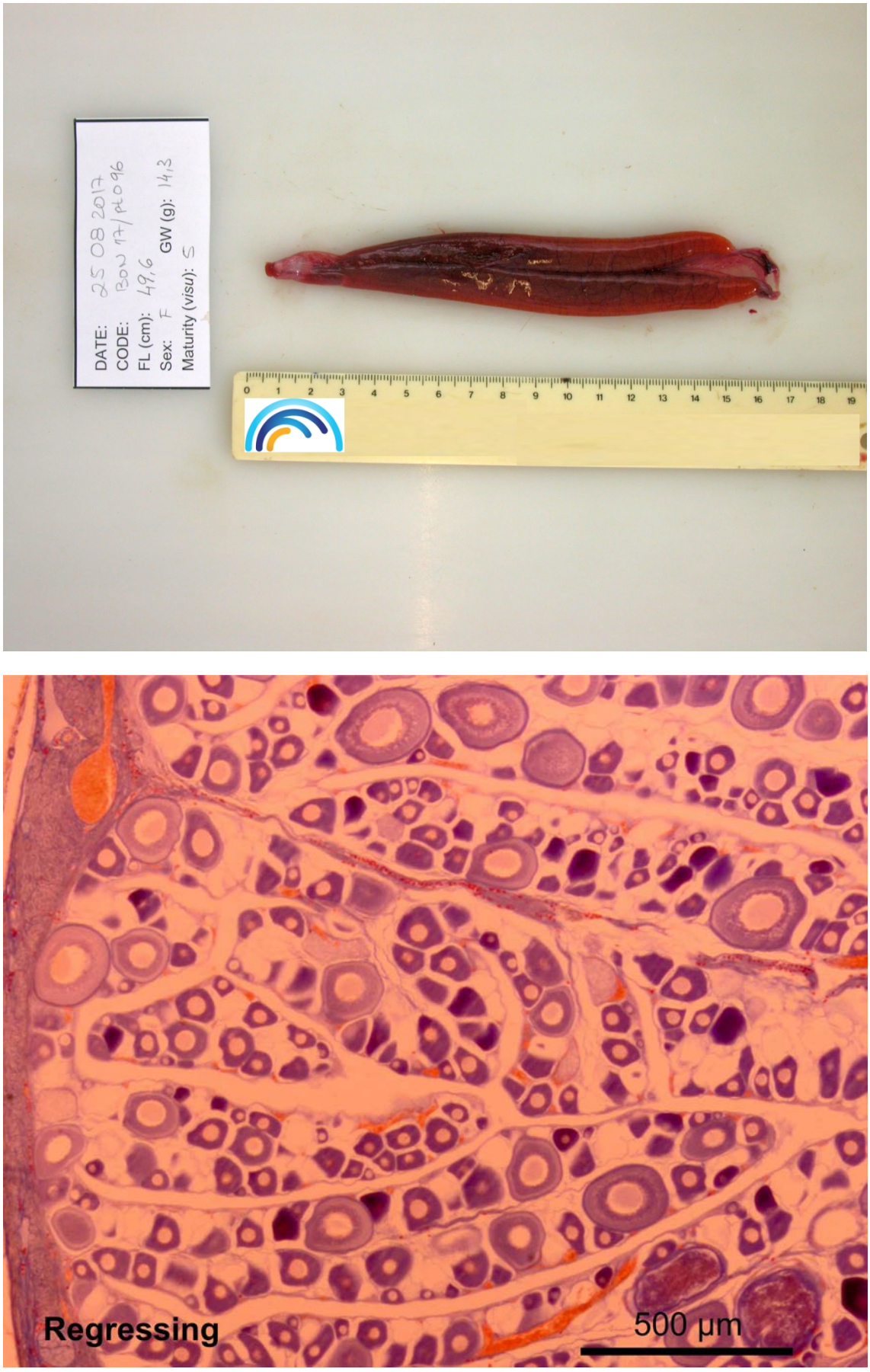


Figure 22. Macroscopic and microscopic photographs for regressing stage of female *S. sarda*.

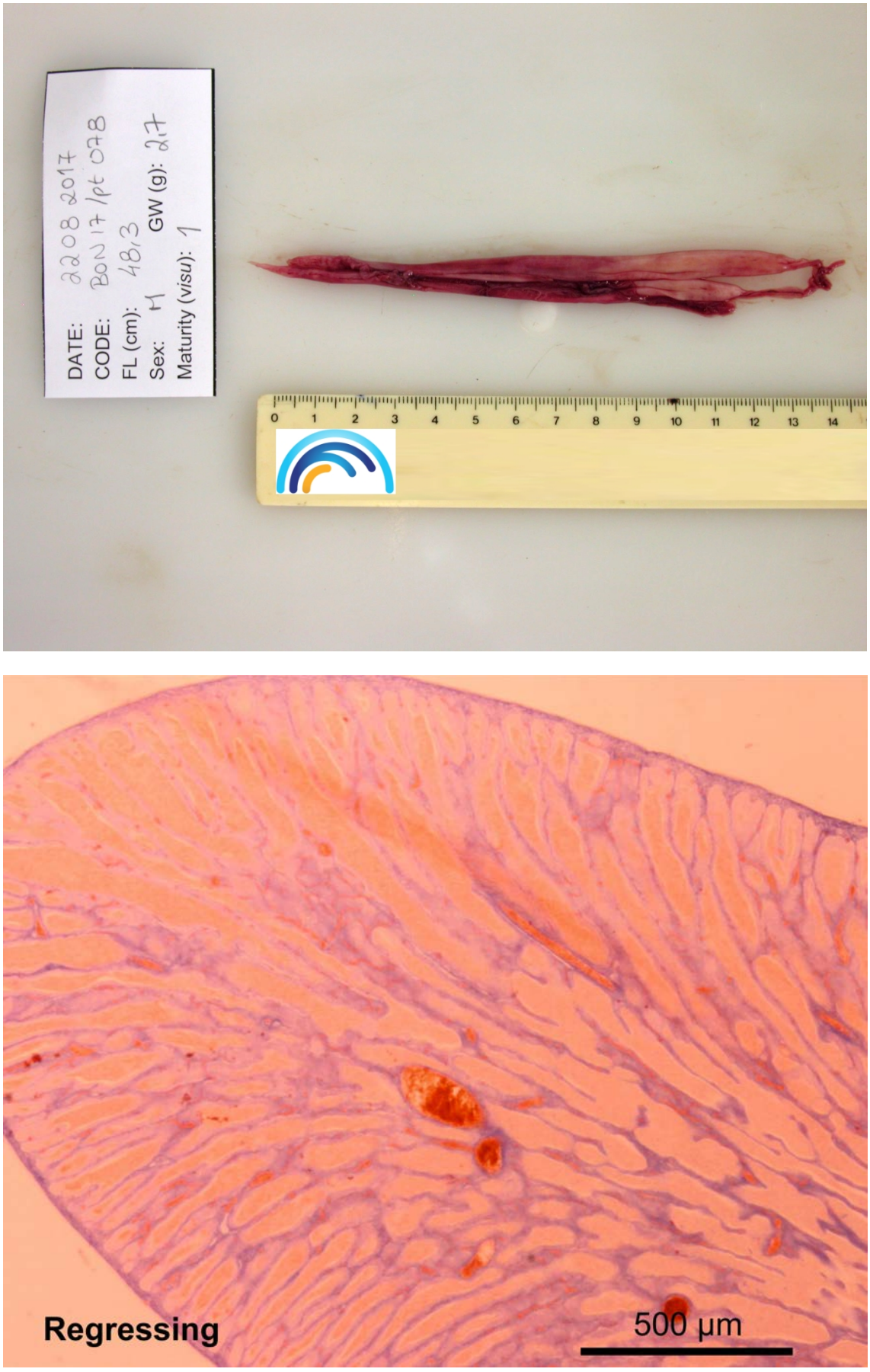


Figure 23. Macroscopic and microscopic photographs for regressing stage of male *S. sarda*.

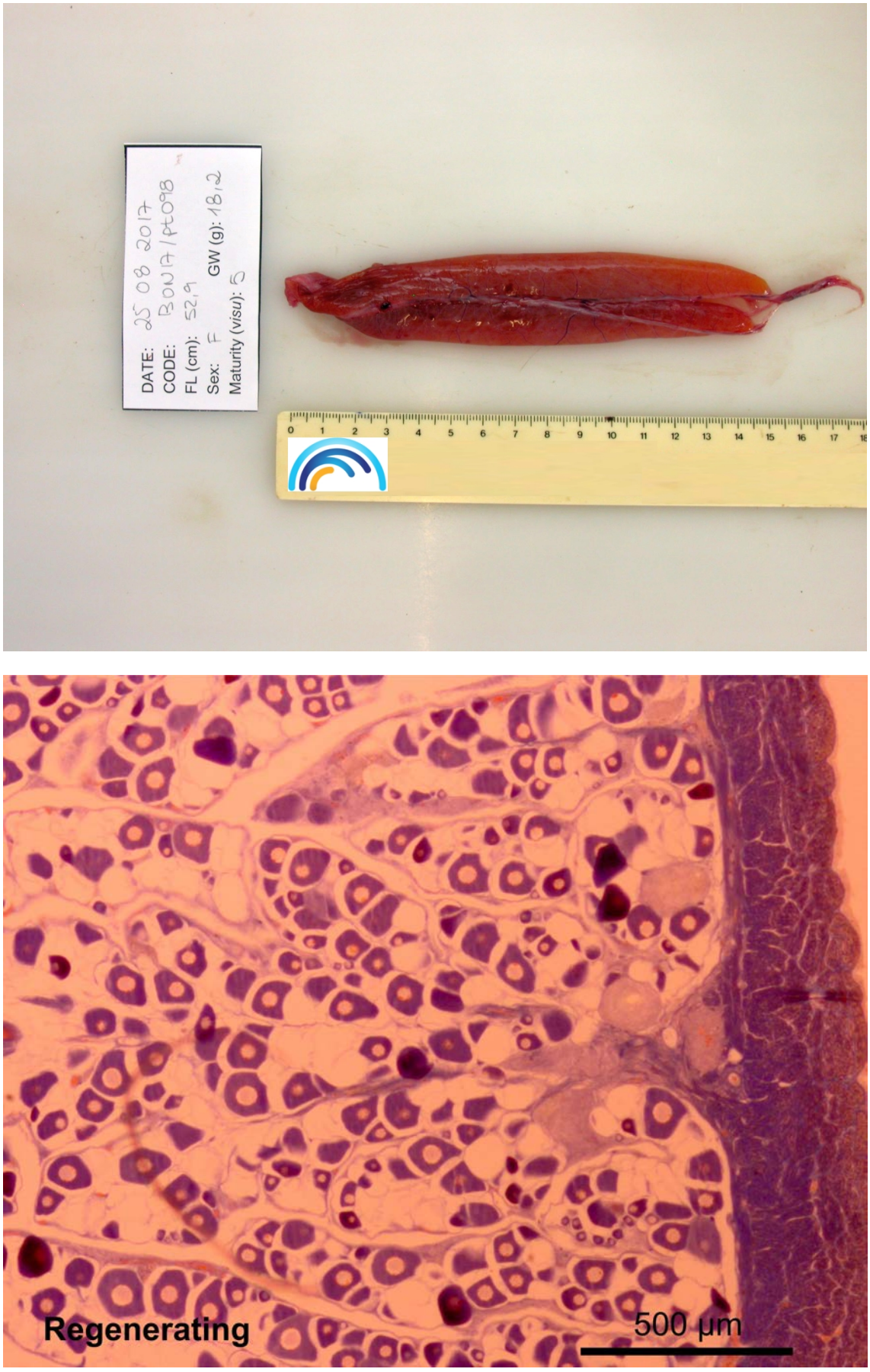


Figure 24. Macroscopic and microscopic photographs for regenerating stage of female *S. sarda*.

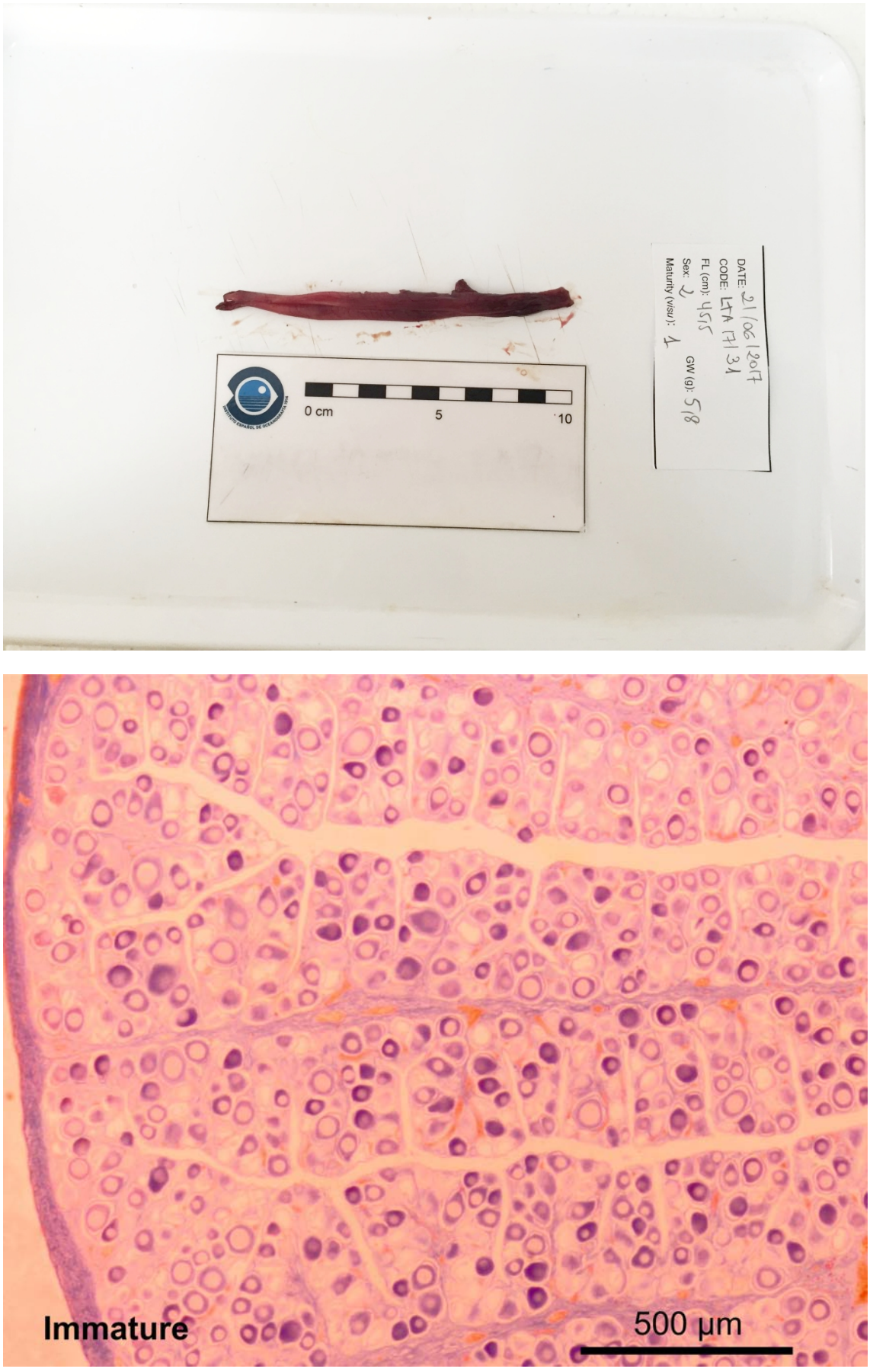


Figure 25. Macroscopic and microscopic photographs for immature stage of female *E. alletteratus*.

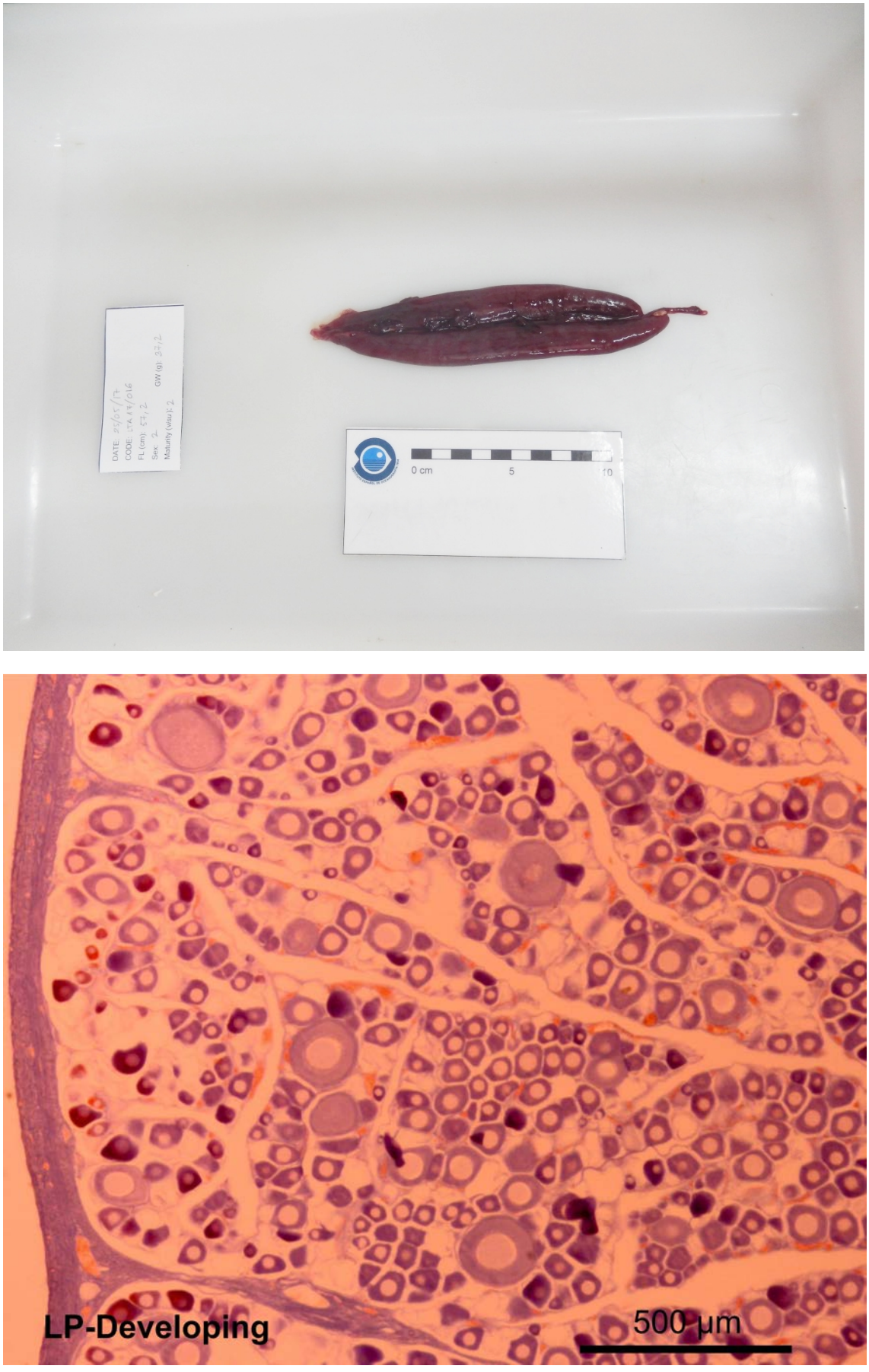


Figure 26. Macroscopic and microscopic photographs for developing stage (2a) of female *E. alletteratus*.

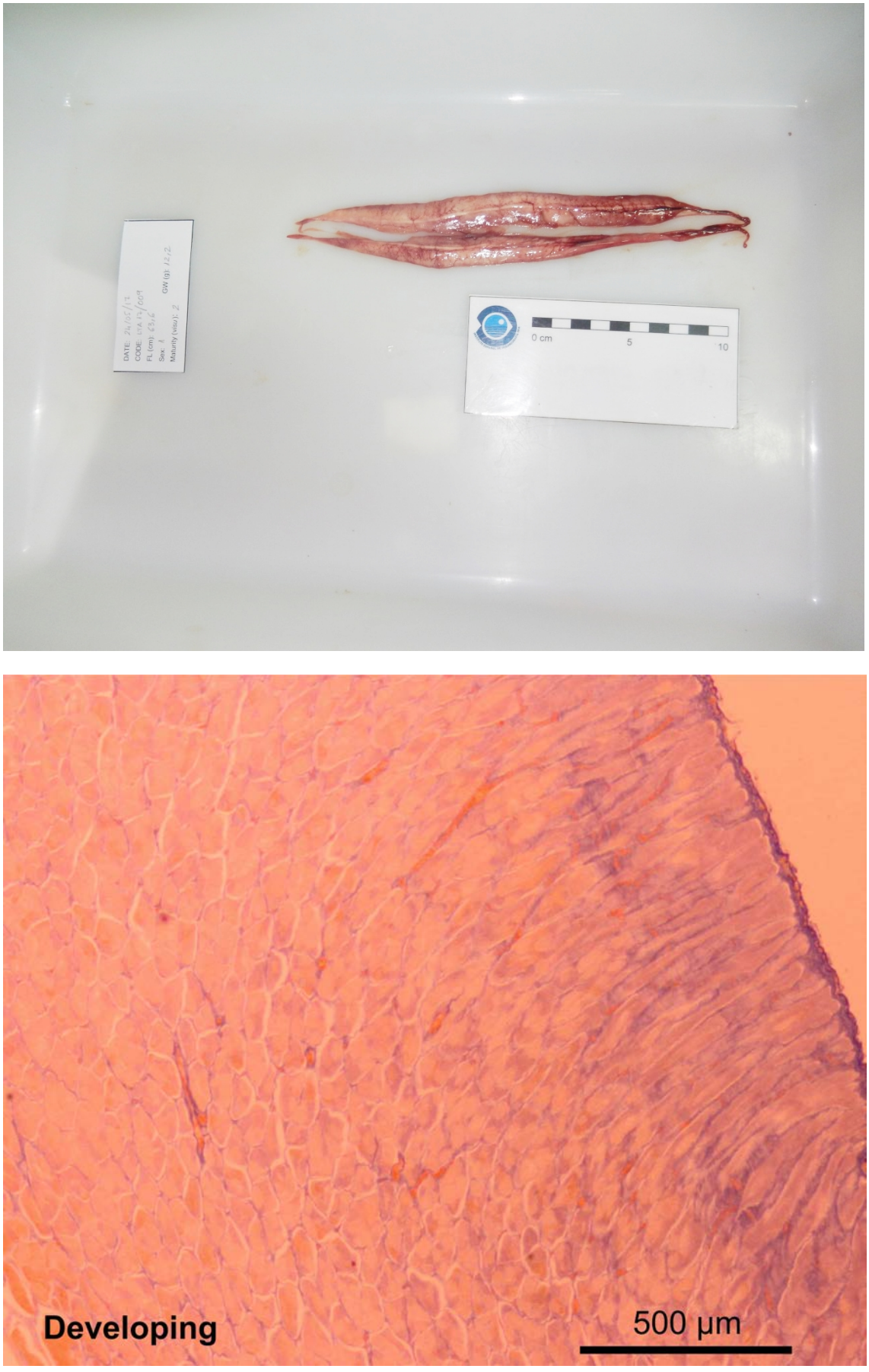


Figure 27. Macroscopic and microscopic photographs for developing stage of male *E. alletteratus*.

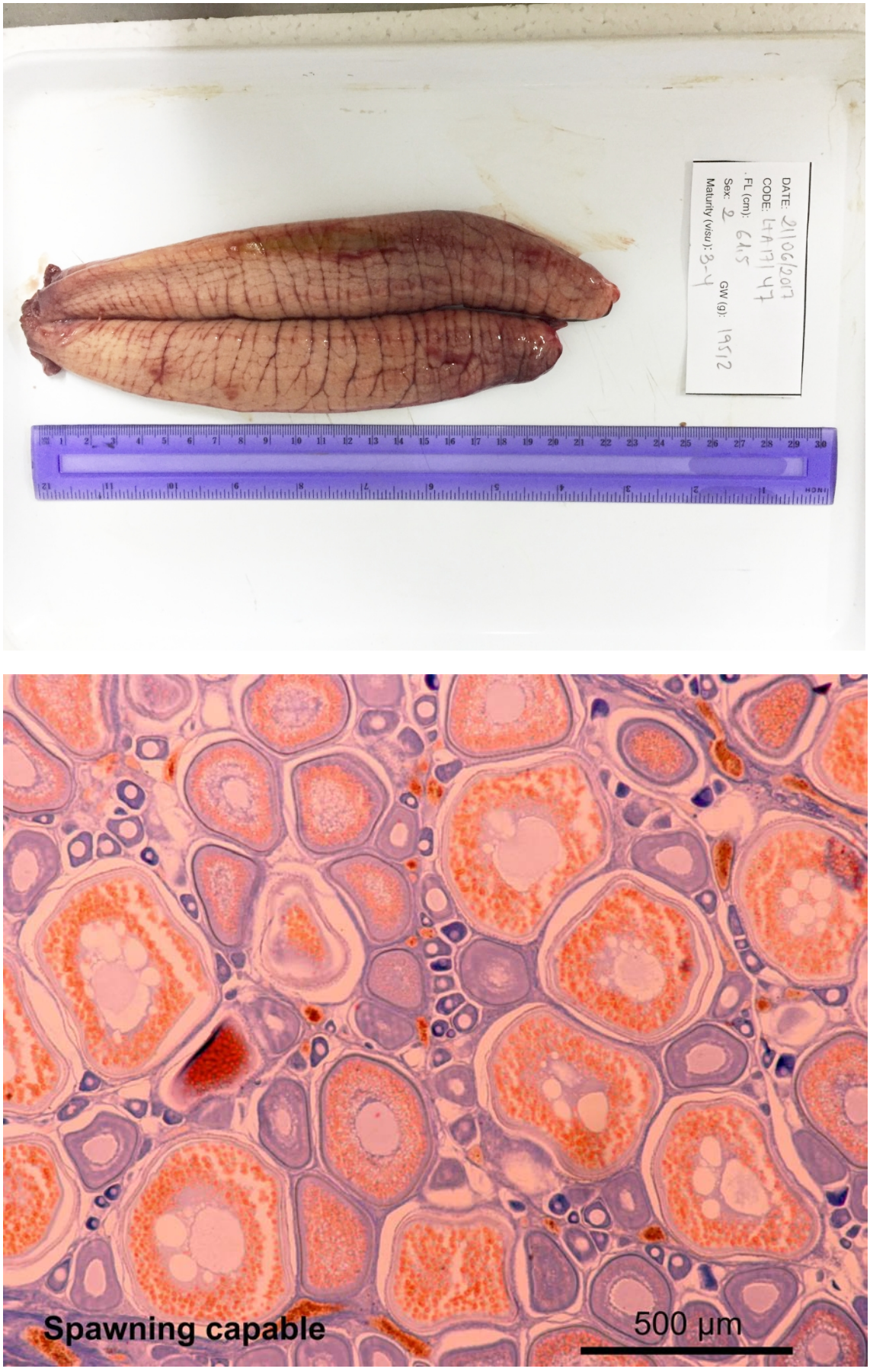


Figure 28. Macroscopic and microscopic photographs for spawning capable stage female *E. alletteratus*.

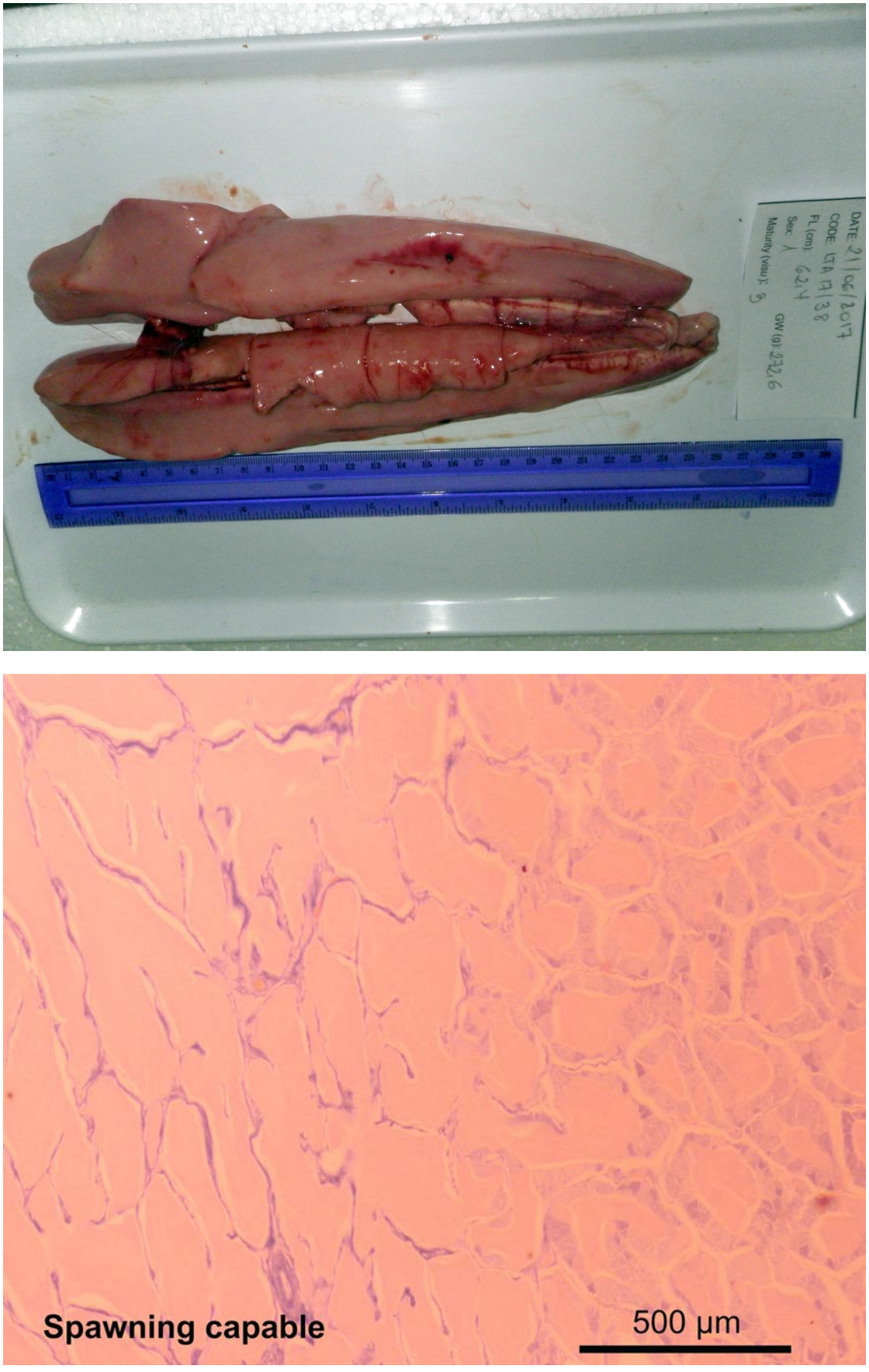


Figure 29. Macroscopic and microscopic photographs for spawning capable stage of male *E. alletteratus*.

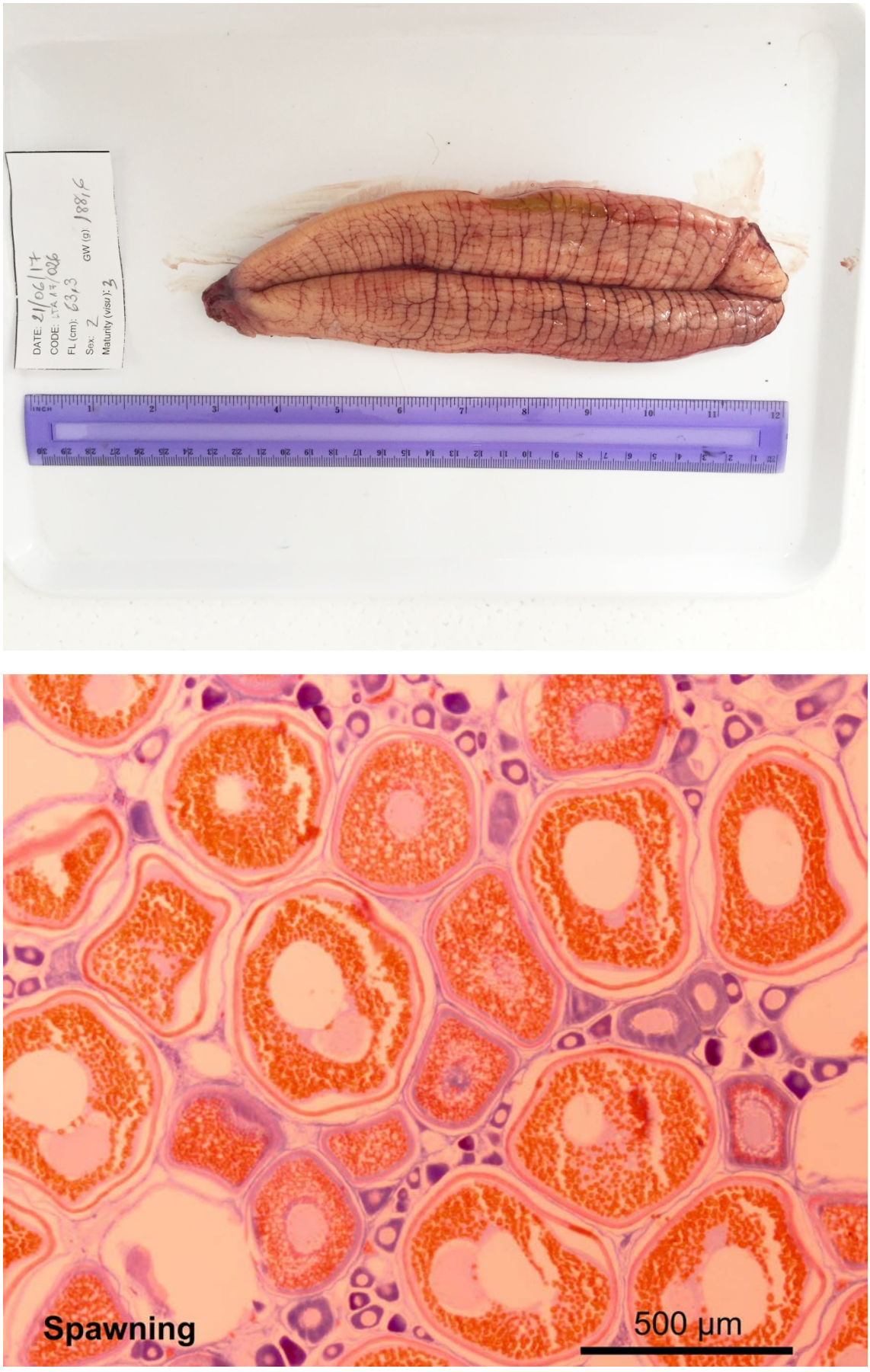


Figure 30. Macroscopic and microscopic photographs for spawning stage of female *E. alletteratus*.

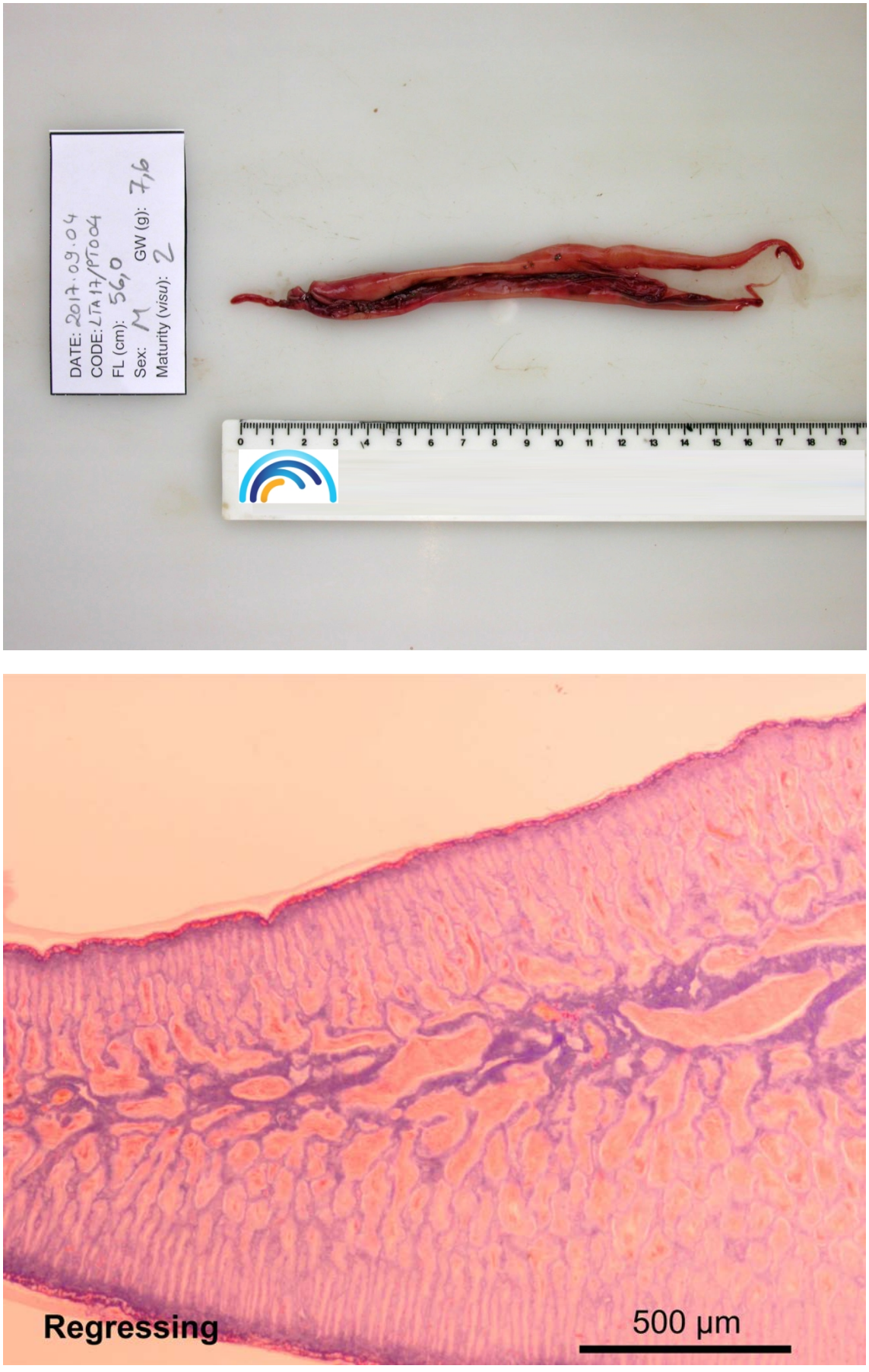


Figure 31. Macroscopic and microscopic photographs for regressing stage of male *E. alletteratus*.

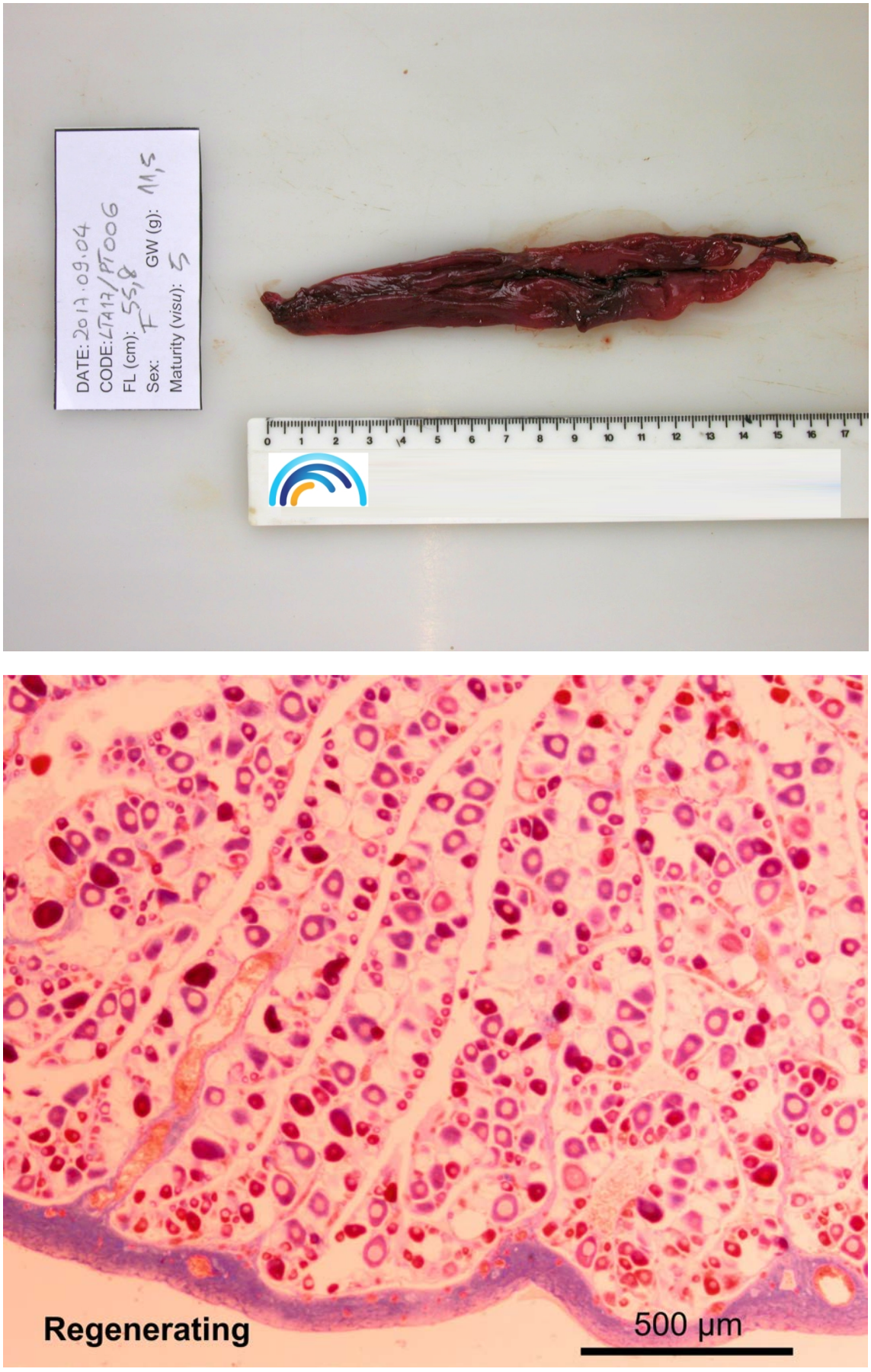


Figure 32. Macroscopic and microscopic photographs for regenerating stage of female *E. alletteratus*.