ASCIDIAN NEWS*

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As always, I thank the large number of AN readers who sent in contributions and for letting me know how important AN continues to be! This marks 49 years that I have been doing this newsletter! There are **93** new publications listed at the end of this newsletter. Please keep in touch and continue to send me contributions for the next issue. To be assured of your new publications being included, always send me the pdf.

*Ascidian News is not part of the scientific literature and should not be cited as such.

NEWS AND VIEWS

1. From **Ayelet Voskoboynik** (<u>ayeletv@stanford.edu</u>): The next International Tunicata Meeting (ITM) will be held at **Univ. of Calif. Santa Cruz Conference Center from July 21-26, 2024.**

The 12th International Tunicate Meeting (ITM-2024) brings together the international community of researchers using tunicates as model organisms for studies encompassing cell and developmental biology, neurobiology and immunity, post-embryonic development and regeneration, as well as genetics, genomics, ecology, taxonomy and evolution. <u>https://www.tunicatemeeting2024.com/</u>

Early registration has passed but you can still register. Final program Release: July 1. The ITM Organizing Committee: Alberto Stolfi, Ayelet Voskoboynik, Billie Swalla, Bill Jeffery, Brad Davidson, Emma Farley, Robert Zeller, Tony De Tomaso, Bill Smith.



2. As announced in the previous newsletter, an Ascidian Taxonomy Workshop is being held June
24-28, 2024, Coastal Carolina University, Conway, South Carolina, USA. Hosted by
Lauren Stefaniak, Susanna López-Legentil, and Marie Nydam. Funded by NSF DEB RUI
#2122475. The workshop will focus on living specimens that we will collect at local marinas. There
will be lectures on ascidian phylogenetics, taxonomy, identification and ecology, as well as extensive
hands-on practice with identification of living and formalin-preserved specimens.

3. The 1st International Symposium on Women in Tunicate Biology that was organized by Anna Di Gregorio (adg13@nyu.edu) and Marie Nydam (mnydam@soka.edu) was held online on March 28-29, 2023 (see Ascidian News #91). This global symposium was attended by 50 researchers from several countries, including Austria, Brazil, India, Italy, Japan, New Zealand, Turkey, and the United States. The 35 manuscripts in this collection include 'tribute' papers that honor women scientists who pioneered and advanced the field of tunicate biology, as well as 'In Her Words' letters, which provided a canvas for women scientists to freely illustrate their research and themselves. The proceedings have been published in a Special Issue in *genesis: The Journal of Genetics and Development*. https://onlinelibrary.wiley.com/toc/1526968x/2023/61/6

This special Issue is expected to become a resource for all scientists interested in tunicate biology, a reference for early and contemporary work in this field of science, and an inspiration for all women scientists. All of the major fields of tunicate biology are represented, including developmental and stem cell biology, regeneration biology, ecology and taxonomy. Many of the papers are Open Access and a pdf of any can be requested directly from the author.

4. Sea squirt burgers: "the future of food"?

13 May 2024, https://thefishsite.com/articles/sea-squirt-burgers-future-food-pronofa-tunicates

Scandinavian startup Pronofa is farming and processing *Ciona* tunicates, commonly known as sea squirts, in a bid to provide an environmentally friendly alternative to mince made from conventional terrestrial livestock. The company says it has developed a way to farm these tunicates, which naturally colonise structures such as nets and require no formulated feed fine-tuning it to encourage production of up to 82 kg of tunicate per m², and enabling an efficient harvesting process.

The production capacity at their current facility in Sweden is approximately 800-1,500 tonnes of minced *Ciona* meat and they are building a second facility in Norway, which should allow them to produce 2,500 tonnes by 2025 and beyond 15,000 tonnes once both facilities are at full capacity. Meanwhile, in terms of processing, they claim to have already emulated the taste, function and nutritional value of traditional meat, giving them "a 10–12-year headstart on future competitors".

See the website for the complete article and photos.

5. From **Billie Swalla** (<u>bjswalla@uw.edu</u>): The Univ. of Washington Friday Harbor Labs is offering several 5 week summer courses at the Labs (FHL). Numerous scholarships are available. <u>https://fhl.uw.edu/courses/course-descriptions/course_quarter/summer-2024/</u>

There is also money for Japanese students, postdocs and faculty to visit FHL through the Japan/U.S. - E.S. Morse Scholar Exchange program. <u>https://depts.washington.edu/fhl/morse/</u>Please contact Dr. Billie J. Swalla for more details <u>bjswalla@uw.edu</u>

6. Japan's newest species is a tiny, panda-like sea creature. There have been numerous photos and videos posted online in the past few years. It has now been described as *Clavelina ossipandae* Hasegawa and Kasigawa 2024. See the New Publications at the end of this newsletter.



https://theowlhouse.fandom.com/f/p/44000000000136202

Work in Progress

1.From **Tito Lotufo** (<u>tmlotufo@gmail.com</u>): I'm sending a brief note about a new habitat formed by a giant cannonball ascidian that we found off Oman. The size of the animal and the thickness of the tunic is really impressive, and they form a sort of reef. It is a new genus that I'm currently describing and should be submitted soon.

Samimi-Namin, K., Lotufo, T., Hoeksema, B. W., Tweedt, S. M., Meyer, C. and Paulay, G. 2024. Unique aggregations of a large undescribed solitary tunicate in the Arabian Sea. Diversity **16**: 1-8.

2. From Serina Lee, Singapore (serina.lee@nus.edu.sg) and Noa Shenkar, Israel (shenkarn@tauex.tau.ac.il) : We are working on a manuscript together describing *Phallusia nigra* culture methods in closed and open aquarium systems. In March, Noa's student will be coming over to Singapore to run environmental tolerance experiments on local population of *P. nigra* juveniles. Serina writes: "I currently have at least 1500 babies sitting inside my aquaria. It is amazingly easy to grow them when I have all the set up ready. Seawater exchange is automated and so is feeding (on dosing pump) All I need to do is provide the microalgae every other day to top up the feed reservoir. And it is quite therapeutic watching the heart pumping blood throughout the transparent babies." Noa's student Amit will be presenting their results in the upcoming Intl. Tunicate meeting in Santa Cruz, Calif. in July.

3. From **Wilfried and Anne Bay-Nouailhat** (<u>mer.littoral@gmail.com</u>): a remarkable new publication by the Museum National d'Histoire Naturelle in Paris: Line, L. G., Corbari, L., Aurelle, D., Bay-Nouailhat, A., Bay-Nouailhat, W. et al. 2024. Bilan scientifique, the planet revisited in Corsica: Volet marin 2019-2021. [in French]. MNHN Paris, 189 pp. The Ascidiacea are on pp. 88-92, with many underwater photos, Figs. 33-42, Table 2. Downloadable: <u>https://mnhn.hal.science/mnhn-04552677</u>

4. From Stephano Tiozzo (stefano.tiozzo@imev-mer.fr):

A pre-print manuscript: **First chromosome-level genome assembly of the colonial tunicate** *Botryllus schlosseri* (<u>https://doi.org/10.1101/2024.05.29.594498</u>

Botryllus schlosseri (Tunicata) is a colonial chordate that has long been studied for its multiple developmental pathways and regenerative abilities and its genetically determined allorecognition system based on a polymorphic locus that controls chimerism and cell parasitism. We present the first chromosome-level genome assembly from an isogenic colony of *B. schlosseri* clade A1 using a mix of long and short reads scaffolded using Hi-C. This haploid assembly spans 533 Mb, of which 96% are found in 16 chromosome-scale scaffolds. With a BUSCO completeness of 91.2%, this complete and contiguous *B. schlosseri* genome assembly provides a valuable genomic resource for the scientific community and lays the foundation for future investigations into the molecular

mechanisms underlying coloniality, regeneration, histocompatibility, and the immune system in tunicates.

5. From Stephano Tiozzo (stefano.tiozzo@imev-mer.fr):

The abstract will be presented at the 12th ITM in July and is part of the Ph.D. thesis of Marie Lebel. Chasing the bud: single-cell RNAseq atlas and chromosome-level genome of *Botryllus* schlosseri.

The colonial tunicate *Botryllus schlosseri* propagates asexually through a developmental process known as palleal budding. This process involves the continuously rebuilding of the entire body from stereotyped regions of the peribranchial epithelia and the overlying epidermis. While the anatomy of palleal budding has been extensively described, the cells involved in bud initiation and their transcriptomic signatures remain elusive. To investigate the cellular and molecular origin of palleal budding, as well as its differentiation dynamics, we generated a single-cell RNA sequencing atlas covering the entire *B. schlosseri* budding cycle and we provided usable haploid and diploid genomes assembled at the chromosome level.

The initial clustering analyses of seven separate and integrated developmental stages led to the identification of respectively 37 and 40 cell clusters. By means of *in situ* hybridization and previously published studies, we were able to identify and annotate different types of blood cells, germline, body wall and cardiac muscles, epidermal and neural cells, gut epithelia and different endostyle cells. The obtained atlas enabled us to target specific clusters, which express known budding markers such as Nk4 and Vimentin. By applying finer sub-clustering and differential expression analyses we were able to identify relatively small populations of cells putatively at the origin of the budding process. Through RNA velocity and pseudotime analyses, we are now able to infer the cell trajectories and extrapolate information on the dynamics of these cells. The datasets obtained provide unprecedented insights into the mechanisms underlying agametic development in the model *B. schlosseri* and potentially offer useful tools for deciphering the fundamental principles governing budding across diverse tunicate species.

Meetings Abstracts

1. XXIV Meeting of the Italian Association of Developmental and Comparative Immunobiology (IADCI), February 14th - 16th, 2024. Accademia Nazionale di Scienze, Lettere e Arti di Modena, Italy.

a) Stress granule related-genes during the blastogenetic cycle of two colonial ascidians: *Botryllus schlosseri* compared to *Botryllus primigenus*. L Drago1, T Sasada2, T Sunanaga2, L Ballarin1 1Dept. of Biol., Univ. of Padua, Padua, Italy; 2Dept. of Chemistry and Biotechnol., Univ. of Kochi, Kochi, Japan.

Colonial ascidians are the only chordates able to reproduce both sexually and asexually. In the present study we used the Italian species *Botryllus schlosseri* and the Japanese *Botryllus primigenus* to investigate the possible role of tiar, ttp and g3bp in the periodical renewal of the colonies, defined as generation changes or takeovers. In this scenario, the above genes, which codify key components for the formation of stress granules, storing specific mRNAs, can play a pivotal role, allowing the regulation of processes such as stress responses, cell proliferation and stem cell development. We started by characterizing tiar, ttp and g3bp sequences in B. schlosseri and B. primigenus, then we analyzed gene expressions by in situ hybridization in hemolymph cells and colony tissues, and we proceeded with quantification of the gene expressions by quantitative real-time PCR, during the colonial blastogenetic cycle. Our results allowed us to assign to the studied genes a role in defense of the germline of the new colonial generations.

b) Underwater noise induces stress on behavioral and physiological performances of the colonial ascidian *Botryllus schlosseri*. G Sabbadin1, L Ballarin1, C Anselmi2, G Zambon3, V Zaffaroni Caorsi3, L Manni1. 1Dept. of Biol., Univ. of Padua, Padua, Italy; 2Stem Cell Biology Regenerative Medicine Institute, Stanford Univ., Stanford, UK; 3Dept. of Earth and Environmental Sciences, Univ. of Milano-Bicocca, Milan, Italy.

The ability of marine invertebrates to cope with anthropogenic underwater noise is mostly unknown although it is of concern in the European Marine Strategy Framework Directive. Therefore, its effects need to be investigated with the purpose of contributing to a sustainable blue growth. Among marine invertebrates, the ascidians are of interest possessing several mechanoreceptors potentially able to respond to water particle vibrations caused by noise. These include receptors on oral tentacles, the coronal cells, considered homologues of vertebrate hair cells of inner ear. The effect of noise produced by maritime traffic on the colonial ascidian Botryllus schlosseri were studied exposing animals to a different continuous noise (for 30 minutes; peak bands 63-125 Hz; at 160 dB; 152,8 dB; 145,5 dB). To evaluate the stress induced by the treatment, we used a behavioral test, the tentacle stimulation test, which specifically monitors coronal cells sensitivity. Their stimulation evokes the atrial siphon closure. Tests were performed in triplicates, before and after noise stimulation. Heartbeats were also counted to assess potential effects on animal physiology. Untreated colonies (genetically identical to treated colonies) were used as control and data were statistically analyzed. Results show that the noise induces stress on animals, decreasing both their sensitivity and heartbeat frequency. Future experiments will verify which is the threshold level that negatively affects animal performances. Moreover, noise effects on immune responses will be evaluated analyzing the ability of phagocytes to ingest target foreign cells and of cytotoxic cells to mount an inflammatory response upon the recognition of nonself.

2. Benthic Ecology meeting, Charleston, South Carolina, US, April 10-14, 2024.

a) Microbial distortion: Delayed preservation decreases microbial diversity and alters microbiome composition in a marine invertebrate. Hutchings TB, López-Legentil S, Stefaniak L, Nydam M, Erwin PM.

Field collections of marine invertebrates are often accompanied by delays in preservation, which may disproportionally impact microbiome studies. Here, we tested the effects of delayed preservation on microbiome diversity and composition in the ascidian *Trididemnum solidum*. Replicate samples collected from Belizean reefs were either (1) immediately preserved in ethanol ("control"), (2) held in ambient seawater for three hours before preservation ("SW"), or (3) held in ambient seawater with menthol (a common way to relax some invertebrates for taxonomic identification) for three hours before preservation ("SW+M"). A 3-hour delay in sample preservation (SW) significantly reduced microbiome richness compared to controls (p=0.039), while menthol treatment (SW+M) mitigated this diversity loss (p=0.157). Microbial composition shifted in both delayed preservation methods compared to controls (SW p=0.054, SW+M p=0.047), largely driven by a bloom of the facultatively anaerobic gammaproteobacteria Catenococcus that was on average 37x (SW) and 195x (SW+M) more abundant in delayed preservations than in the control. There were 170 microbial taxa (2.3% of total) that were significantly differential between control and SW, and 96 taxa (1.3%) between control and SW+M. Together, our results show that brief delays in preservation can impact microbiome characterization accuracy, with common pre-preservation practices like menthol usage counteracting these short-term effects.

b) Ascidian diversity and abundance in North Carolina shellfish farms. López-Legentil

S, Monteith G, Pilcher J, Hutchings B.

Ascidians are marine invertebrates that are particularly abundant on artificial structures, where they often overgrow native species and compete with other filter feeders. In aquaculture facilities, ascidian

overgrowth also causes significant economic losses. However, no study has described their diversity and abundance in North Carolina (NC) shellfish farms. Here, we surveyed five farms and four nearby marinas (within 48 km) to compare their ascidian communities. At each site, photo-quadrat surveys were conducted to determine ascidian diversity and abundance per m². Distribution patterns were assessed using presence-absence and relative abundance matrices with 'marina' and 'shellfish farm' as factors. Six species were observed in the farms: four native, and two cryptogenic (unknown origin). The most abundant species were the cryptogenic *Styela plicata* and *Distaplia bermudensis*, followed by the native species *Clavelina oblonga*. All species observed in shellfish farms were also observed in marinas and statistical analyses revealed no significant difference in diversity or abundance between them. Except for one, all native species observed in shellfish farms had worldwide distributions. Independently of their introduction status, species that survive in a wide range of habitats are more adaptable and better suited to establish long-term populations on artificial substrates.

3. SCAR Open Science Conference 2024, "Antarctic Science: Crossroads for a New Hope," August 19-23, 2024, Pucón, Chile. Members of the Instituto de Diversidad y Ecología Animal (IDEA; CONICET and National University of Córdoba, Argentina) will be presenting the following abstracts:

a) Delving the knowledge on biodiversity and biogeographic connections of the subantarctic MPA Namuncurá/Burdwood Bank: the case of ascidians, diverse and frequent animals of macrobenthic communities. Anabela Taverna; M. Carla de Aranzamendi; Luciana Plum; Tamara Maggioni; M. Soledad Acosta; Camila Neder; Marcos Tatián.

The plateau of the Namuncurá/Burdwood Bank Marine Protected Area (NMPA/BB) -located in the Scotia Arc region, southeastern of Tierra del Fuego, Argentina- is isolated by deep waters, suggesting a particular importance in terms of endemic species of benthic organisms. With predominance of rocky bottoms and coarse biogenic sand, the NMPA/BB sustains a high biodiversity that requires protection, with the presence of vulnerable structure-forming species such as ascidians (Chordata, Tunicata), important components of macrobenthic communities. The richness of ascidians may be underestimated due to incorrect species identification or a poor understanding of their distribution. In relation to the NMPA/BB, although the diversity of ascidians has been little studied, it is relatively high compared to other areas of the Scotia Arc, with 25 species previously reported. The aim of this study is to deepen the knowledge of ascidian diversity, based on morphological and molecular analyses, in the NMPA/BB and its relationships with adjacent areas: Tierra del Fuego (including Isla de los Estados) and the slopes of the Namuncurá/Burdwood Bank II MPA (NMPA/BB-II). The capture frequency of ascidians at 27 stations and 52 trawls (BO "Puerto Deseado" Campaign, 2016) was high (92%). So far, we have identified 42 species and obtained mitochondrial COI gene sequences (29 species) and nuclear 18S gene sequences (13 species). The ascidian affinities between different zones of the NMPA/BB (Core, Buffer, and Transition), are high (Kulczynski similarity index, 50-66%). Considering the three zones together, the similarity with stations of Tierra del Fuego reaches 50%. The similarity of these three zones is lower with respect to the slope area (NMPA/BB-II) south of the bank (16-19%), suggesting differences with depth. This information will contribute to the establishment of conservation values and the ongoing rezoning necessary for this important MPA management strategies.

b) The Antarctic ascidian *Cnemidocarpa verrucosa* and the ecological significance as a substrate for epibiotic communities in a context of environmental changes. Clara Rimondino; Gastón Alurralde; Emilia Calcagno; Tamara Maggioni; M. Carla de Aranzamendi; Camila Neder; Marcos Tatián.

In coastal marine environments, the availability of free substrate significantly influences the distribution and abundance of sessile benthic organisms. The presence of organisms acting as

biological substrates can facilitate the establishment of other benthic organisms, in a phenomenon known as epibiosis. The epibiosis intensity (i.e. the richness and abundance of epibionts) varies across latitudinal and depth gradients, and basibiont characteristics. Recent increases in sedimentation from melting coastal glaciers threaten local biodiversity in the West Antarctic Peninsula. Ascidians stand despite changing environmental conditions, providing a stable surface for a variety of epibionts to attach and grow. Thereby, ascidians are expected to enrich biodiversity and create habitat heterogeneity. We analysed macroepibiotic assemblages occurring on the abundant ascidian Cnemidocarpaverrucosa from two areas within Potter Cove (South Shetland Islands, Antarctica): one characterized by soft substrate and high sediments level ("brown water") and, the other one, characterized by hard substrate and low sediments level ("blue water"). The hypothesis was that epibiosis intensity varies according to environmental conditions such as substrate type and sedimentation level, as well as, individual characteristics of the occupied body area on the basibiont. We observed diverse epibionts, including algae, sponges, bryozoans, and even other ascidians. In addition, a higher epibiosis intensity was found on ascidians living on soft substrate and brown water with further effects associated to properties and characteristics of the body zones parts within the same basibiont. In a context of increasing temperature and consequent higher sediment discharge in coastal marine zones, the composition of benthic communities is expected to be further affected. In this regard, ascidians emerge as key organisms in the structure of these shallow Antarctic communities.

Thesis Abstracts

1. An omics world: colonisation and adaptation processes of Styela plicata. Carles Galià-Camps PhD dissertation, University of Barcelona (Catalonia, Spain). Supervisors Marta Pascual, Carles Carreras, and Xavier Turon.

In the last decades biological invasions have emerged as a global concern, as they impact local biodiversity by disrupting ecosystem functioning and ultimately affect human health, economy and wellness. In a climate change scenario, catalyzed by globalization, the issue of invasive species is expected to become magnified in the near future. However, little is known about the intrinsic mechanisms allowing invasive species to overcome new conditions and become a major threat worldwide. This PhD thesis aims to study the colonization and adaptive processes of the invasive tunicate S. plicata worldwide using '-omic' approaches, including population genomics and microbiome. By combining multiple sequencing techniques such as PacBio Long Reads, Illumina Whole Genome Sequencing (WGS), Illumina RNAseq and Illumina amplicon sequencing, we have adopted multiple approaches to the study of this species. First, by using 80 publicly available genomes, we evaluated general output trends when using the 2b-RADseg technique depending on the genomic architecture of target species, demonstrating that overall the technique does not generate biases although it slightly enriches exonic regions. Furthermore, by empirically testing the effect of 2b-RADseq base-selective adaptors on genotyping using four Styelaplicata individuals, we paved the way for cost-effective population studies on this species. Next, we built and annotated a "de-novo" reference genome for Styelaplicata, and combined it with Illumina WGS of 24 individuals worldwide. The resulting pangenome revealed the presence of 4 potentially adaptive inversions, the first evidence of population structure, SNPs related to local adaptation, and mito-nuclear interactions. These methodological improvements and the generation of a reference genome assembly allowed us to analyze 87 individuals from 18 localities worldwide using 2b-RADseq to further reveal its population genomic structure, finding clear signals of local adaptation and recent demographic bottlenecks related to invasive events. Finally, using Illumina sequencing of the V4 region of the 16S gene of several tissues of juvenile and adult individuals and water samples from three harbors, in addition to adult samples on two of these harbors over two years, we shed light on the dynamics of the microbiome hosted by Styelaplicata. We revealed that Methylocaeanibacter sp. ASV0 is the most

important bacteria in the tunic, whereas Gammaproteobacteria ASV1 and the unidentified bacteria ASV2 are so in the gill for holobiont survival. Moreover, we found evidence of abundance shifts through adulthood for these bacteria, suggesting active enrichment by *S. plicata* towards its symbiotic bacterial community. Finally, we revealed potential adaptive bacteria responding to seasonality and trace elements such as *CandidatusHepatoplasma* sp. ASV11 and *Endozoicomonas* sp. ASV50, among others. This multilevel approach provides a solid basis to properly understand the mechanisms of *Styelaplicata* and other invasive marine species to colonize and quickly adapt to new habitats.

NEW PUBLICATIONS

- Alvarez, S., Gestoso, I., Ramalhosa, P. and Canning-Clode, J. 2024. Exploring visual methods for monitoring marine non-indigenous species colonizing artificial structures in the Madeira archipelago (NE Atlantic). Regional Studies in Mar. Sci. 69: epub.
- Al-Yaqout, A., Nithyanandan, M., Issaris, Y. et al. 2024. In-situ observations of swarming pelagic tunicate *Pegea confoederata* (Forskål, 1775) (Tunicata: Thaliacea) in coral reef habitats of Kuwait. Kuwait J. Sci. **51**: epub.
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- Ballarin, L. 2023. Ascidian cytotoxic cells: from zero to hero. Invert. Survival J. 20: 79-88.
- Batista, P. J., Nuzzo, G., Gallo, C. et al. 2024. Chemical and pharmacological prospection of the ascidian *Cystodytes dellechiajei*. Mar. Drugs **22**: epub.
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- Braconcini, M., Gorrasi, S., Fenice, M., Barghini, P. and Pasqualetti, M. 2024. *Rambellisea gigliensis* and *Rambellisea halocynthiae*, gen. et spp. nov. (Lulworthiaceae) from the marine tunicate *Halocynthia papillosa*. J. Fungi **10**: epub.
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- Carlton, J. T. and Schwindt, E. 2024. The assessment of marine bioinvasion diversity and history. Biol. Invasions **26**: 237–298.
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- Chen, Y., Ni, P., Fu, R., Murphy, K. J., Wyeth, R. C., Bishop, C. D., Huang, X., Li, S. and Zhan, A. 2024. (Epi)genomic adaptation driven by fine geographical scale environmental heterogeneity after recent biological invasions. Ecol. Appl. 34: epub.
- Cheng, J. W., Li, S. G., Li, X. and Zhan, A. B. 2024. Influence of calcium concentration on larval adhesion in a highly invasive fouling ascidian: From morphological changes to molecular mechanisms. Mar. Pollution Bull. **200**: epub.

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- Damian-Serrano, A. 2024. Yellow tails in *lasis cylindrica* (Salpida: Salpidae) chains suggest zooidtype subspecialization in salp colonies. Ecology **105**: epub.
- Daric, V., Lanoizelet, M., Mayeur, H., Leblond, C. and Darras, S. 2024. Genomic resources and annotations for a colonial ascidian, the light-bulb sea squirt *Clavelina lepadiformis*. Genome Biol. & Evol. **16**: 1-8.
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- Di Gregorio, A., Locascio, A., Ristoratore, F. and Spagnuolo, A. 2023. Women researchers in tunicate biology at the Stazione Zoologica Anton Dohrn in Napoli. Genesis **epub**: 1-9.
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