















Amphioxus (*Branchiostoma lanceolatum*) in the North Adriatic Sea: ecological observations and spawning behavior

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Abstract

The European amphioxus (*Branchiostoma lanceolatum*) is a member of the chordate subphylum Cephalochordata, and, as such, a key model organism for providing insights into the origin and evolution of vertebrates. Despite its significance and global distribution, detailed characterizations of natural populations of cephalochordates are still very limited. This study investigates the abundance, habitat, and spawning behavior of amphioxus in the North Adriatic Sea. Across 32 sampled sites, adult amphioxus were consistently present, reaching densities exceeding 300 individuals m⁻². DNA barcoding confirmed the species as *B. lanceolatum*, and environmental analyses revealed an amphioxus preference for slightly gravelly sand with low silt content and a correlation between amphioxus density and the presence of specific macroinvertebrate taxa. Remarkably, the amphioxus population was breeding in early spring and possibly late fall, in contrast to the typical late spring/early summer spawning season described for other populations of European amphioxus. Amphioxus adults kept in captivity maintained the spawning seasonality of their place of origin, suggesting the possibility of extending the overall spawning season of European amphioxus in laboratory settings by exploiting populations from diverse geographic origins. This study thus expands our understanding of *B. lanceolatum* ecology and reproduction in the Mediterranean Sea, emphasizing the role of the North Adriatic Sea as a substantial reservoir.

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INTRODUCTION

The European amphioxus (*Branchiostoma lanceolatum* Pallas, 1774) is a chordate species of the subphylum Cephalochordata that is commonly found in shallow waters of temperate and tropical seas (Poss & Boschung 1996). Members of the Cephalochordata are generally referred to as amphioxus or lancelets, which is chiefly due to the shape of their bodies, which are elongated, laterally flattened, and pointy at both their anterior and posterior ends. While the larva is planktonic, adult cephalochordates live burrowed in the sediment, typically in the sublittoral zone at depths of up to 30 to 50 m, with some species exceptionally found at greater depths (Poss & Boschung 1996; Nishikawa 2004). They prefer soft bottoms composed of sand, coarse shelly debris, or gravel and lie buried within this substrate, often with their mouths protruding above the surface, allowing them to ingurgitate water, which is subsequently filtered to trap suspended food particles.

Cephalochordates, with a simple but vertebrate-like body plan, are considered prototypical chordates, bearing a striking resemblance to early chordate fossils like *Pikaia* and *Haikouella* (Shu *et al.* 2003; Morris & Caron 2012). As the earliest-branching chordates and the sister group of the tunicate + vertebrate clade (Delsuc *et al.* 2006; Putnam *et al.* 2008), they play a pivotal role in the study of vertebrate origins (Holland & Holland 2021). Several resources and protocols have been developed to harness amphioxus as a model system, for example, to obtain (Fuentes *et al.* 2007) and pharmacologically manipulate (Bertrand *et al.* 2017; Bozzo *et al.* 2020) large quantities of embryos. Additionally, high-resolution immunofluorescence and *in situ* hybridization analyses (Bozzo *et al.* 2017b, 2023) as well as microinjection techniques for gene manipulation (Hirsinger *et al.* 2015) have been established. Importantly, the genome of three amphioxus species has been fully sequenced (Putnam *et al.* 2008; Marlétaz *et al.* 2018; Bi *et al.* 2020), and the generation of mutant lines has been proven feasible, although technically challenging (Su *et al.* 2020). Amphioxus has, therefore, become an important model organism in evolutionary developmental biology (evo-devo), particularly for the comparative study of neural development (Bozzo *et al.* 2017a, 2021a, b), developmental signaling pathways (Bertrand *et al.* 2017; Bozzo

et al. 2023), and regenerative processes (Somorjai 2017; Zullo *et al.* 2020; Bozzo *et al.* 2022).

The cephalochordates comprise three genera: *Branchiostoma*, *Epigonichthys*, and *Asymmetron* (Poss & Boschung 1996; Nishikawa 2004), the latter two being characterized by unpaired gonads. The current classification is based on meristic criteria and distinguishes 29 species, the majority belonging to the genus *Branchiostoma* (Poss & Boschung 1996). However, molecular taxonomy analyses have revealed the presence of cryptic species (Zhang *et al.* 2006). The only cephalochordate so far reported in the Mediterranean Sea is *B. lanceolatum* (Tortonese 1963; Poss & Boschung 1996; Caccavale *et al.* 2021), which is also found in the northeastern Atlantic Ocean and in the western Indian Ocean (Poss & Boschung 1996). The presence of *B. lanceolatum* is well documented along the northern shores of the Mediterranean Sea, including several locations along the Italian coastline in the Ligurian, Tyrrhenian, and Adriatic Seas (reviewed in De Biasi & Boni 2002). Although the species can be very abundant locally in some regions of the Mediterranean Sea (Fuentes *et al.* 2004), the densities reported for amphioxus populations along the coast are typically very low, allowing the sampling of only very limited numbers of specimens.

Despite its importance as a model organism in evo-devo, only a few natural populations of amphioxus have been the object of a detailed characterization. Prompted by a recent report of high densities of *B. lanceolatum* (Renzi *et al.* 2018), we investigated the occurrence of this species along the Italian coastline of the North Adriatic Sea. We found that *B. lanceolatum* adults were indeed abundant in the area, with particularly high densities observed off the mouth of the river Tagliamento. To provide an initial characterization of this amphioxus population, we carried out analyses of both the habitat and the seasonal spawning behavior of *B. lanceolatum* adults.

MATERIALS AND METHODS

Sampling area and amphioxus collection

Adult amphioxus were collected on August 7–9, 2018, June 27 and 28, 2019, January 27–30, 2020, and March 28–30, 2023 about 5.6 nautical miles (10.5 km) off the mouth of river Tagliamento (Italy) in the North Adriatic

Sea (GPS coordinates are listed in Table S1, Supporting Information).

The collection was performed using a Van Veen dredge (0.1 m², maximum volume 17 L) from a research vessel of the CNR-ISMAR of Venice (Italy). For each collection site, adult animals were sieved out from the sediment using a 0.5-mm mesh, counted, and photographed for subsequent measurements in Fiji (Schindelin *et al.* 2012). The population density was calculated as individuals m⁻². After collection, animals were transported to the Acquario di Genova (Genoa, Italy) for husbandry. Along with amphioxus adults, sediment was sampled at specific collection sites.

Recording of water parameters

In June 2019, January 2020, and March 2023, measurements of temperature, depth, and salinity at the sea floor of the collection sites were recorded using an Idronaut 304 multiparameter probe (Idronaut).

Granulometric analyses

Particle size analyses were carried out in the laboratory according to the standard techniques defined by the Italian Ministry of Environment and Land Protection (Cicero & Di Girolamo 2001). Sediment samples were aliquoted to obtain lower-volume representatives for each collection site. Each aliquot was pretreated with a solution of 30% hydrogen peroxide and distilled water (2:8) for 48 h at room temperature to facilitate granule separation. Following this step, the coarser fraction was isolated by wet sieving with distilled water using a 63 μm mesh. The two fractions obtained for each aliquot were dried at 60°C and weighed. The arenitic fraction (>63 μm) (sand and gravel) was dry sieved by a vibrating sieve with stacks of sieves from -1 to 4 phi with a 0.5-phi interval. The obtained individual fractions were then weighed to determine their relative weight. The pelitic fraction (<63 μm) (silt and clay) was subjected to an initial phase of mechanical disaggregation using mortar and pestle. Subsequently, an aliquot from each sample was diluted in 100 mL of distilled water and treated with ultrasound before analysis by a LISST-100X laser granulometer (Sequoia Scientific), which discretized the particle size spectrum into 32 classes between 1.90 and 381 μm . The obtained concentration values were processed and integrated with the data obtained from the sieves. The primary statistical parameters of the distribution and the relative abundances (%) of gravel, sand, and pelitic fraction, classified accord-

ing to Udden and Wentworth classifications (Udden 1914; Wentworth 1922), were determined using the GRADIS-TAT software (Blott & Pye 2001).

Macrobenthos sampling and classification

In January 2020, a transect in the study area was chosen to collect and evaluate the macroinvertebrate community. Sediments were sieved with a 0.5-mm mesh and the fraction retained was fixed in 70% alcohol and subsequently sorted at the CNR-ISMAR of Venice (Italy) under a stereomicroscope (Olympus SZ40, magnification range: 0.65 to 4X). To obtain a species list, macroinvertebrates were identified to the species level (wherever possible) using a compound microscope (Zeiss Axioskop 40, magnification 10X, 20X, 40X, and 100X, ocular GF-Pw 16X) to observe the fine diagnostic characters. We referred to the checklist of marine fauna for taxonomical organization and to the World Register of Marine Species for a cross-check of accepted family, genus, and species names following the latest revisions (Relini 2008; Ah Yong *et al.* 2023). Data were elaborated with spreadsheets and using R (R Core Team 2021).

DNA extraction, amplification, and sequencing

A selection of amphioxus adults was fixed in 100% ethanol on-site and stored at -20°C. In the laboratory, the posterior half of the body was dissected, minced with a scalpel blade, and digested in SNET buffer (20 mM TRIS, pH 8.2; 5 mM EDTA; 400 mM NaCl; 1% wt/v SDS) containing proteinase K (400 $\mu\text{g mL}^{-1}$), overnight at room temperature. Digested tissues were ground with a disposable pestle, and the DNA (genomic and mitochondrial) was subsequently extracted with a standard phenol/chloroform protocol followed by ethanol precipitation (Green & Sambrook 2012). The extracted DNA was resuspended in TE buffer (10 mM Tris HCl, pH 8.2; 2 mM EDTA).

Fragments of the *cytochrome c oxidase subunit I* (COXI), *12S ribosomal ribonucleic acid* (12S rRNA), and *16S ribosomal ribonucleic acid* (16S rRNA) genes were amplified from the mitochondrial DNA by PCR using primers previously published for *B. lanceolatum* (Caccavale *et al.* 2021). The PCR reaction was performed in a final volume of 25 μL containing 12.5 μL Hot Start Master Mix (Biotech rabbit), 50 ng template DNA, and 0.4 μM of each primer in a Mastercycle thermal cycler (Eppendorf) for 35 cycles. The following PCR profile was used: preheating at 94°C for 2 min, denaturing at

94°C for 30 s, annealing at 57°C for 45 s, and extension at 72°C for 1 min. The PCR products were purified from unannealed primers and free nucleotides using the Illustra ExoProStar Kit (Cytiva) according to the manufacturer's indications and then sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequence data have been submitted to the GenBank database under accession numbers: PP049451 to PP049454 (*COXI*), PP033674 to PP033677 (*12S rRNA*), and PP025941 to PP025944 (*16S rRNA*) (see also Table S2, Supporting Information).

Identification of nucleotide sequences and multiple sequence alignment

Partial sequences of *COXI*, *12S rRNA*, and *16S rRNA* from different amphioxus species were identified in the NCBI database (<http://www.ncbi.nlm.nih.gov>) (Table S2, Supporting Information). Sequences were retrieved from complete mitochondrial genomes of the genus *Branchiostoma* (Table S3, Supporting Information) as well as from that of *Epigonichthys maldivensis* (NC_006 465), which was used as the outgroup. To construct the dataset for phylogenetic analyses, the sequences of *COXI*, *12S rRNA*, and *16S rRNA* were aligned separately using MegAlign in DNA star (Lasergene 7). The alignments were subsequently concatenated, creating a single locus for each sample (Table S4, Supporting Information).

Phylogenetic analyses

Phylogenetic trees were inferred from the aligned, concatenated sequences using both the neighbor joining (NJ) and maximum likelihood (ML) methods in MEGA11 (Tamura *et al.* 2021), with a total of 1605 positions in the final dataset. For the NJ tree, evolutionary distances were computed using the Tamura–Nei model method and were expressed as numbers of base substitutions per site. The ML tree was also calculated using the Tamura–Nei model, and the rate variation among sites was modeled with a gamma distribution (five categories) with the shape parameter 0.5997 in an analysis involving 67 nucleotide sequences. The rate variation model allowed for some sites to be evolutionary invariable ($I = 30.03\%$). Bootstrap branch support confidence values were calculated in 500 replicates for both the NJ and ML analyses. The final phylogenetic tree files were visualized with FigTree v1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Maintenance and spawning of amphioxus in captivity

The collected amphioxus adults were transported to the Acquario di Genova (Genoa, Italy) and maintained according to previously published protocols (Carvalho *et al.* 2017). Briefly, the animals were housed in 30- to 40-L tanks with about 2 cm of sediment on the bottom. The tanks were connected to a closed water circulation system running on locally sourced seawater (pH 8.0 to 8.1, salinity 35.5 to 36.5 ppt). Water quality was ensured by mechanical and biological filtration and UV sterilization as well as weekly changes of 20% of the total seawater volume of the system. The temperature was set to 16°C in fall/winter and to 21°C in spring/summer, with gradual changes between the two seasons. Animals were subjected to a 12:12 light-dark cycle and were fed daily with 500 mL of live phytoplankton (*Tetraselmis suecica*, *Isochrysis galbana*, *Tisochrysis lutea*, and *Rhodomonas salina*) per tank, by isolating the tank from the closed water circulation system for a few hours. Spawning was induced using a mild heat shock protocol, as previously described (Fuentes *et al.* 2004, 2007; Bozzo *et al.* 2020).

Amphioxus measurements and statistical analyses

After collection, amphioxus adults were placed in a small plastic tank and photographed. From these photos, the total length of each animal was measured from the rostrum to the tip of the tail in Fiji (Schindelin *et al.* 2012), using the size of the tank for calibration. Animals kept at the Acquario di Genova (Genoa, Italy) were periodically taken out of the sediment, counted, and photographed for length measurements, as described above, to monitor their growth and survival in captivity over an extended period. All statistical analyses were performed using GraphPad Prism 9 (Dotmatics).

RESULTS

In the study area of the North Adriatic Sea, we sampled 32 sites forming a grid covering a total surface of 675 000 m² (Fig. 1).

Density of the amphioxus population and DNA barcoding

During all four campaigns, from 2018 to 2023, amphioxus adults were consistently found in the sampled

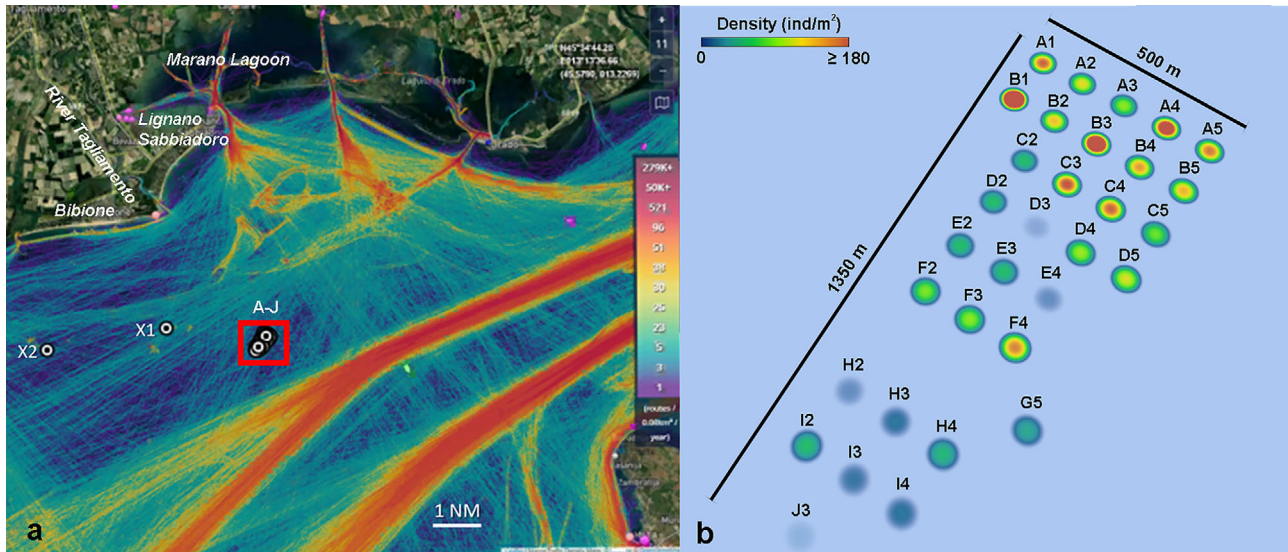


Figure 1 (a) Satellite image showing the position of the sampling grid of *Branchiostoma lanceolatum* adults in the North Adriatic Sea (A–J) and two additional sample sites (X1 and X2) further away. The density of nautical vessel traffic for the years 2021 and 2022 is also shown (source: marinetransport.org). NM, nautical mile. (b) Heatmap of the sampling grid with the color-coded density of recorded amphioxus adults. ind, individuals. For further information see Table S1, Supporting Information.

area, with an estimated average density of about 80 individuals m^{-2} (Fig. 1b; Table S1, Supporting Information). Throughout the study area, however, we observed marked differences in the density of the amphioxus population, ranging from 5 individuals m^{-2} (D3, January 2020) to over 300 individuals m^{-2} (B1, January 2020) (Table S1, Supporting Information). Of note, the density measured for the same sample site during different campaigns was not necessarily constant (compare, e.g. site B1 in January 2020 and March 2023) (Table S1, Supporting Information). In addition, amphioxus adults were also found at two sites outside the sampling grid, with a density of 10 individuals m^{-2} (site X1, January 2020) and 40 individuals m^{-2} (site X2, March 2023) (Table S1, Supporting Information), but not in locations where the seabed was markedly different from that of the study area (composed predominantly of silt or characterized by anoxic conditions, for instance). The animals sampled in 2019 were 50% males and 50% females, while those sampled in 2020 were 51.5% males and 48.5% females. This sex ratio of almost 1:1 is consistent with previous observations in wild populations of both *B. floridae* and *B. belcheri* (Stokes & Holland 1996; Henmi & Yamaguchi 2003).

We unambiguously confirmed by DNA barcoding that the amphioxus species present in the study area was the European amphioxus, *B. lanceolatum* (Fig. S1, Supporting Information).

Environmental characteristics

The study area presented an even seabed with a depth of about 14 m (ranging between 13.3 and 16.5 m). The environmental parameters recorded were constant throughout the study area: the average water temperature at the bottom was 17.8°C (June 2019), 11.5°C (January 2020), and 11.6°C (March 2023), and the salinity ranged between 37.43 and 37.97 ppt.

The sea floor within the study area consisted of slightly gravelly sand, characterized by a predominance of sand (92.9% to 97.7%) with small proportions of very fine gravel (1.4% to 3.74%) and silt (0.8% to 1.4%) (Fig. 2) and was rich in biotic material (crushed shells and coralline algae). The most notable differences were recorded at site D3, where the proportion of gravel was significantly higher (5.4%, mainly shell debris) at the expense of the sand fraction (92.9%). Consequently, the sediment texture at site D3 was classified as gravelly sand.

Associated macrobenthic community

From a first macroscopic observation of the benthic community, the presence of *Callista chione* and *Ophiotrix* sp. was observed, which were particularly recurrent as components of the megabenthic fauna (by convention > 1 cm). We carried out a comprehensive sampling and classification of the entire macrobenthic community

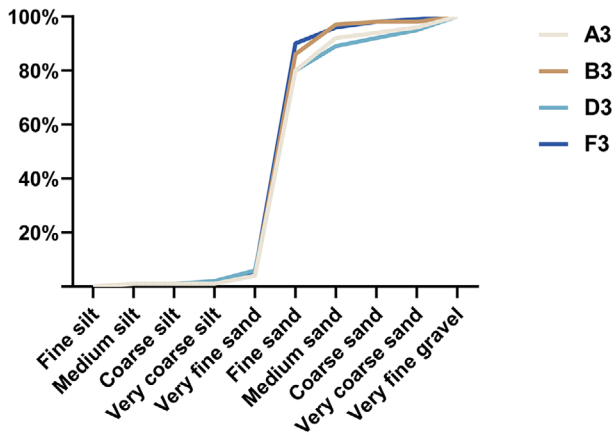


Figure 2 Cumulative percentages of grain size fractions sampled along the A3–F3 transect (see Fig. 1).

(> 1 mm) at specific sampling sites (A3, B3, D3, and F3), reaching the species level whenever possible (see Table S5, Supporting Information, for the complete list). A total of 2130 individuals were collected, belonging to 90 families ascribed to eight phyla (Annelida: 27 families; Arthropoda: 16 families; Bryozoa: 6 families; Chordata: 2 families; Cnidaria: 2 families; Echinodermata: 7 families; Mollusca: 29 families; Sipuncula: 1 family), for a total of 137 species.

Mollusca and Annelida were the most abundant phyla, especially at site D3, where these groups were the most highly represented in terms of both number of species and quantity of individuals (Fig. 3). Considering the limited number of sampling sites, we searched for relationships between the density of amphioxus adults and the composition of the macrobenthic community. A non-metric multidimensional scaling analysis carried out on a matrix of the abundances of dominant species (defined as species that make up at least 80% of the total abundance) revealed that the highest densities of *Branchiostoma* were associated with taxa, such as *Callista*, *Lentidium*, *Dialychone*, *Aponuphis*, and *Spio* (Fig. 4). The sampling sites A3, B3, and F3, with the highest densities of *Branchiostoma*, were more closely associated with each other than to site D3, which was characterized by the presence of the mollusks *Loripes* and *Abra* and of the polychaetes *Sigambra* and *Aricidea*. These results indicate that *Branchiostoma*, and the mentioned genera of mollusks and polychaetes, reach high density at the same sites.

Size-frequency distribution in the population

The total length of wild amphioxus adults collected from the study area in the North Adriatic Sea was

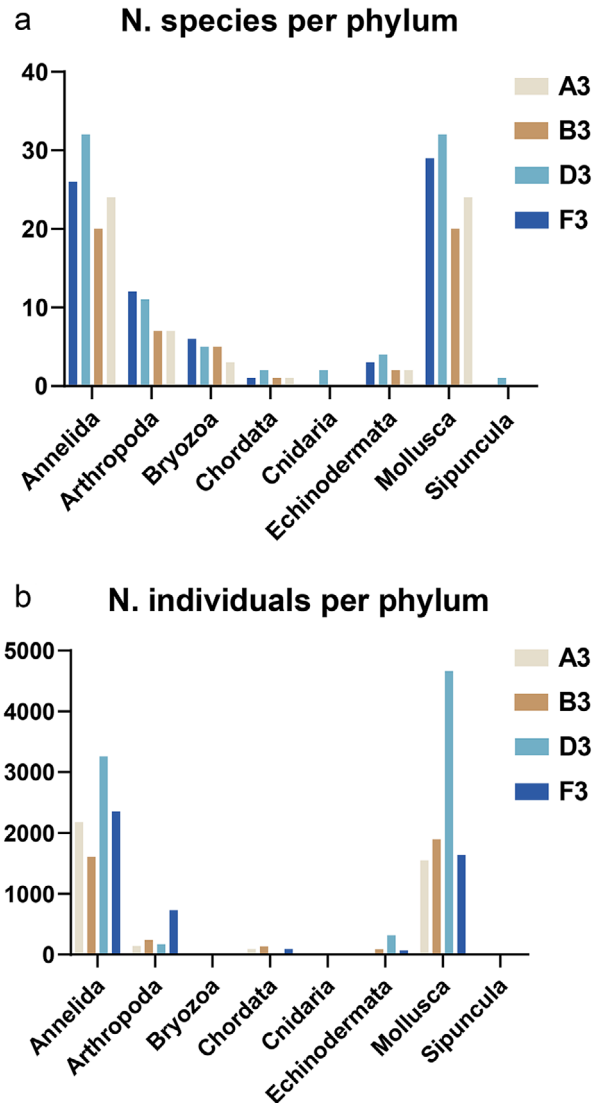


Figure 3 Distribution of the abundance of species (a) and individuals (b) of the different phyla per m² along the transect A3–F3 (see Fig. 1). N., number.

measured twice over a period of about 3 years (between January 2020 and March 2023) (Table 1). In January 2020, we found animals ranging from a minimum of 19.01 mm to a maximum of 39.60 mm, with a mean of 31.29 mm that almost coincided with the median value of 31.33 mm. In March 2023, a wider range of lengths was observed, from 13.21 to 41.59 mm, with a mean of 28.62 mm, which was significantly smaller than the mean of the total length of the population measured in January 2020 (*t*-test, $P < 0.0001$). Moreover, the mean of 28.62 mm more markedly deviated from the median value of 29.50 mm.

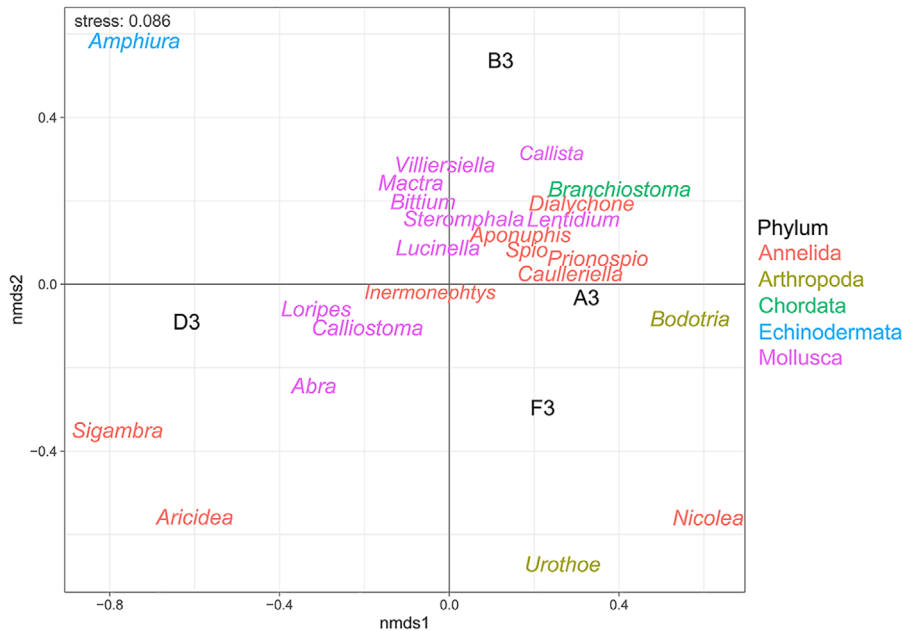


Figure 4 Non-metric multidimensional scaling (nMDS, Bray–Curtis distance metric) analysis carried out on a matrix of the abundances of dominant species. Species are shown with the names of their respective genus for graphic representation purposes. Colors indicate animal phyla.

Table 1 Statistical analysis of the sizes of *Branchiostoma lanceolatum* adults from the North Adriatic Sea

	January 2020	March 2023
Sample size	213	135
Minimum length	19.01 mm	13.21 mm
Maximum length	39.60 mm	41.59 mm
Range	20.59 mm	28.38 mm
Mean	31.29 mm	28.62 mm
Median	31.33 mm	29.50 mm
Variance	18.32	39.60
Skewness	−0.3233	−0.07361
Kurtosis	−0.3632	−0.9081

The January 2020 measures were consistent with a Gaussian distribution according to the Anderson–Darling, D’Agostino–Pearson, and Kolmogorov–Smirnov tests ($P > 0.05$). In contrast, the March 2023 dataset significantly failed the three normality tests ($P = 0.0002$, $P = 0.0007$, and $P = 0.0005$, respectively). Consequently, we proceeded to analyze the size-frequency distribution using a bin size of 0.5 mm. As expected, a Gaussian curve could be fitted to the January 2020 dataset (Fig. 5a), while

the March 2023 dataset was consistent with a bimodal distribution (Fig. 5b).

Growth in captivity and spawning seasonality

A subset of amphioxus adults, consisting of 178 individuals sampled in January 2020, were maintained in captivity for over 30 months and were periodically monitored to assess their mortality, growth rate, and spawning seasonality. The number of animals remained constant for about 2 years (Fig. 6a), before starting to decrease due to mortality following a sigmoidal curve. The last surviving animals ($n = 17$) were fixed for experimental purposes in September 2022, after spending 32 months in captivity. At the time of fixation, they appeared healthy and gravid.

To monitor growth, the total length of all individuals was measured periodically (Table S6, Supporting Information). The average length of the population significantly increased over time (ANOVA, $P < 0.0001$), with a growth rate of 0.56 mm per month in the first year and of 0.16 mm per month in the second year. Therefore, a growth curve was plotted using the average length of the population (Fig. 6b). A logistic model fitted the data better than a linear model ($P < 0.0001$), and a sigmoidal growth

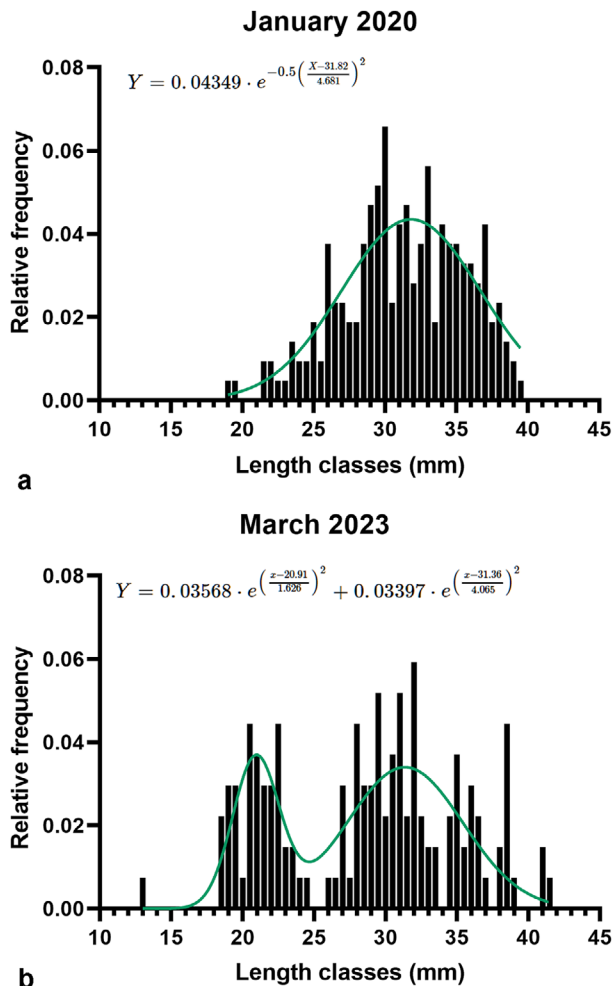


Figure 5 Size-frequency distribution of *Branchiostoma lanceolatum* adults sampled in January 2020 and March 2023. (a) The population sampled in January 2020 showed a normal distribution best described by the Gaussian equation provided in the panel. (b) The population in March 2023 showed a bimodal distribution best described by the sum of two Gaussian curves provided in the panel.

curve could be extrapolated, indicating a typical bundled growth. The equation of the best-fitting curve was:

$$Y = 39.47 \cdot \frac{31.41}{8.06 \cdot e^{-2.050 \cdot x + 31.41}}$$

In the field, most amphioxus adults had full gonads in late January (2020) and March (2023), while they were completely empty in late June (2019) and early August (2018). To monitor sexual maturation in captivity throughout the year, we classified the animals into three classes based on the size of the gonads, which are visible

through the translucent body wall (Fig. 7a): empty animals (with no visible gonads), half-full animals (with visible gonads but unlikely to be ready to spawn), and full animals (with well-developed gonads and ready to spawn). In captivity, the animals started to develop gonads in August, and most animals were ripe in December (Fig. 7b,c). Consistently, most animals had empty gonads between April and September, with the latter being the month with the highest fraction of animals presenting half-full gonads (Fig. 7b). These observations suggest that the development of gonads begins at the end of the summer and that spawning takes place in spring and possibly fall. Consistent with this notion, we observed spontaneous spawning of amphioxus adults in captivity in March, April, and November, and successfully induced spawning of full animals by applying a mild heat shock (Fuentes *et al.* 2004, 2007; Bozzo *et al.* 2020) in March, April, September, and November.

DISCUSSION

In this study, we characterized a population of amphioxus in the North Adriatic Sea. DNA barcoding analyses based on three mitochondrial genes identified the species as *B. lanceolatum*, which, so far, is the only amphioxus species documented in the Mediterranean Sea (Tortonese 1963; Poss & Boschung 1996; Caccavale *et al.* 2021).

The North Adriatic Sea is a shallow basin, with an average depth of about 40 m (Bellafiore & Umgiesser 2010; Janeković *et al.* 2014; Vrdoljak *et al.* 2021), which makes it particularly hospitable for amphioxus (Poss & Boschung 1996). Previous work reported that the properties of the seabed constitute a critical factor for the distribution of amphioxus (Webb 1975). It has further been shown that amphioxus adults have a strong preference for medium to coarse sediments containing small debris (such as shattered shells) that facilitates the flow of water (Poss & Boschung 1996), with the key factors being size and smoothness of sediment particles rather than their chemical composition (Rossi & Orel 1968). Amphioxus adults are thus found in a variety of different sediments that are not strictly classifiable as “amphioxus sands” (De Biasi & Boni 2002).

Amphioxus adults generally avoid sediments with a silt fraction exceeding 1.5% (Webb & Hill 1958), although a few specimens of *B. lanceolatum* were found in fine sands composed of over 20% silt/clay off the city of Piombino in the North Tyrrhenian Sea (Italy) (De Biasi & Boni 2002). However, the seabed in the sampled location off Piombino was also rich in calcareous biogenic detritus (De Biasi &

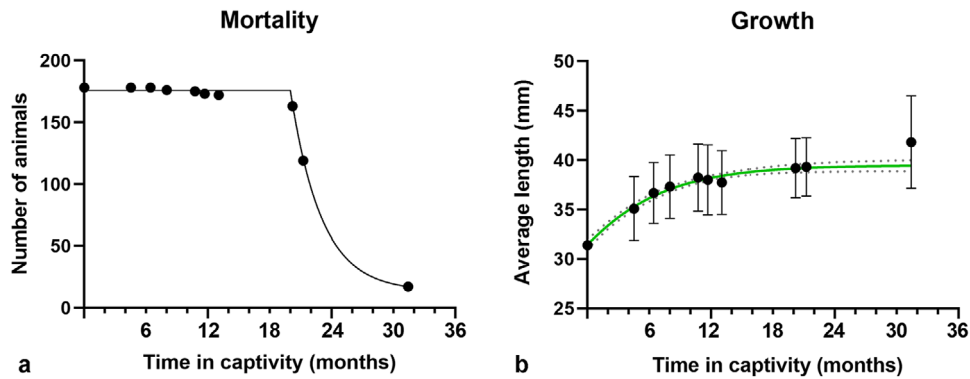


Figure 6 (a) Mortality of *Branchiostoma lanceolatum* adults in captivity. Each point represents the number of animals alive at a given time point. The curve was extrapolated using the plateau followed by a one-phase decay model. (b) Growth in size of *Branchiostoma lanceolatum* adults in captivity. Each point represents the mean length of the population of animals at a given time point. The error bars represent the standard deviation. The growth curve (green, $R^2 = 0.94$) was empirically determined with 95% confidence bands (dotted gray lines).

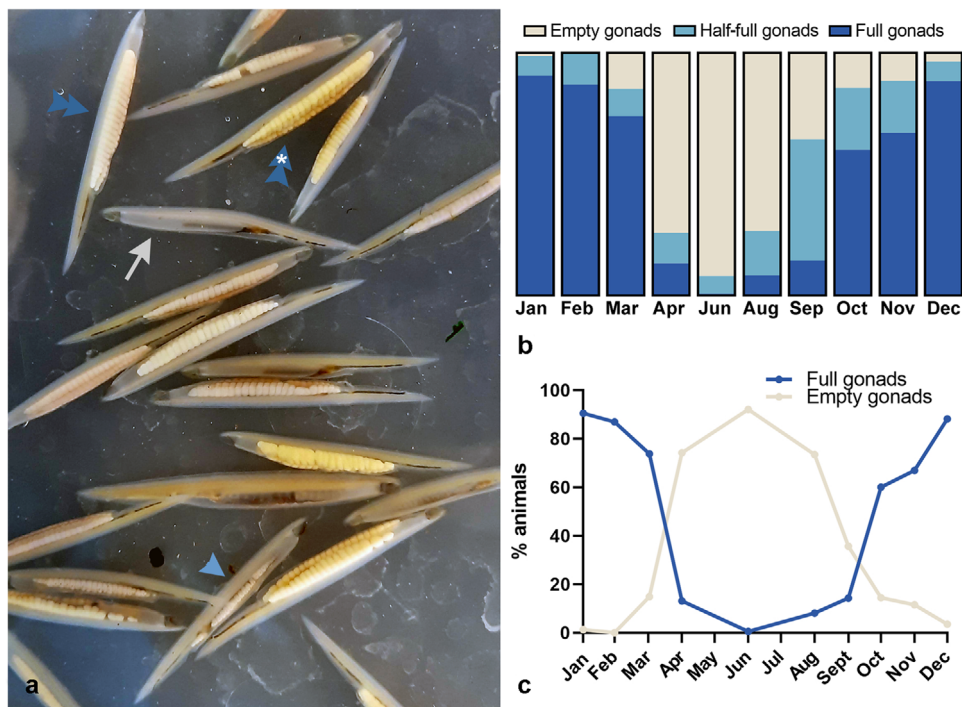


Figure 7 Gonad maturation of *Branchiostoma lanceolatum* adults in captivity. (a) Picture of a tank with animals with empty (beige arrow), half-full (light blue arrowhead), and full gonads (blue tandem arrowheads, without and with asterisk). Ripe males (blue tandem arrowhead without asterisk) and females (blue tandem arrowhead with asterisk) are distinguishable by the color of the gonads, which are more distinctly yellow in females. (b) Relative proportion of animals with empty, half-full, and full gonads in different months of the year (amphioxus adults were not sampled in May and July). (c) Variation in the percentages of animals with full and empty gonads throughout the year (amphioxus adults were not sampled in May and July).

Boni 2002), which increased the average size of sediment particles, thereby facilitating water circulation and oxygenation of the substrate. We found that the amphioxus population in the North Adriatic Sea lives in sediments with a silt percentage of less than 1.4%, which is in line with previous observations of amphioxus habitats. Furthermore, we confirmed the preference of amphioxus for smoother sediments, since we found the population density of amphioxus to be higher at sites where the coarse fraction was mainly composed of coralline algae rather than shell debris.

Amphioxus populations exhibit significant variations in their densities. The study area characterized in this study presents an average density of *B. lanceolatum* adults of 80 individuals m^{-2} with local peaks up to 330 individuals m^{-2} , which is significantly higher than what has been reported for other collection sites along the Italian coast, with only 3 individuals m^{-2} in the Ligurian Sea, for example (De Biasi *et al.* 2003). However, the average population density reported for the North Adriatic Sea is lower than the average of 230 individuals m^{-2} reported for the *B. lanceolatum* population at Racou Beach in Argelès-sur-Mer (France) (Desdevises *et al.* 2011), which is the major source of *B. lanceolatum* specimens for the scientific community.

The presence of amphioxus has previously been reported in 20 of 170 sites along a transect of over 100 km from Chioggia to Punta Sdobbà in the North Adriatic Sea of Italy (Rossi & Orel 1968). Compared to this collection campaign that transected our study area, we found the amphioxus population to cover a significantly larger area, including zones located much closer to the shoreline. Considering that the amphioxus population described in this study can be estimated to include over 50 million specimens in less than 1 km^2 , the North Adriatic Sea promises to be an important reservoir of *B. lanceolatum*. As our observations on the macrobenthic community are coherent with previous studies that have yielded comparable species lists (Antoniadou *et al.* 2004), we further expect *B. lanceolatum* adults to be present in other regions of the Adriatic Sea, as long as the sediment type of the seabed is consistent with the substrate preferences of adult amphioxus.

The structure of the amphioxus population described here is indicative of an established and healthy population. The size–frequency distribution was described by a Gaussian curve that was difficult to divide into sub-cohorts. In contrast, four adult cohorts were identifiable in the *B. lanceolatum* population at Racou Beach in Argelès-sur-Mer (France) (Desdevises *et al.* 2011), with the maximum size exceeding 56 mm in length, which

is 1.3 times larger than the largest amphioxus adult we found in the North Adriatic Sea population. Several hypotheses can be proposed to explain this difference: (1) a potentially longer reproductive period for the North Adriatic population coupled with lower nutrient availability; (2) significant variations in environmental parameters, such as temperature, salinity, and depth; and/or (3) potential differences in sampling methodology. Despite these considerations, the growth rate of *B. lanceolatum* adults from the North Adriatic Sea that we observed in captivity was very similar to that observed for *B. lanceolatum* adults in the wild at Racou Beach in Argelès-sur-Mer (France) (Desdevises *et al.* 2011).

This study also expands our knowledge of the ecology of *B. lanceolatum* in the Mediterranean Sea, which has so far been investigated chiefly at Racou Beach in Argelès-sur-Mer (France) (Fuentes *et al.* 2004, 2007; Desdevises *et al.* 2011). We found that the spawning seasons of the two *B. lanceolatum* populations differ significantly. Amphioxus adults collected in the North Adriatic Sea were characterized by full gonads in January, February, and March, and massive spawning was observed in April. Although the possibility of a second spawning in May or June cannot be ruled out, all animals had empty gonads by the end of June. By October, a large percentage of adults had visible gonads again. Importantly, we succeeded in inducing spawning in the laboratory in March, April, September, and November. These observations strongly suggest that the *B. lanceolatum* population in the North Adriatic Sea reproduces in early spring and possibly in late fall. In contrast, *B. lanceolatum* from Racou Beach spawns between the end of May and the end of June (Desdevises *et al.* 2011).

The stark difference in the spawning behavior between the two *B. lanceolatum* populations is likely due to differences in the water temperature. The yearly range of water temperatures at Racou Beach is between 10°C and 24°C (Desdevises *et al.* 2011), while the surface water temperature in the North Adriatic Sea can easily reach 28°C for several days in July and August. Although adult *B. lanceolatum* can survive temperatures peaking up to 41°C (Bert 1867), embryos and larvae fail to develop normally above 24°C (Fuentes *et al.* 2004). In comparison, embryos of the subtropical species *B. floridae* do not develop normally, if the temperature drops below 20°C (Fuentes *et al.* 2007). *B. lanceolatum* very likely has a significantly higher resistance to low temperatures, being able to thrive in the North Sea (Helgoland, Germany) as well as in the Baltic Sea (Kristineberg, Sweden) (Poss & Boschung 1996). Taken together, we interpret the spawning behavior of *B. lanceolatum* adults in the North

Adriatic Sea as an adaptation to avoid exposure of the most heat-sensitive developmental stages to the extreme water temperatures of the summer months.

Our results suggest that the animals maintain the spawning seasonality of their place of origin even after years in captivity if kept under standardized conditions in a controlled environment. This has a profound implication for the community of developmental biologists using *B. lanceolatum* as a laboratory model system. By using amphioxus adults from the two different populations, Racou Beach and the North Adriatic Sea, the spawning season in the laboratory can be extended to cover most of the year: from early spring, through the summer, into late fall.

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SUPPLEMENTARY MATERIALS

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Phylogenetic tree of concatenated mitochondrial sequences of the *cytochrome c oxidase subunit I (COXI)*, *12S ribosomal ribonucleic acid (12S rRNA)*, and *16S ribosomal ribonucleic acid (16S rRNA)* genes from different amphioxus species.

Table S1 Density of *Branchiostoma lanceolatum* adults at different points of the sampling area determined during the collection campaign

Table S2 GenBank accession numbers of the *cytochrome c oxidase subunit I (COXI)*, *12S ribosomal ribonucleic acid (12S rRNA)*, and *16S ribosomal ribonucleic acid (16S rRNA)* sequences used for carrying out the phylogenetic analyses

Table S3 GenBank accession numbers of the mitochondrial genomes used to extract the sequences of *cytochrome c oxidase subunit I (COXI)*, *12S ribosomal ribonucleic acid (12S rRNA)*, and *16S ribosomal ribonucleic acid (16S rRNA)* used for phylogenetic tree reconstruction

Table S4 FASTA file of the alignment of the concatenated *cytochrome c oxidase subunit I (COXI)*, *12S ribosomal ribonucleic acid (12S rRNA)*, and *16S ribosomal ribonucleic acid (16S rRNA)* sequences used for inferring phylogenetic trees

Table S5 Species list of the macrobenthic community on the transect A3-F3 (see Fig. 1) indicated as individuals m^{-2} , except for Bryozoa that are scored as presence (“+”)

Table S6 Mean body length of *Branchiostoma lanceolatum* adults measured in captivity

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