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Plasticity in reproduction and nutrition in wood-boring bivalves (*Xylophaga atlantica*) from the Mid-Atlantic Ridge

Gaudron, SM[1,2*], Haga, T[3], Wang, H[4], Laming, SR[4], Duperron, S[4,5].

[1]UMR 8187 Laboratoire d'Océanologie et Géosciences (Univ. Lille, CNRS, Univ. Cote d'Opale), 62930 Wimereux, France

[2]Sorbonne Universités, Université Pierre et Marie Curie Paris 06, UFR927, 4 place Jussieu, 75005 Paris, France

[3]Research Group, Toyohashi Museum of Natural History, 1−238 Oana, Oiwa-chô, Toyohashi, Aichi 441- 3147, Japan

[4]Sorbonne Universités, Université Pierre et Marie Curie Paris 06, UMR 7208 BOREA (CNRS), 75052 Paris, France

[5]Institut Universitaire de France, Paris, France

*** Corresponding author:** sylvie.gaudron@upmc.fr

Dr Sylvie Marylène Gaudron

UMR8187 Laboratoire d'Océanologie et de Géosciences (LOG)

Station marine de Wimereux (Université de Lille)

28 Avenue Foch

B.P.80

62930 Wimereux (FRANCE)

Tel : 0033 (0)321992961

Fax : 0033 (0)321995801

Abstract

High densities of the wood-boring bivalve *Xylophaga atlantica* colonized pine wood cubes in colonization devices deployed at 2279 m depth for 414 d (14 July 2007−31 August 2008) near hydrothermal vents at the Rainbow site on the Mid-Atlantic Ridge (36°13.7454'N/33°54.0513'W). Histological and biometric observations on specimens with shell lengths (SL) of 0.5−4.2 mm revealed three cohorts in this dioecious population. The first cohort was dominated by mature females, each with an estimated fecundity of ~ 450 oocytes with a mean diameter of 28.0 ± 3.9 µm (maximum diameter 40.0 µm); an intermediate cohort was a mix of males and females with SL at first maturity of ≤ 1.7 mm; the third cohort was exclusively morphologically distinct, mature, dwarf males, SL ~ 500 µm. These dwarf males were attached to the dorsal shell surfaces of females in the first cohort. The difference in the SL of Prodissochonch I (~60 µm) and Prodissoconch II (500-530 µm) confirmed planktotrophy. Based on their carbon and nitrogen stable-isotope ratios, and a paedomorphic morphology suggesting they are ill-equipped to bore wood, it seems likely that the dwarf males are heterotrophic filter-feeders. Fluorescence in situ hybridization showed, however, that dwarf males hosted a few Gammaproteobacteria in their gills. The absence of a bacterial signal in the germ cells and developing oocytes of females implies that direct trans-ovarial inheritance of symbiotic bacteria does not occur in *X. atlantica*.

Key-words: colonization, chemosynthetic habitat, bacterial symbiont, food availability, spatial niche, sex determination, growth rate, settlement, paedomorphism, mollusc.

Introduction

The fragmented distribution of highly ephemeral wood-fall habitats in the deep sea (here defined as > 200 m depth) depends on the initial introduction of wood into the marine environment (e.g. during extreme weather events), the offshore transport of this wood by oceanographic processes and ultimately, once waterlogged, the deposition of this wood within the deep ocean (Thiel and Gutow 2005; Canals et al. 2006; Tyler et al. 2007). Despite a patchy deep-sea distribution, naturally occurring and artificially deployed wood logs are colonized at an astonishing speed by molluscan

wood-boring bivalves of the family Xylophagaidae (Knudsen 1961; Turner 1973; Voight 2008; Gaudron et al. 2010; Cunha et al. 2013; Romano et al. 2013; Amon et al. 2015). In a pioneering study (Turner 1973), high densities of mature individuals of *Xylophaga* n. sp. were recorded in wood panels after a short 3-month deployment at 1830 m in the Northwest Atlantic (39°46'N/70°41'W). This study led her to hypothesize that xylophagaid larvae were abundant, transported by bottom currents and guided by an ability to detect wood over considerable distances, with the capacity to delay metamorphosis in the absence of wood and to settle rapidly following appropriate environmental cues. Culliney and Turner (1976) went on to confirm that pediveligers of *X. atlantica* (from a shallow water population) in laboratory-based larval studies were indeed able to delay metamorphosis for up to 6 months, indicating a potential to considerably extend the pelagic larval duration (PLD) (see review on PLD, Hilário et al. 2015). Recent short-term colonization experiments (10 d) carried out in the Mediterranean Sea at 1700 m (Gaudron et al. 2010) and at hydrothermal vents on the Mid-Atlantic Ridge (2275 m, Gaudron unpublished), from which post-larval stages of *Xylophaga* spp. were recovered, appear to confirm Turner's predictions. Haga and Kase (2013) measured the relative size differences between the post-embryonic and post-settlement larval shells of *X. supplicata* – i.e. the Prodissoconch I (PdI: ~40 µm) and the Prodissoconch II (PdII: ~310 µm; shell that is deposited during pelagic larval dispersal) –and found that larvae were planktotrophic for long periods spent in the water column, according to the criteria of Jablonski and Lutz (1983).

Only a few reproductive studies have been carried out on the genus *Xylophaga* (reviewed by Voight 2015), describing a diversity of reproductive modes. There are contradictory findings for *X. dorsalis*, with evidence for both protandric hermaphroditism (Purchon 1941) and dioecism (Tyler et al. 2007). The latter mode was also identified in *X. depalmai* where simultaneous hermaphrodites were observed occasionally (Tyler et al. 2007). In Haga and Kase (2013), many (though not all) specimens of *X. supplicata* were identified as protandric, first developing as males and then changing into females after temporary simultaneous hermaphroditism. However, a number of small, paedomorphic specimens attached to the shells of larger females were ultimately found to be dwarf males and not brooded juveniles, as had previously been suggested (Knudsen 1961; Turner 2002). Extrapolating from this misidentification, Haga and Kase (2013) surmised that some or all *Xylophaga* spp. known to harbor brooded juveniles (19 of the 53 species described), could instead be carrying dwarf males. Using histology, Ockelmann and Dinesen (2011) identified functional dwarf males in *Xyloredo ingolfia* but misidentified the species according to Haga and Kase (2013), because it was actually *Xylophaga clenchi*. The relatively frequent occurrences of this phenotype in deep-sea wood-boring species are considered by some authors to be an adaptation to a relative scarcity of wood substrata in deeper waters.

Wood falls are a locally important input of energy in an otherwise nutrient-depleted deep sea. Various processes contribute to wood-fall degradation. One of these is xylotrophy. Teredinid and xylophagaid families capable of burrowing into wood do so by repeated rotations of their specialized shells which are adorned with rasping denticles (Voight 2015). The wood fragments are then swallowed and stored within a caecum in the foregut of the bivalve (Distel et al. 2011). Though many wood-boring bivalve species are believed to host gill-associated symbionts, only the Gammaproteobacteria (e.g. *Teredinibacter turnerae*) found in the gills of teredinid shipworms have been well-categorised metabolically (Waterbury et al. 1983; Distel et al. 2002a, b; O'Connor et al. 2014). These Gammaproteobacteria can perform both cellulolysis and fix nitrogen. In the xylophagaids, Distel and Roberts (1998) first identified bacterial symbionts within the gills of *Xylophaga atlantica* and *X. washingtona*. Based on 16S rRNA high-throughput sequencing, Fagervold et al. (2014) have since identified bacterial OTUs (Operational Taxonomic Units) within the gills of *Xylophaga* spp. that resemble those identified in shipworms. However, the exact location of these bacteria within host tissues, their abundance and their activity remain undetermined.

Xylophaga atlantica were collected from 2279 m depth on the Mid-Atlantic Ridge and those found carrying potential dwarf males were investigated. We conducted a comprehensive analysis of their reproduction and nutrition. We tried to answer several questions in this study: 1) Are the biology and ecology of this deep *X. atlantica* population similar to that described for shallow-water *X. atlantica*? To answer this we examined gametogenesis, fecundity, growth rate, size at first maturity and larval shell dimensions (PdI and II), using histology, scanning electron microscopy and image analysis; 2) What could have driven dwarfism in this *X. atlantica* population? 3) Being ill-equipped to bore wood, do dwarf males host symbiotic bacteria in their gills and is there evidence of symbiont assimilation? To test this, we used Fluorescence in situ Hybridization (FISH) to look for bacterial symbionts within gill filaments, and employed stable isotope analyses ($\delta^{13}C$ and $\delta^{15}N$) to identify whether xylotrophy might contribute to host nutrition; 4) Is there any evidence for maternal transmission of bacterial symbionts during oogenesis? FISH was applied to reproductive tissues to confirm whether putative bacterial symbionts were present within oocytes at various stages of their development within the female acini.

Materials and methods

Collection and identification

Wood-boring bivalves were collected from numerous 2 cm-sided pine wood cubes housed in a single **CHEMECOLI** (**CHEM**osynthetic **E**cosystem **CO**lonization by **L**arval **I**nvertebrates) colonization

device which was deployed on 14 July 2007 for 414 d on the Mid-Atlantic Ridge at the Rainbow hydrothermal vent field (36°13.7454'N and 33°54.0513'W), at 2279-m depth near some small vent chimneys (Online resource 1). The device contained 87 cubes for a total wood volume of 1.539 dm^3. Once on board, cubes were randomly selected from the bottom, middle and top of the device, and fixed in different fixatives in a cold room. Specimens from cubes fixed in 4% buffered formaldehyde in twice-filtered seawater (TFSW) were used for taxonomy. Specimens from cubes fixed in 95% ethanol were used for biometry and those frozen at -20°C were used for stable isotope analyses. Specimens from cubes fixed in 4% buffered formaldehyde in TFSW for 4 h at 4°C, rinsed three times in TFSW and then transferred into 50/50 Ethanol/TFSW were used for both Fluorescence in situ hybridization (FISH) and for reproductive histology, following the same protocol as Gaudron et al. (2012). Wood cubes were dissected aseptically with sterile razor blades in order to collect all wood-boring bivalves. Specimens were identified morphologically based on descriptions by Turner (2002).

DNA was extracted from 13 large individuals and 15 dwarf males using the QiaQuick Dneasy Kit (Qiagen, USA). Partial sequences of the gene encoding 28S rRNA were amplified by PCR using primers 28S-C1 ACCCGCTGAATTTAAGCAT and 28S-C2TGAACTCTCTCTTCAAAGTTCTTTTC, as described in Williams et al. (2004). Sequences were compared with GenBank using the Blast tool (Altschul 1997) and deposited to GenBank with accession KU684449 (one larger specimen) and KU684450 (one dwarf male).

Haematoxylin / Eosin staining and Fluorescence in situ Hybridization (FISH)

A total of 16 *Xylophaga atlantica* (Online resource 1) of various sizes (1.7−4.2 mm shell lengths, SL) from the smallest to the largest individuals identified from sorting (except dwarf males) were measured using an Olympus SZX12 binocular equipped with SPOT software before being dissected. Tissues were rinsed in butan-1-ol and Histoclear (Euromedex Ltd.) and embedded in Paraffin Embedding Wax (melting point 52°C). The soft tissues (bivalves without shell) were cut into 8 µm-thick sections using a microtome (Microtome Finesse 325, Thermo Fisher Scientific) and deposited on SuperFrost Plus slides (Euromedex Ltd.). A standard haematoxylin/eosin (HE) staining protocol (Gaudron et al. 2012) was used on one in every ten slides and observations were performed using an Olympus BX61 light microscope (Olympus, Japan) equipped with ImagePro software. The sex was identified in these 16 specimens. Oocyte diameters were measured in 7 female specimens and partial fecundity was estimated by counting the total number of fully-grown oocytes within all acini seen in histological sections of four females.

Potential dwarf males (SL < 500 µm, *n* = 5) were blotted dry and infiltrated (8 x 30-min infiltrations) in a gelatine capsule (size 00, Electron Microscopy Sciences, UK) filled with LR-White resin (London Resin Company, UK), transferred to a fresh resin-filled gelatine capsule, orientated appropriately, capped, and polymerised at 55°C (20 h minimum). Gelatine was then removed with hand-hot water. Resin blocks were wet-sectioned (glass knife) on a Leica EM Ultra Cut R Ultramicrotome (Germany) to thicknesses of 350 nm−1 µm, deposited on Superfrost-plus slides, and stained in Toluidine Blue while others were unstained for FISH.

FISH was employed on the non-stained slides of both large specimens and potential dwarf males to reveal the presence of possible symbiotic bacteria in gills and female gametes following the protocol of Gaudron et al. (2012). Due to the small size of the bivalves, a cross-section of the entire individual, including the digestive tract, acini with gametes, and the gill could be visualized on most slides. The paraffin wax was removed using a serial gradient of ethanol and Histoclear prior to FISH. The LR-White slide-mounted sections were only rehydrated in phosphate buffered saline (PBS 1x). FISH was performed using Cy3- and Cy5-labeled probes as described previously (Duperron et al. 2005) with a hybridization buffer containing 30% formamide. Probes Gam-42 (5'-GCCTTCCCACATCGTTT-3'; Manz et al. 1992), targeting the 16S rRNA (small subunit) of most Gammaproteobacteria, EUB-338 targeting most Eubacteria (5'-GCTGCCTCCCGTAGGAGT-3'; Amann et al. 1990) and NON-338, a control for non-specific binding (5'-ACTCCTACGGGAGGCAGC-3'; Wallner et al. 1993) were used. After washing, slides were mounted in Slow-Fade Gold (Invitrogen) containing DAPI, which stains nucleic acids. Fluorescence microscopy was performed using an Olympus BX61 light microscope (Olympus, Japan).

Size-distribution, bivalve density and growth rate

SLs of 100 large borers and 68 dwarf males were measured with an Olympus SZX12 binocular microscope equipped with SPOT software. The protocol followed that of Romano et al. (2013), using the Bhattacharya method and the specific routine in FISAT II package (FAO 2002) to identify each *Xylophaga atlantica* cohort that recruited into the CHEMECOLI during the 414-d deployment. SL frequency distributions in 0.25-mm SL intervals were used to accommodate all individuals in this population, including the dwarf males.

The relative density of individuals was estimated by counting the number of wood-borers recovered from a stratified, random subsample of 15 cubes from the top, middle and bottom of the CHEMECOLI (5 cubes zone^{-1}). Minimal mean growth rate was estimated by subtracting the mean SL of PdII from the computed mean SL of the first cohort from FISAT II, dividing the result by the number of days of deployment, minus 10 d (404 d); post-larval stages of *X. atlantica* had already

colonized an identical, short-term, 10-d deployment (Gaudron unpublished data) at the same site (Rainbow 2275m; 36°13.7553N and 33° 54.11W).SLs of PdII were measured in randomly selected individuals (n = 5) in each cohort. Mean SL for each cohort were compared using a student test (MINITAB v.15).

Stable isotope analyses

Frozen wood-boring bivalve females (n = 3) bearing dwarf males (pool of 30 individuals) were identified under a dissecting microscope and dissected to remove the shell when possible. Frozen wood cubes (n = 3) from CHEMECOLI were first sliced into pieces with a sterile scalpel, then rinsed in distilled water, and finally screened under a dissecting microscope to check for the presence of metazoan material (tissue, faeces, eggs and larvae). Of these, bivalve tissues (autonomous and dwarf males), pieces of wood and one amphinomid polychaete (control) were rinsed in distilled water and then dried (2 d, 60 °C). Some dried subsamples of female wood-borer tissues and a pool of whole dwarf males were treated with 1 N HCL (~3 h) to remove inorganic carbon and then rinsed with distilled water.

1 mg (± 0.1mg) of dried bivalve, polychaete tissues or wood pieces were analysed using a CHN elemental analyser (EuroVector, Milan, Italy) for particulate organic carbon (POC) and particulate nitrogen (PN) in order to calculate their C/N atomic ratio (Cat/Nat). The resultant gas from the elemental analysis was introduced into a GV IsoPrime (UK) stable isotope mass spectrometer at Iso-Analytical (Crewe, UK). Values of $\delta^{13}C$ and $\delta^{15}N$ were determined and expressed as relative per-mil (‰) differences between samples and Pee Dee Belemnite (PDB) for carbon and air for nitrogen according to the following equation:

$$\delta(X) = \left[\left(\frac{R_{sample}}{R_{standard}} \right) - 1 \right] * 1000$$

where X (‰) is ^{13}C and ^{15}N abundance and R is the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios.

Scanning Electron Microscopy (SEM)

The dwarf males and the autonomous wood-boring females were dehydrated using an ethanol series and critical-point dried, then coated with gold before observations with a SEM (Cambridge S260 at 15 kV). PdI was observed and measured. The internal anatomy of a dwarf male was observed.

Results

Identification

Identification of wood borers was based on taxonomically-relevant features of the inhalant and exhalant siphons, the shape of the mesoplax (Online resource 1) and the posterior adductor scar, which all matched the description of *Xylophaga atlantica* (Turner 2002). The 28S rRNA partial sequences of all specimens (including putative dwarf males, see Fig. 1b, c) were identical (base to base). Compared with GenBank, each returned sequences with 98% similarity scores to five 28S rRNA sequences attributed to *Xylophaga atlantica* deposited by Romano et al. (2014). Figure 1b shows dwarf males attached ventrally by the byssus threads on the dorsal side of an autonomous female (Online resource 2). Dwarf males did not have the denticles on their shells needed to bore and rasp wood (Fig. 1c; Online resource 2). The SL of the PdI was ~ 60µm (Online resource 2). Dwarf males had mature spermatozoa (Online resource 2). No dissoconch deposition (adult shell) was observed in dwarf males, despite the presence of mature spermatozoa and a ligament, which indicated that metamorphosis into adults had occurred (Online resource 2). Thus dwarf male SL, 540 ± 2.2 µm (n = 68), essentially represented their SL at settlement (i.e. PdII).

Size structure of the population

The SL frequency distributions were trimodal (Online resource 3), but there were only two true cohorts since dwarf males lacked adult dissoconch shell despite being sexually mature (Online resource 2). The cohort with the individuals with the greatest SL (3.4 ± 0.5 mm) were mostly large females bearing dwarf males (Online resource 3), and the eight specimens in this size range used in histology were all females (Fig. 2). The intermediate cohort (Online resource 3) had a mean SL of 2.6 ± 0.4 mm: the sex ratio for the eight specimens used in histology in this size range was 1:1 (Fig. 3). The remaining cohort with the smallest SLs had a mean SL of 0.6 ± 0.1 mm, and was composed exclusively of dwarf males examined using both SEM (Online resource 2) and histology (Fig. 4). The density of all *Xylophaga atlantica* (including dwarf males) was estimated at 6853 individuals dm^{-3}. The minimal shell growth rate was estimated at 7 µm d^{-1} for the first cohort (Online resource 4). Mean SL of PdII for the three cohorts were not significantly different using a *t*-test (Cohort 1: 500 ± 10 µm; Cohort 2: 530 ± 30 µm; cohort 3: 530 ± 30 µm).

Gametogenesis, fecundity, size at first maturity and spawning behaviour

All specimens examined using SEM or HE staining of histological sections had mature gametes, so no immature specimens were observed. Wood-boring females (n = 12) carrying mature oocytes were 1.7−4.2 mm SL. Non-dwarf wood-boring males (n = 4) and dwarf males, both carried all stages of gametes (spermatogonia to mature spermatozoids), and were 1.7−2.3 mm SL and 0.48−0.58 mm SL respectively. Partial fecundity was estimated at 450 ± 130 oocytes $female^{-1}$ (n = 4). Mean oocyte

diameter was 28.0 ± 3.9 µm (n = 7) for mature females in the first cohort (Fig. 2a, c) with a maximum diameter of 40 µm. A few mature oocytes in Prophase I (unfertilized), similar to those of the first-cohort wood-boring females, were found in the gill chambers of two second-cohort males (Fig. 2a, c). These spawned oocytes were probably captured following their release by a female, suggesting that spawning was occurring when the colonization device was retrieved. There was no evidence of hermaphroditism.

Symbiotic bacteria

In females and non-dwarf males, gill filaments were occupied by dense populations of symbiotic bacteria in gill cells devoid of cilia (Fig. 2b, d; Fig. 3b). Depending on sectioning angle and region (see HE sections [Fig. 3c; Fig. 3a]), the abundance of ciliated versus bacterium-loaded gill epithelial cells varied but no quantification was attempted. Signals from the Gammaproteobacteria- and the Eubacteria-targeting probes almost fully overlapped in both sexes, indicating that gill-associated bacteria were predominantly Gammaproteobacteria. Histological sections of female gametes at different stages of oogenesis were available, their nuclei stained with DAPI (Fig. 2b). Germ cells gave strong DAPI blue signals (as did the nuclei of gill-filament epithelial cells), allowing the boundaries of acini to be discerned, within which fully-grown vitellogenic oocytes were packed (Fig. 2a). No bacterial signal was ever found on, or inside germ cells and fully-grown vitellogenic oocytes, where the unambiguous bacterial signal in gill tissue served as a positive control (Fig. 2b). Bacteria are therefore believed to be absent from reproductive tissues in these *X. atlantica*.

In dwarf males, gill filaments were less numerous and much smaller (Fig. 4b). Nevertheless, unambiguous Gammaproteobacteria FISH signals were detected in association with the non-ciliated abfrontal regions of gill filaments (white arrow in Fig. 4c). Visually, bacterial densities were lower than those in larger specimens. Bacteria appeared to be extracellular, but higher resolution would be needed to confirm this.

Wood-based diet and trophic level

Stable isotope analyses performed on female wood-borers yielded $\delta^{13}C$ values of ~22‰ close to the $\delta^{13}C$ values of the wood itself (~23‰) (Table 1). Dwarf male tissues were more enriched in ^{13}C (~20‰), resembling values obtained for the amphinomid polychaete control (Table 1). Despite the paucity of nitrogen within the wood itself (C/N ~160; Table 1) C/N values of the overall fauna were 3−5 (Table 1). The $\delta^{15}N$ values (~4‰; Table 1) of the females were just at the upper limit of primary producers in food webs (Minagawa and Wada 1984), while $\delta^{15}N$ values (~6.5‰; Table 1) of the dwarf males resembled nitrogen stable-isotope signatures corresponding to primary consumers

(Minagawa and Wada 1984). For comparison, amphinomid polychaetes, usually seen as predators, had $\delta^{15}N$ values of ~8‰ (Table 1). This reflects the lowest limit for secondary consumers within food webs (Minagawa and Wada 1984).

Discussion

Wood-boring bivalve specimens in this study were identified as *Xylophaga atlantica* Richards 1942 based on morphology (Turner 2002) and 28S rRNA sequences with a 98% similarity to sequences of *X. atlantica* in Genbank. The 28S rRNA sequences of large females in the present study matched (100% similarity) with 28S rRNA sequences of dwarf males, which confirms that individuals belong to the same species. *Xylophaga atlantica* has previously been collected in the Western Atlantic at depths of 15−1242 m (Turner 2002) and more recently, *X. atlantica* ('morphotypes C, D and E') was collected from 900− 2000 m in the Western Mediterranean Sea and Eastern Atlantic (Romano et al. 2013; 2014; Online resource 4). However, none of these studies reported the presence of dwarf males. One may wonder what could have induced this phenotypic plasticity in morphology and reproductive strategy.

Traits of *Xylophaga atlantica* from deep and shallow populations

Specimens of *X. atlantica* in the current study were gonochoristic; there was no evidence of sex change through a hermaphroditic phase. The mature oocytes (~ 40 µm) in this study are very similar to those of a shallow population of *X. atlantica* (45 µm, Culliney and Turner 1976). Large females and their associated dwarf males both carried mature gametes, while spawned oocytes were observed within the pallial cavity of non-dwarf males and female specimens. This could indicate spawning synchronicity within this xylophagaid population at the time of sampling (end of August 2008), and suggests that some instances of fertilization might occur within the pallial cavity of one, or the other parent. At the end of the 414-d deployment three cohorts of veligers had recruited (or two, if we exclude dwarf males). Shallow-water *X. atlantica* has been shown to recruit seasonally (Berg et al. 1987). Culliney and Turner (1976) found ripe *X. atlantica* in late summer, and suggested metamorphosis and settlement occurred from autumn to winter. Our data cannot detect whether seasonality played a role in structuring size-frequency distributions in the MAR population.

Fecundity and size (age) at first maturity in xylophagaids have only been recorded previously in *X. depalmai* from colonization devices deployed at 500 m (Tyler et al. 2007; Online resource 4). In that study, fecundity was stated to be 'high' but no data were provided. Gametogenesis was already occurring in specimens collected from 59-d colonization experiments, with size at first

maturity > 2 mm SL (Tyler et al. 2007; Table 1). In the present study, the size at first maturity was ≤ 1.7 mm SL for non-dwarf males and females (Online resource 4). Dwarf males had SL at first maturity of ≤ 0.5 mm almost immediately after settlement. It is difficult to classify the fecundity as low, moderate, or high in this study (~ 450 oocytesfemale^{-1}), given the paucity of other data. Fecundity in the opportunistic gastropod *Cocculina rathnuni*, colonizing deep-sea wood substrates, was only 40 oocytes female^{-1} (Young et al. 2013), who argued that low fecundity was still compatible with opportunism, as larval predation pressure in the deep sea is likely less than in shallow water. It is not clear whether this is true for larvae arriving at reducing habitats, such as sunken wood, where faunal densities are much higher. However, only one specimen of a wood-fall species known to feed on xylophagaid larvae (Ockelmann and Dinesen 2011) was collected from the CHEMECOLI (see Rodrigues et al. 2015), the small larvivorous mussel *Idas argenteus*. Wood-fall habitats are highly ephemeral. Tyler et al. (2007) argued that biological traits such as the early reproductive development of *X. depalmai*, typically rare in deep-sea organisms, may represent adaptations to ephemeral reducing habitats (e.g. hydrothermal vents, whale falls and wood falls). Similar hypotheses have since been proposed for other molluscs that colonize wood, which are also small at first maturity. These include the bathymodiolin mussels *Idas modiolaeformis* (2.35 mm SL, Laming et al. 2014) and *I. simpsoni* (1.8 mm SL, Génio et al. 2015), and several wood-colonizing limpet species (e.g. *Cocculina rathbuni*: <1.5 mm SL, Young et al. 2013).

In our study, the estimated growth rate of *X. atlantica* (7 μm d^{-1}) was relatively low when compared with previous wood colonization experiments (Online resource 4). This may reflect spatial constraints imposed by the high densities of individuals collected in a low temperature habitat. Physical crowding has been proposed as contributing to unexpectedly low growth rates in shallow-water *X. atlantica* (Romey et al. 1994; Online resource 4) and in deep-water, dense populations of *X. alexisi* (Voight and Segonzac 2012; Online resource 4). These authors argued that at low population densities, *X. alexisi* might have much higher potential growth rates.

Larval biology of a deep *Xylophaga atlantica* population

The SL of the PdII for the three cohorts (~ 500−530 μm), and the small post-embryonic PdI of the earliest veliger (SL ~ 60 μm), provide insights into the planktotrophic larval mode and its duration (Jablonski and Lutz 1983).The size of the PdII measured in this study is nearly twice that recorded for larvae of laboratory-reared, shallow-water *X. atlantica* (Culliney and Turner 1976).This suggests that veligers of the wood-borers recovered within CHEMECOLIs at 2279 m, have probably spent an extended period of time in the water column. Such extended periods may be an adaptation to very fragmented habitat distributions. This hypothesis was proposed for *X. alexisi* larvae from an abyssal-

366 plain population (4626 m) as a means of surviving long periods in the plankton (Voight and
367 Segonzac 2012). The relatively cool (~ 4°C) water temperature in the deep Atlantic Ocean may
368 lengthen PLDs, as lower temperatures are likely to induce lower metabolic rates.
369 PdII SLs in *X. atlantica* mirror sizes recorded previously in the cold-seep bathymodiolin
370 mussels *Idas modiolaeformis* and *'Bathymodiolus' childressi*, where PLDs of up to 5 and 13 months
371 respectively have been demonstrated (Gaudron et al. 2012; Arellano et al. 2014). Under favourable
372 oceanographic transport conditions, extended PLD could explain the broad geographical distribution
373 of *X. atlantica*, spanning the Eastern Atlantic, into the Western Atlantic, and to the Western
374 Mediterranean (Romano et al. 2014). *Xylophaga atlantica* may thus be considered an amphi-Atlantic
375 candidate species with a potential for long-distance dispersal comparable to that documented in some
376 *Bathymodiolus* species (Olu et al. 2010).
377
378 Epigenetic factors influencing sex-determination in *Xylophaga atlantica*?
379 The existence of large females and dwarf males in *X. atlantica* suggests a kind of epigenetic sex-
380 determination in this species, rather than complete genetic sex regulation. There may be
381 environmental drivers similar those in other metazoans, such as food availability, population density
382 and temperature (e.g. Santerre et al. 2013). In the present study, two epigenetic or 'proximate' factors
383 may apply: food availability and the diminution of the spatial niche. Consider the following scenario:
384 at the beginning of the colonization experiment in 2007, deployed wood represented nascent habitat
385 for settling wood-borer larvae, where optimal food availability favoured a female bias in the sex-
386 ratio (first cohort) as seen in other bivalves where oogenesis requires a considerable energy
387 investment (Chávez-Villalba et al. 2011). During the course of the 414-d deployment, the wood was
388 progressively consumed by this first cohort so food and space availability decreased with time. New
389 colonists that arrived and settled (second and third cohorts) shifted the population towards a male
390 bias, spermatogenesis having a lower metabolic demand (Chávez-Villalba et al. 2011). In this
391 scenario, the initial epigenetic factor driving sex-determination is food availability, favouring
392 females. The second, a smaller spatial niche, selects for non-dwarf males and ultimately, dwarfism
393 with larvae settling directly on female shells. Romano et al. (2014) did not find dwarf males in their
394 samples of *X. atlantica*. However, their colonization experiments used pieces of wood ten times
395 bigger than those in the current study, with wood-borer densities 60 times lower than those described
396 here, so there were no constraints on food or space.
397 Male dwarfism has evolved in species with small population sizes and in which the female is
398 sedentary or hard to find, such as the echiurian, *Bonellia viridis* (Berec et al. 2005). Haga and Kase
399 (2013) recorded a similar trend in wood-associated *X. supplicata* with low population densities,

where the direct association of females with dwarf males would increase fertilisation success, while dwarfism was argued to compensate for a rarity of wood logs (resource limiting). Voight (2015) pointed out that dwarf males offer a dioecious strategy that minimizes crowding and resource consumption. In the opportunistic polychaete, *Osedax* spp., which colonize decomposing whale carcasses (another sulfidic deep-sea organic-fall habitat), most males are dwarves with a high density of female bone-eating worms (Rouse et al. 2004). Occurrence of dwarf males in *X. atlantica* and *Osedax* spp. seems to be a convergence that may reflect similar evolutionary constraints acting on these species in habitats that share many characteristics (ephemeral existence, patchiness, rarity, but with the potential to sustain a very productive population locally, despite resource limitations).

Xylotrophy and symbiosis in wood-boring adults versus associated dwarf males of *Xylophaga atlantica*

Xylotrophy was examined in shallow-water *X. atlantica* (recovered from oak in lobster traps at 100-m depth in the Western Atlantic), but it is not clear whether Gammaproteobacteria in the gills of *Xylophaga* spp. play any role in cellulolytic (synthesis of cellulase enzyme) or/and nitrogen-fixing activities (Distel and Roberts 1997). In our study, the shape and sculpture of xylophagaid shells (e.g. such as in cohorts 1 and 2) allows them to physically bore wood, with the potential to assimilate the pulp with the aid of hypothesized cellulolytic symbiotic bacteria (metazoans are rarely able to produce cellulase). Stable isotope values suggest that large specimens (1st cohort) had a diet based on direct wood consumption, with ~ 1‰ fractionation (DeNiro and Epstein 1978) of the ^{13}C between the wood and bivalve tissues. The C/N (~160) of the wood is a consequence of low nitrogen levels, a feature common to most sunken-wood habitats (Nishimoto et al. 2009; Duperron et al. 2013). That means that xylotrophic metazoans need a separate source of nitrogen, and symbiosis with nitrogen-fixing bacteria may be one alternative. Gammaproteobacteria were very abundant in the gills of large *X. atlantica* specimens in this study but were rare by comparison in the gills of dwarf males. To date, the role of these putative gill bacterial symbionts in both dwarf males and full-sized specimens has yet to be confirmed.

Stable carbon isotopes (~ 20‰) recorded in the dwarf males did not reflect the consumption of wood in the current study. δ^{15}N ratios (Table 1) were more in line with the levels of primary consumers in a classical food web and much higher than those of large specimens (Minagawa and Wada 1984). Fagervold et al. (2014) described specific bacterial communities found in association with the faecal pellets of *Xylophaga* spp., which accumulate within their burrows. Given the high densities of *X. atlantica* in this study, it is likely that the resulting faecal matter and by-products generated through burrowing would have formed a component of the dwarf males' diet. Thus, not

434 only do the dwarf males retain paedomorphic traits, but they are likely predominantly heterotrophic
435 filter-feeders. Unfortunately stable isotope analyses in the current study did not include the
436 associated detritus, so the exact source of nutrition in dwarf males cannot be confirmed.
437
438 Bacterial transmission in *Xylophaga atlantica*
439 The absence of Gammaproteobacteria in oocytes suggests that bacterial symbionts of *X. atlantica* are
440 not transmitted directly from the parent organism during gametogenesis or prior to spawning. The
441 presence of a low number of bacteria in dwarf males measuring ~ 500 µm SL indicates that symbiont
442 acquisition had already occurred at this stage in this sex-related phenotype. Since shell development
443 following metamorphosis cannot be defined, it remains unclear whether acquisition occurred prior to,
444 during, or after settlement, as reported for some Bathymodiolinae mussels (Laming et al. 2014;
445 2015). The potential for horizontal bacterial transmission may be supported by recent 454-
446 pyrosequencing analyses of the bacterial communities associated with our pine wood cubes, which
447 identified close relatives of shipworm symbionts (Szafranski et al. 2015). It is possible that free-
448 living forms of these symbionts (or the same symbionts living in other proximal hosts) may be
449 acquired directly from the environment. Although it is possible that mature oocytes acquire bacteria
450 while entrained within the bacteria-loaded gill filaments of females and large males (i.e. before or
451 around the time of fertilization), no bacteria were found attached to oocytes in the pallial cavity of
452 specimens from the current study.
453

454 ## Conclusion

455 Here we have presented evidence that higher population density in *Xylophaga atlantica* may be
456 accompanied by shifts in reproductive mode, probably in response to epigenetic influences. Rapid
457 settlement, moderate fecundity and small size at first maturity may be adaptations to the ephemeral
458 and patchy nature of wood-fall habitats, while male dwarfism, an extreme form of sexual
459 dimorphism, is believed to be an adaptation to the inherently finite nutritional and spatial resources
460 available. The apparent disparity between PLDs of shallow- and deep-water *X. atlantica* likely
461 relates to the relative paucity of suitable habitats in the deep sea leading to long larval dispersal. The
462 small sizes and different nutrition of dwarf males permit the opportunistic use of a unique spatial
463 niche. This adaptation allows *X. atlantica* populations to persist through resource partitioning,
464 despite their finite availability. Regarding symbiosis we confirm that large specimens of *X. atlantica*
465 host Gammaproteobacteria within their gills but with no occurrence in germ lines, which likely rules

out trans-ovarial inheritance, suggesting environmental bacterial transmission is the principal mode for symbiont transmission.

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Compliance with Ethical Standards

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Table 1 Mean stable C and N isotope values (± SD) and C/N ratios for female and associated dwarf male *Xylophaga atlantica* collected after 414 d at 2279 m depth at Mid-Atlantic Ridge. Values for one co-occurring polychaete added as positive control.

Samples	*n*	$\delta^{13}C$	$\delta^{15}N$	C/N
Wood	3	-23.1 (± 0.1)	-	159.6 (± 17.3)
Female autonomous wood-borers	3	-	4.6 (± 0.5)	5.3 (± 0.3)
Female autonomous wood-borers acidified	3	-21.7 (± 0.3)	-	-
Dwarf males acidified	30 (pool)	-20.2	6.4	3.8
Amphinomid polychaete	1	-19.9	8.0	4.4

Figure captions

Fig. 1 SEMs of *Xylophaga atlantica* females bearing dwarf males. a) Prodissoconch I (PdI) with edge marked by arrow (scale bar 50 µm); b) female with attached dwarf males (outlined) (scale bar 1 mm); c) dwarf males from b) magnified (scale bar 500 µm)

Fig. 2 Microscopic images of autonomous wood-boring females *Xylophaga atlantica* (~3 mm SL). a) transverse section of tissues stained with haematoxylin/eosin (HE) highlighting female reproductive features, mature oocytes on right and gill on left, with possible spawned oocytes (scale bar 500 µm); inset shows mature oocytes at higher magnification (scale bar 50 µm); b) fluorescence in situ hybridization (FISH) image showing bright green gill (probe EUB-338 [Cy-3]) and DAPI-stained nuclei of gill filament and germinal cells. Note absence of bacterial signal in acini in germinal cells and oocytes compared to positive control in gill (arrow) (scale 50 µm); c) HE-stained section showing gill filament with pink ciliated region (arrow) and non-ciliated region (purple); also showing oocytes, germinal cells and spawned oocytes (scale bar 50 µm); d) FISH image showing overlapping EUB-338 (Cy-3; green) and Gam-42 (Cy-5; red) staining (white arrow) and DAPI-stained nuclei in gill (blue); note bacterial signal in non-ciliated gill region (scale bar 50 µm); be?, spawned oocyte; gc, germinal cell; gl, gill filament; ov, mature oocyte

Fig. 3 Microscopic images of autonomous wood-boring males *Xylophaga atlantica* (~2 mm SL). a) transverse section of tissues stained with haematoxylin/eosin (HE) highlighting male reproductive features and gill filament of two types: ciliated (blue arrow) and non-ciliated (red arrow); b) fluorescence in situ hybridization image showing overlapping EUB-338 (Cy-3; red) and Gam-42 (Cy-5; green) yielding few bright dots (white arrow) and DAPI-stained nuclei in gill (blue arrow). Scale bars 50 µm; spc, spermatocyte; gl, gill filament

Fig. 4 Microscopic images of dwarf males *Xylophaga atlantica* (~0.5 mm SL). a) longitudinal serial section of tissues stained with haematoxylin/eosin highlighting male reproductive features (scale bar 200 µm); b) and c) high magnification of a closely similar LR-White semi-thin sections (300 nm); b) section stained with Toluidine blue showing two gill filaments with ciliated region (arrow) (scale bar 15 µm); c) section hybridized by fluorescence, where Gam-42 (Cy-3) yield some bright green dots in gill (arrow) and DAPI-

753 stained nuclei (scale bar 15 µm); ft, foot; gl, gill filament; spc, spermatocyte; sg,
754 spermatogonia; msp, mature spermatozoa; re, rectum; te, testis; pa, posterior adductor
755 muscle
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